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EDITORIAL

Current treatment of chronic hepatitis C in China: Dilemma and potential problems

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Abstract

Major advances have been made in the treatment of chronic hepatitis C virus (HCV) infection with the advent of direct-acting antiviral agents (DAAs). China has the most cases of HCV infection worldwide, but none of the DAAs has been approved in mainland China so far, and interferon (IFN)-α-based treatment remains the standard of care. HCV patients without response or with contraindications to IFN-based therapy have no alternative options. However, many patients buy DAAs, especially the generic forms of sofosbuvir, from other countries or areas. Under these circumstances, the use of these drugs may cause many predictable and unpredictable problems in ethics, law and medical practice. Given the obstacles of legal accessibility to DAAs and the potential problems of obtaining and using DAAs in China, the early launching of the DAAs in China or the legalization of buying drugs from areas outside China and using these drugs in China is an urgent issue and needs to be dealt with as soon as possible, in the interest of the patients.

Key words: Hepatitis C virus infection; Treatment; Direct-acting antiviral agent; Generics; China

Core tip: This article describes the current treatment situation of chronic hepatitis C virus infection in China and discusses the potential problems pertinent to the access and the use of direct-acting antiviral agents (DAAs), especially the use of generic DAAs from various sources.

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INTRODUCTION

Infection with hepatitis C virus (HCV) is a leading

cause of liver disease. Worldwide, an estimated 130-170 million people have HCV infection, and China has the most cases of HCV infection worldwide, with an estimated 29.8 million people[1]. A high proportion of people with HCV infection have developed advanced chronic liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC).

The primary goal of treating chronic HCV infection is to achieve a sustained virologic response (SVR), which is defined as the absence of serum HCV RNA 12-24 wk after cessation of treatment. Patients achieving an SVR are considered cured in that 99% of patients who achieve an SVR remain undetectable for virus during long-term follow-up[2]. Achievement of SVR is associated with improved clinical outcomes. Pegylated interferon (Peg-IFN)-α 2a or 2b in combination with ribavirin (RBV) has been the standard of care for chronic HCV infection. However, treatment with Peg-IFN-α and RBV has limited efficacy. For instance, 48 wk of Peg-IFN and RBV therapy may achieve SVR in only 40% of patients with HCV genotype 1 infection[3]. Significant adverse events may accompany the duration of treatment[3-5], resulting in poor adherence and premature treatment discontinuation. Moreover, patients with decompensated liver disease, patients with HIV/HCV co-infection, patients who have comorbidity such as heart disease or chronic kidney diseases, patients who have had renal failure and renal transplantation, and patients who have undergone liver transplantation for HCV-associated liver disease may be contraindicated to or ineligible for the regimen of IFN and RBV. Patients who have a null or low response to the regimen of IFN and RBV and patients who are unwilling to take the drugs have no alternative effective treatments. Therefore, novel treatments that have more potent antiviral activity and fewer adverse effects and are eligible and compatible for patients with complex comorbidity in real life settings are urgently required.

Fortunately, major advances have been made in the treatment of chronic HCV infection, with the advent of direct-acting antiviral agents (DAAs) in recent years. Many regimens free of IFN or free of both IFN and RBV have been devised based on combination of new DAAs. These new regimens provide excellent efficacy with higher SVR rates and good safety profile with fewer side effects, and are of shorter duration of treatment. The patients also have a better treatment experience, higher adherence to treatment, and substantial improvement of health-related quality of life during treatment[6]. DAA combination regimens also provide high SVR rates in patients with various HCV genotypes, disease conditions and treatment experiences, including cirrhosis associated with HCV genotype 1[7,8], liver and kidney transplant recipients[9], HCV-genotype-1-infected patients with compensated cirrhosis who had not achieved SVR after successive treatments with Peg-IFN and protease-inhibitor regimens[10], and treatment-naïve and treatment-experienced patients co-infected with HIV and HCV genotypes 1-4[11].

CURRENT TREATMENT OF CHRONIC HCV INFECTION IN CHINA

Because of the unavailability of the novel DAAs, IFN-α or Peg-IFN-α in combination with RBV remains the current standard of care for chronic HCV infection in mainland China. Under these circumstances, HCV patients, especially some important and difficult to treat HCV patients, such as nonresponders to IFN and RBV treatment and those with relapse; patients with renal failure or heart disease; patients intolerant to the adverse events of and with contraindications to INF and RBV; patients with HCV-related cirrhosis and/or HCC; and patients with HIV/HCV co-infection have no other treatment options. In reality, however, the patients themselves are pragmatic. They try to seek help from other sources and find ways possible to obtain the drugs for the treatment of their disease. The high cost used to be one of the obstacles for some patients because of the unaffordability, but the cost is not an issue with the launching of generic drugs in some countries such as India. The price of the generic drugs is much lower than their brand-name counterparts. As a result, many HCV patients have bought or are going to buy DAAs, mainly sofosbuvir, a "blockbuster drug", from various regions or countries such as India and Bangladesh through different means, including through brokers, relatives and friends who have the opportunity to buy the drugs. The HCV patient population using DAAs, mainly sofosbuvir, from the above-mentioned sources is rapidly increasing in China.

DISCUSSION

Undoubtedly, most patients may benefit from the use of these drugs. Ethically and responsibly, doctors would be pleased to see that the patients have access to effective medicines and the probable cure of their disease. However, some problems may be encountered.

First, none of the DAAs including sofosbuvir, whether generics or brand name, has been approved by the China Food and Drug Administration. In this respect, the use of these drugs appears to be illegal in China. There is a similar but not identical example. Lu Yong, a Chinese man, who was diagnosed with chronic myeloid leukemia when he was aged 34 years in 2002, was arrested, jailed and then released by the police authorities in China early this year because of purchasing the Indian generic drug imatinib mesylate for himself and other patients. Although he appeared to be accused of selling "fake drugs", he knows nothing about the reasons for either being arrested or being released[12]. It is suggested that there are some legal issues and gaps.

Second, physicians may feel frustrated and embarrassed when they are consulted by patients with chronic HCV infection regarding the treatment of the disease. The doctors may tell the patients that there are many new drugs that are effective and have fewer adverse effects and may be suitable for their condition when they advise patients who are unsuitable for IFN and RVB, but none of the drugs is lawfully available in China. Of course, the doctors can let the patients wait for the availability of these drugs. However, some of the patients, such as those with decompensated liver disease and those awaiting organ transplantation, cannot wait because of the rapid progress of their disease and its life-threatening potential. Another situation is that the patients consult doctors about using drugs that they have bought from other countries, mostly from illegal sources and by illegal means, and the drugs may thus be regarded as "fake drugs". Additionally, the quality of the drugs may also not be guaranteed. In this situation, the doctors may place themselves at risk because they appear to guide their patients to use "fake drugs".

Third, because of the lack of other DAAs for rational combination, most of the patients take sofosbuvir in combination with RBV and some patients even use sofosbuvir alone for the treatment of their HCV infection with various treatment durations, irrespective of the HCV genotypes involved, the underlying liver disease (hepatitis or cirrhosis) and comorbidity. In reality, the situation may be rather more complex than expected. HCV genotype 1 infection accounts for most cases of HCV infection in China, but there are also other genotypes in China, including 2, 3 and 6[13].

Although the combination of sofosbuvir and RBV is a pan-genotypic regimen and may be applied for HCV genotypes 1-6, and this regimen remains the standard of care for genotypes 2 and 3, its efficacy was suboptimal in patients with HCV genotype 1, with an SVR of 54% for genotype 1b treatment-naïve hepatitis and 60% for genotype 1 treatment-naïve cirrhosis after 24 wk of treatment[14]. In the United States, the regimen of sofosbuvir and RBV for 24 wk in patients with genotype 1 infection is not recommended because of the longer treatment duration and lower expected SVR rates compared with other regimens[15]. The efficacy of this regimen for treatment-experienced genotypes 2 and 3 infection, with or without cirrhosis, was also suboptimal, with an SVR of 72% for genotype 2 treatment-experienced cirrhosis after 12 wk of treatment, an SVR of 77% for genotype 3 treatment-experienced hepatitis after 24 wk of treatment, and an SVR of 60% for genotype 3 treatment-experienced cirrhosis after 24 wk of treatment[14]. Based on these data, it is indicated that a proportion of patients with HCV genotype 1 or genotypes 2 and 3 treatment-experienced hepatitis or cirrhosis is using an non-optimal regimen for treatment. Therefore, it is anticipated that higher non-response and relapse rates may result from the use of this regimen.

Another concern is the possibility of treatment-emergent variants that may confer resistance to the antiviral treatment, although sofosbuvir has high genetic barriers to resistance[14]. Of note, a DAA used as monotherapy is not recommended because of the strong likelihood of treatment failure and the potential to induce resistance[15].

Moreover, the drug safety and drug-drug interactions pertinent to the use of sofosbuvir and RBV or sofosbuvir alone cannot be completely ignored. Sofosbuvir has a good safe profile and few drug-drug interactions. However, the safety of sofosbuvir in patients with some comorbidity, such as severe renal impairment (an estimated glomerular filtration rate < 30 mL/min/1.73 m^2) or end-stage renal disease on dialysis, is not well established, in that the levels of sofosbuvir and its metabolite are substantially elevated in such patients[15]. In contrast, co-administration of P-glycoprotein inhibitors, including anticonvulsants such as phenobarbital, antimycobacterials such as rifampin, ritonavir-boosted tipranavir, and St John's wort can decrease sofosbuvir concentrations[16]. Additionally, although sofosbuvir is not supposed to have significant pharmacological interactions with tacrolimus or cyclosporine[17], unexpected tacrolimus/cyclosporine reduction, which needs dosage adjustment, was observed in transplant recipients during sofosbuvir/RBV treatment for severe HCV infection recurrence. This raises the importance of awareness in the post-transplant HCV-recurrence setting when sofosbuvir is administered[18]. All these may place the patients with some comorbidity in unsafe conditions or bring new problems for the retreatment of patients if necessary.

CONCLUSION

As doctors, we hope for early launching of the DAAs, not only sofosbuvir but also other agents that can be rationally combined with sofosbuvir, in China, and the legalization of buying drugs including generic drugs from areas outside China and using these drugs in China, without further delay, in the interest of the patients. Presently, regulations concerning the usage of drugs, including generic drugs from other areas and international routes, should be established by the health authorities in China to meet the urgent needs. In the long run, China's health authorities need to reform fully their drug import policy and distribution system to satisfy legitimately and sensibly the therapeutic requirement of patients. In particular, the authorities need to guarantee the timely availability of novel drugs, even if they may be generics, for the urgent need of severely ill patients to improve survival and quality of life. At the same time, doctors may become confident to advise the use of such drugs without fear of violating the law or regulations concerning the quality of the drugs. We wish for the early arrival of a time when patients can easily and legally have access to effective drugs, and doctors can lawfully and

unimpeachably play their role in the treatment of HCV infection.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

HER2 testing in gastric cancer: An update

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Abstract

Human epidermal growth factor receptor 2 (HER2) overexpression is increasingly recognized as a frequent molecular abnormality in gastric and gastroesophageal cancer. With the recent introduction of HER2 molecular targeted therapy for patients with advanced gastric cancer, determination of HER2 status is crucial in order to select patients who may benefit from this treatment. This paper provides an update on our knowledge of HER2 in gastric and gastroesophageal cancer, including the prognostic relevance of HER2, the key differences between HER2 protein expression interpretation in breast and gastric cancer, the detection methods and the immunohistochemistry scoring system.

Key words: Human epidermal growth factor receptor 2 testing; Gastric cancer; Immunohistochemistry; Scoring system; Trastuzumab

Core tip: It is clear that human epidermal growth factor receptor 2 (HER2) protein over-expression and gene amplification are much more heterogeneous in gastric cancer compared to breast cancer. Gastric and gastroesophageal tumors require a unique immunohistochemistry scoring system and interpretation expertise. We aimed to clarify the key differences in immunohistochemistry interpretation of gastric cancer, providing a practical update on HER2 testing and scoring.

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INTRODUCTION

Human epidermal growth factor receptor 2 (HER2), also known as CerbB-2 and ERBB2, is a proto-oncogene located on chromosome 17q21 that encodes a transmembrane protein with tyrosine kinase activity,

a member of the HER receptor family and is involved in signal transduction pathways, leading to cell growth and differentiation[1].

Amplification of the HER2 gene and overexpression of its product were first discovered in breast cancer and are significantly associated with worse outcomes[2]. Many studies have demonstrated that HER2 is also present in several other malignancies, including colorectal cancer, ovarian cancer, prostate cancer, lung cancer and, particularly, gastric and gastroesophageal cancer[3].

In gastric and gastroesophageal cancer, the frequency of HER2 overexpression varies widely in the literature; studies have yielded inconsistent findings regarding its prognostic relevance[4-12]. With the recent introduction of trastuzumab for the treatment of patients with advanced gastric cancer, the clinical demand for HER2 assessment is rapidly increasing. However, HER2 testing in gastric cancer differs from testing in breast cancer because of inherent differences in tumor biology, intratumoral heterogeneity of HER2 expression and incomplete membrane staining that are commonly observed in gastric tumors[13].

This paper aims to summarize the current evidence regarding HER2 in gastric and gastroesophageal cancer and to provide a practical update on HER2 testing and scoring that is essential for appropriate selection of patients who are eligible for treatment with trastuzumab.

RELEVANCE OF HER2 IN GASTRIC AND GASTROESOPHAGEAL CANCER

The frequency of HER2 overexpression in gastric and gastroesophageal cancer ranges from 4.4% to 53.4%, with a mean of 17.9%[4-14].

Although some small-scale studies have not demonstrated the prognostic properties of HER2[4,5,9,12], a larger number of studies indicate that HER2 is a negative prognostic factor, showing more aggressive biological behavior and higher frequencies of recurrence in HER2-positive tumors[1,6-8,11,14].

Given this controversy of HER2 prognostic values, a systematic review of a large number of studies was recently conducted in order to address this issue[14]. Forty-two publications with a total of 12749 patients were reviewed; the majority (71%) of the publications showed that a HER2-positive status was associated with decreased survival and clinicopathological features of tumor progression, such as serosal invasion, metastases and higher disease stage[14]. The results clearly set HER2 as a negative prognostic factor, suggesting that HER2 overexpression/amplification is a molecular abnormality that might be associated with the development of gastric cancer[7,14].

HER2 MOLECULAR TARGETED THERAPY

Trastuzumab is a monoclonal antibody directed against HER2; as one of the first molecular-targeted drugs to be developed, it was first introduced for the treatment of HER2-positive advanced breast cancer[2].

There is no consensus on the mechanism in which trastuzumab acts in cancer cells, but the evidence is that in addition to preventing dimerization of HER2 with other HER family members and stimulating endocytosis, it seems to induce cell mediated immunity and inhibit angiogenesis[15].

In the ToGA trial, patients with HER2-expressing unresectable gastric and gastroesophageal tumors were treated with chemotherapy and trastuzumab or with chemotherapy alone. A statistically significant increase in overall survival was observed in patients who received trastuzumab[16].

Although only a modest improvement of 2.7 mo in the median overall survival was observed in HER2-positive patients with the addition of trastuzumab, according to the ToGA trial, there was an improvement of 4.2 mo in the median overall survival in a post-hoc analysis[14,16-18].

Other molecular HER2-targeted agents have been tested or are currently being tested such as pertuzumab, lapatinib, the antibody-drug conjugate trastuzumab-emtansine (TDM-1)[19-23] and afatinib (NIH study trial registration number NCT01522768; ClinicalTrials.gov). However, the efficacy of these agents has been shown to be either unsatisfactory or as modest as trastuzumab[22,24]. Trastuzumab is the first molecular targeted agent approved as a standard treatment in gastric cancer, but it remains under investigation for more potent utilization.

Thus, it is imperative to determine the HER2 status in advanced gastric or gastroesophageal junction adenocarcinoma in order to select patients who may benefit from this promising treatment.

HER2 TESTING METHODS

HER2 status is mainly assessed by immunohistochemistry (IHC) or *in situ* hybridization (ISH) assays. Both methods can be done on formalin-fixed and paraffin-embedded biopsy tissues or surgical specimens and occasionally, cytological samples[25]. Fluorescent *in situ* hybridization (FISH) is regarded to be the gold standard; however, because of its higher cost and time consumption, as well as the need for a fluorescence microscope, generally only equivocal cases are subjected to this technique. Furthermore, the high concordance between FISH and IHC that is reported in the literature supports the use of IHC, the most familiar and readily accommodated method in most surgical pathology laboratories[26-29].

Thus, IHC should be used as the first screening method for HER2 evaluation and those cases with results considered equivocal for HER2 overexpression (2+) should be referred for FISH analysis or other alternative *in situ* hybridization method[28] (Figure 1). A simple and practical alternative to FISH for these

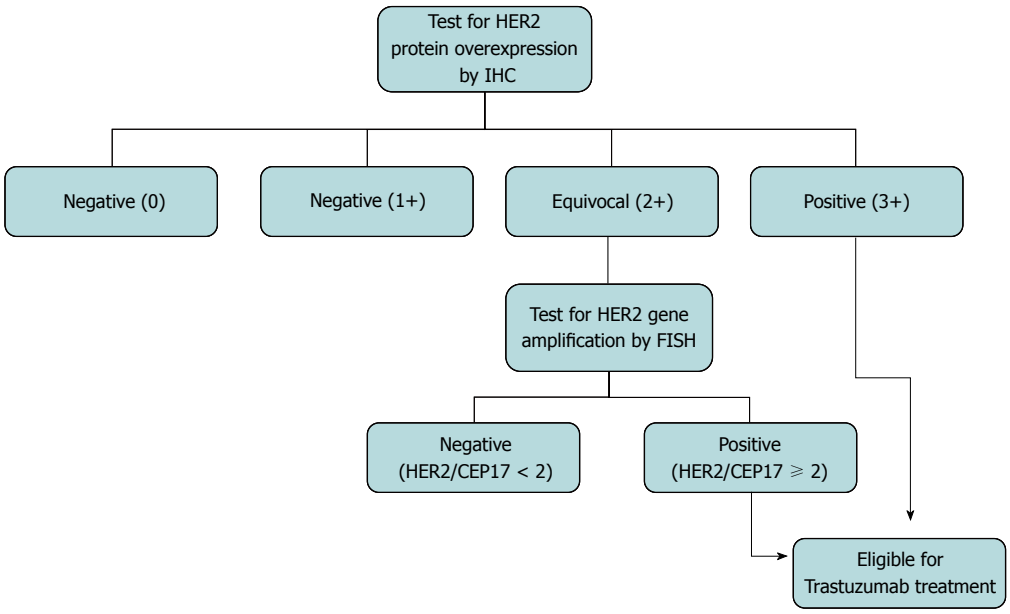

Figure 1 Human epidermal growth factor receptor 2 testing algorithm. HER2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; FISH: Fluorescent *in situ* hybridization; CEP17: Chromosome 17.

Table 1 Advantages and disadvantages of the human epidermal growth factor receptor 2 testing methods

Method	Advantages	Disadvantages
IHC	Quick to perform; Most laboratories use fully automated processes; Widely used and familiar to all pathologists; Results can be viewed using a conventional bright-field microscope; Permits parallel viewing of tumor cell morphological features; Stained tissues do not degrade over time	Equivocal cases (2+) need another method for conclusion; Accuracy is more dependent on pre-analytic variables
FISH	Very objective and accurate; Actual copies of HER2 genes can be counted; Considered the golden standard of HER2 testing	Technically more demanding; Usually performed only in large laboratories/institutions; Costs are substantially high; Requires the use of fluorescence microscope and dark room; Comparatively more time consuming; Reagents degrade over time
SISH/CISH/ DDISH	Quick to perform; Very objective and accurate; Technique is fully automated; Results can be viewed using a conventional bright-field microscope; Permits parallel viewing of tumor cell morphological features; Slides can be stored because the signal is stable; Double-stranded probes labeled with two haptens can detect both markers on a single slide (DDISH)	More expensive than IHC; Unfamiliar to non-specialist pathologists

IHC: Immunohistochemistry; FISH: Fluorescent *in situ* hybridization; SISH: Silver *in situ* hybridization; CISH: Chromogenic *in situ* hybridization; DDISH: Dual-color dual-hapten *in situ* hybridization.

equivocal cases is provided by the employment of other *in situ* hybridization techniques such as silver *in situ* hybridization (SISH), chromogenic *in situ* hybridization and dual-color dual-hapten *in situ* hybridization. These three methods can be easily analyzed under a conventional bright field microscope and have shown excellent correlation with results obtained by FISH[30-32].

Because IHC is the easiest, least expensive and most widespread method of HER2 assessment, this paper focuses on IHC. Table 1 shows the different HER2 methods and their advantages and disadvantages.

Differences between HER2 expression in breast and gastric cancer

The key differences between HER2 expression in breast and gastric and gastroesophageal cancer are listed[17,30]: (1) the membranous distribution of the antibody in the neoplastic cells of breast cancer is

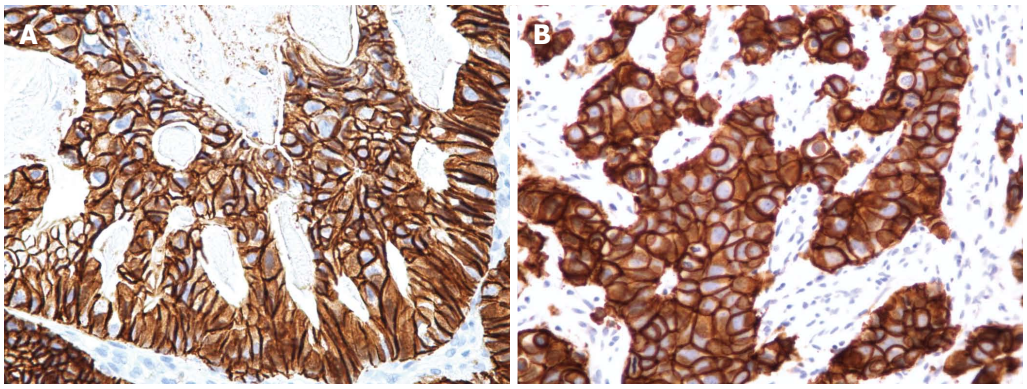

Figure 2 Human epidermal growth factor receptor 2 expression in gastric and breast tumors. A: A HER2-positive (3+) case of gastric adenocarcinoma; the cytoplasmic membranous immunostaining is incomplete and predominantly basolateral (× 400); B: A HER2-positive (3+) case of invasive ductal carcinoma of the breast; the cytoplasmic membranous staining is fully circumferential (× 400). HER2: Human epidermal growth factor receptor 2.

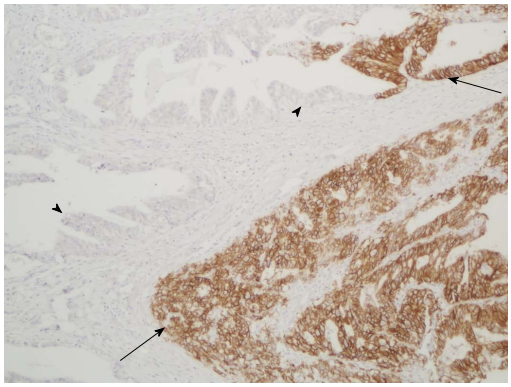

Figure 3 Representative image of the intratumoral heterogeneity of HER2 expression. Arrows indicate areas with strong continuous membranous staining (score 3+) and arrowheads indicate negative areas (score 0) (× 100). HER2: Human epidermal growth factor receptor 2.

predominantly circumferential, whereas in gastric cancer, it is generally incomplete, predominantly basolateral ("U"-shaped) or lateral (parallel lines) (Figure 2). Thus, unlike for breast cancer, circularity of IHC staining is not a criterion for HER2 IHC scoring in gastric cancer; (2) intratumoral heterogeneity, defined as the presence of areas with different HER2 scores within the same tumor, *i.e.*, focal or patchy positivity, is a common pattern encountered in gastric tumors but is only rarely seen in breast cancer (Figure 3). It may cause sampling errors when randomly sampled biopsies are examined (see below). Although the causes of intratumoral heterogeneity of HER2 expression are not yet fully understood, some studies indicate that it could be explained merely by tumor inherent genetic heterogeneity[33,34]. Since *Helicobacter pylori* (*H. pylori*) is widely accepted as the main causative agent of gastric cancer[35], we speculate whether among the diverse bacterial factors, concomitant infection with different strains and diverse host responses there could be a reasonable link with HER2 intratumoral heterogeneity. Interestingly, Tegtmeyer *et al*[36] showed that some *H. pylori* strains could in fact activate HER2, while infection with other strains suppressed HER2 activity. However, this correlation of the bacterium with HER2 intratumoral heterogeneity is still a matter of debate and requires further studies; and (3) variation of the incidence of HER2 expression with anatomic location does not occur in breast cancer, whereas it is more frequent in the proximal stomach, including the esophageal-gastric junction, than in the distal stomach. With the introduction of the seventh edition of TNM classification, a large number of tumors that were formerly categorized as gastric are now considered as esophageal and gastroesophageal junction tumors instead, with relatively high HER2-positivity rates in these primary neoplasms[37].

IHC score system

Given these differences between HER2 expression in breast and gastric cancer, an appropriate scoring system, exclusive for gastric tumors, was developed, because just transferring the breast cancer IHC scoring roles to gastric cancer could lead to a significant loss of patients. The system proposed by Hofmann *et al*[38] that has been assimilated by CAP and FDA, besides being specific for gastric tumors, also distinguishes biopsies from surgical specimens[17]. Table 2 shows the IHC score system for HER2 in gastric cancer and Figure 4 illustrates it.

Differences among samples

As mentioned above, mainly because of intratumoral heterogeneity, the size of the tissue sample might interfere in HER2 analysis. Although Hofmann's HER2 scoring system was formulated for evaluating HER2 status in biopsy and surgical specimens, discordant HER2 results in paired specimens were observed in a small percentage of tumors[39]. Intratumoral heterogeneity appears likewise to be the subject of conflicting results of HER2 expression in primary and metastatic tumor samples[33]. Moreover, in a previous study, we showed a significant difference in sensibility when analyzing HER2 expression in whole-tissue sections and in tissue microarrays[13]. Our personal

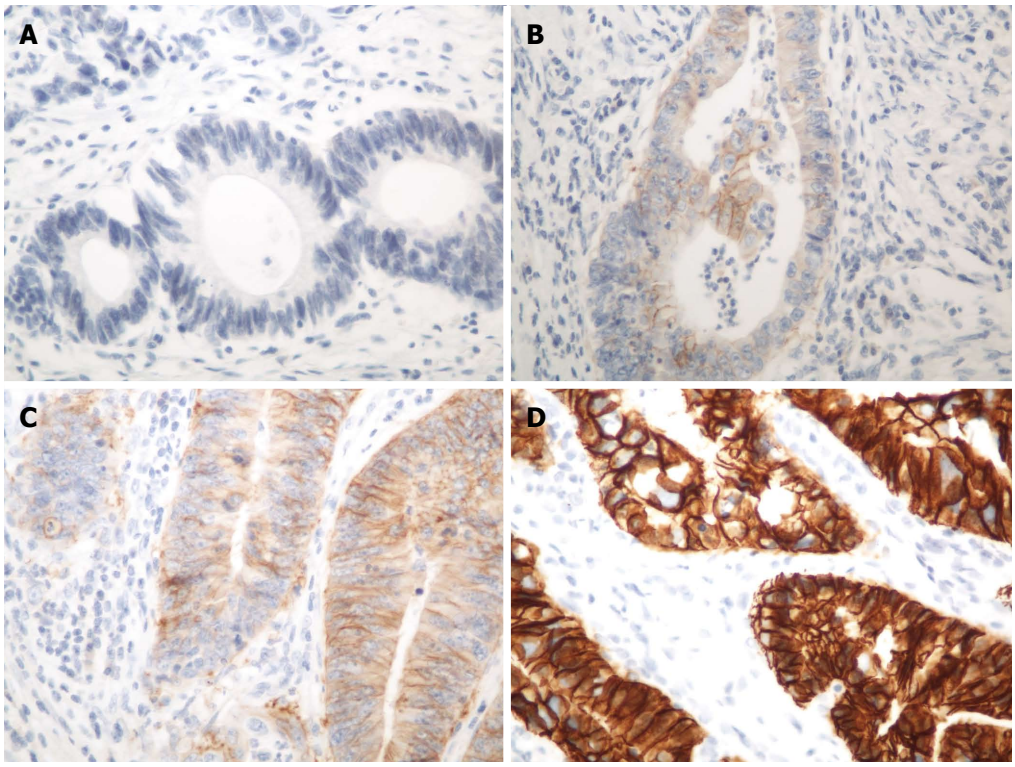

Figure 4 Human epidermal growth factor receptor 2 protein expression in gastric and gastroesophageal tumors. A: A negative (0) case; B: A negative (+1) case; C: An equivocal (2+) case; D: A positive (3+) case. HER2: Human epidermal growth factor receptor 2.

Table 2 Immunohistochemistry scoring for human epidermal growth factor receptor 2 expression in gastric and gastroesophageal junction cancer[17]

Score	Surgical specimen	Biopsy	HER2 overexpression assessment
0	No membranous staining or staining of < 10% of the tumor cells	No membranous staining or staining only in rare cells (less than 5 cohesive cells)	Negative
1+	Staining is weak or detected in only one part of the membrane in ⩾ 10% of the cells	Staining is weak or detected in only one part of the membrane of at least 5 cohesive cells	Negative
2+	Moderate/weak complete or basolateral membranous staining in ⩾ 10% of the cells	Moderate/weak complete or basolateral membranous staining of at least 5 cohesive cells	Equivocal
3+	Strong complete or basolateral membranous staining in ⩾ 10% of the neoplastic cells	Strong complete or basolateral membranous staining of at least 5 cohesive cells	Positive

HER2: Human epidermal growth factor receptor 2.

experience suggests that it is prudent to extend the evaluation to more than one sample and, if feasible, to also evaluate metastatic foci. In fact, testing all available specimens should be considered so that discrepancies can be excluded. When only biopsies are available, it is recommended to have at least four fragments containing tumor cells[40]. We also recommend that all surgical specimens from patients that previously obtained HER2-negative results in biopsies should also be tested to increase the chance of finding HER2-positive tumors.

IHC antibodies

The results of the HER2 test might differ according to the antibody used and, consequently, the antibody might considerably influence therapeutic decisions. An optimal IHC antibody should be adequately sensitive to select the greatest possible number of candidates for treatment and should have the lowest possible false-positive rate in order to avoid overtreatment.

The commercial antibodies currently available are the HercepTest and A0485 (Dako, Glostrup, Denmark), SP3 (Labvision; Thermo Fisher Scientific, Fremont, CA, United States), 4B5 (Ventana Medical Systems, Tucson, AZ, United States) and CB11 (Novocastra, Newcastle upon Tyne, England). Some studies have shown substantial divergence among the antibodies regarding the results of HER2 expression in gastric tumors[13,29,41]. Our previous study compared HercepTest, SP3 and 4B5. We observed that the 4B5 and SP3 antibodies showed similar good performance, with high NPV (negative predictive value) and AUC (area under the ROC curve) values that indicated higher accuracy compared to the HercepTest[13]. Based

on these results and on our personal experience, we believe that 4B5 and SP3 antibodies are more reasonable for first-line tests than the HercepTest in gastric tumors.

CONCLUSION

Given the recent introduction of trastuzumab for the treatment of patients with advanced gastric cancer, assessment of HER2 status is now mandatory for selecting patients eligible for this treatment. Although the development of automated platforms and image analysis should broaden the availability of *in situ* hybridization technologies, immunohistochemistry continues to play an essential role in HER2 status assessment. The overall reliability of HER2 evaluation by IHC, however, can be affected by diverse pre-analytical, analytical and post-analytical variables. Therefore, gastric and gastroesophageal cancer requires a unique scoring system, but above all, it requires expertise in interpretation.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Minimally invasive surgery for upper gastrointestinal cancer: Our experience and review of the literature

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Abstract

Minimally invasive surgery (MIS) for upper gastrointestinal (GI) cancer, characterized by minimal access, has been increasingly performed worldwide. It not only results in better cosmetic outcomes, but also reduces intraoperative blood loss and postoperative pain, leading to faster recovery; however, endoscopically enhanced anatomy and improved hemostasis *via* positive intracorporeal pressure generated by CO_2 insufflation have not contributed to reduction in early postoperative complications or improvement in long-term outcomes. Since 1995, we have been actively using MIS for operable patients with resectable upper GI cancer and have developed stable and robust methodology in conducting totally laparoscopic gastrectomy for advanced gastric cancer and prone thoracoscopic esophagectomy for esophageal cancer using novel technology including da Vinci Surgical System (DVSS). We have recently demonstrated that use of DVSS might reduce postoperative local complications including pancreatic fistula after gastrectomy and recurrent laryngeal nerve palsy after esophagectomy. In this article, we present the current status and future perspectives on MIS for gastric and esophageal cancer based on our experience and a review of the literature.

Key words: Stomach neoplasms; Esophageal neoplasms; Minimally invasive surgical procedures; Postoperative complications; Robotic surgical procedures

Core tip: Minimally invasive surgery (MIS) for upper gastrointestinal cancer reduces intraoperative blood loss and postoperative pain, leading to faster recovery. It also results in better cosmetic outcomes. The impact of MIS on postoperative complications and long-term outcomes has been under debate. We have recently demonstrated that use of da Vinci Surgical System might reduce postoperative local complications including pancreatic fistula after gastrectomy and recurrent laryngeal nerve palsy after esophagectomy.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer death in the world in 2012[1]. Surgical resection remains the only curative treatment option, and regional lymphadenectomy is recommended as part of radical gastrectomy[2]. According to the Japanese Gastric Cancer Association (JGCA) Gastric Cancer Treatment Guidelines, D2 gastrectomy is recommended for advanced gastric cancer (AGC)[3,4]; however, D2 lymphadenectomy, especially when combined with splenectomy or pancreaticosplenectomy, has been reported to increase morbidity and mortality[5-8].

Esophageal cancer (EC) is the eighth most common malignancy and the sixth leading cause of cancer death in the world in 2012[1]. Similar to GC, surgical resection remains the primary curative treatment option, and regional lymphadenectomy is recommended as part of radical esophagectomy[9-12]. Esophagectomy, which requires thoracolaparotomic manipulation, is one of the most invasive operations in gastrointestinal (GI) surgery, being associated with significant morbidity and mortality[13,14].

Minimally invasive surgery (MIS), which was launched in the late 80's[15], has been characterized by minimal access using laparoscope or thoracoscope with CO_2 insufflation[16]. Although the impact of MIS on postoperative inflammatory response has still been unclear, it has been increasingly used for upper GI malignancies in an attempt to improve postoperative outcomes[13,17,18].

This article provides the updates on laparoscopic gastrectomy (LG) for GC and video-assisted thoracoscopic surgery esophagectomy (VATS-E) for EC, particularly focusing on our twenty-year experience in this field along with a review of previously reported and ongoing large prospective studies.

GASTRIC CANCER

LG for early gastric cancer

Since the first report of LG by Kitano *et al*[19] in 1994, LG for GC has gained popularity because of its beneficial short-term effects leading to improved quality of life (QoL) in comparison with open gastrectomy (OG), although many controversies still exist due to the lack of solid evidence on its long-term outcomes[20-24]. Therefore, LG had long been recognized as an investigational treatment even for early gastric cancer (EGC) but not as a standard procedure in Japan[25]. However, based on the results of the following multicenter phase Ⅱ trial conducted by the Japanese Clinical Oncology Group (JCOG) (JCOG0703)[26], the new Japanese Gastric Cancer Treatment Guidelines (ver. 4, issued in 2014) has turned to allow laparoscopic distal gastrectomy (LDG) for clinical stage Ⅰ disease as a standard treatment option[4].

JCOG0703

JCOG0703[26] was conducted to assess the safety of LDG with D1+ lymph node (LN) dissection for clinical stage Ⅰ GC. In this well-designed phase Ⅱ study, to control for the quality of surgery, only surgeons who had performed 30 or more LDGs and 30 or more open distal gastrectomies (ODGs) participated. A central review of the surgical procedure in all the patients was conducted by evaluating photographs taken during the procedure. Between 2007 and 2008, 176 eligible patients from 14 hospitals were enrolled. The incidence of anastomotic leakage or pancreatic fistula was primarily determined, resulting in only 1.7 % (3/173), which was much lower than the pre-specified threshold of 8%. Moreover, morbidity (Common Terminology Criteria for Adverse Events, CTCAE v3.0 Grade 3 or 4)[27] was 5.1%. Thus, the safety of LDG for clinical stage IA/IB disease was securely confirmed.

JCOG0912

On the basis of JCOG0703, a multicenter phase Ⅲ RCT of LDG *vs* ODG with D1+ nodal dissection for clinical stage Ⅰ GC (JCOG0912) has currently been conducted to determine the non-inferiority of LDG to ODG in terms of overall survival[28,29]. For quality control of surgery, surgeons were required to have experience with at least 30 LDGs as well as certification (or its equivalent) from the Japan Society for Endoscopic Surgery (JSES) according to the Endoscopic Surgical Skill Qualification System[30]. Between 2010 and 2013, 921 patients (LDG 462, ODG 459) were enrolled from 33 institutions. Regarding short-term outcomes of this study, LDG significantly improved blood loss, postoperative pain and recovery of bowel movement irrespective of extended operative time. There were no grade 3 or 4 (CTCAE v4.0[31]) intraoperative adverse events in either arm. No difference was observed in the overall proportion of in-hospital, non-hematological

Table 1 Ongoing randomised controlled trials on laparoscopic distal gastrectomy for advanced gastric cancer

	JLSSG0901	KLASS02	CLASS01
Country	Japan	Korea	China
Start year	2010	2011	2012
Phase	Ⅱ/Ⅲ	Ⅲ	Ⅲ
Intervention	LDG *vs* ODG	LDG *vs* ODG	LDG *vs* ODG
Inclusion criteria	cT2-4a cN0-2 (except bulky N2)	cT2-4a cN0/1	cT2-4a cN0-3 (except bulky LN)
Sample size	Ⅱ:180, Ⅲ:500	1050	1056
Primary endpoint	Ⅱ: morbidity Ⅲ: 3-year RFS	3-year RFS	3-year RFS

JLSSG: Japanese Laparoscopic Surgery Study Group; KLASS: Korean Laparoscopic Gastrointestinal Surgery Study; CLASS: Chinese Laparoscopic Gastrointestinal Surgery Study; LDG: Laparoscopic distal gastrectomy; ODG: Open distal gastrectomy; LN: Lymph node; RFS: Relapse-free survival.

grade 3 or 4 adverse events excluding biochemical data (LDG *vs* ODG, 3.3% *vs* 3.7%). The proportion of grade 3 or 4 serum AST/ALT increased was higher in LDG than ODG (16.4% *vs* 5.3%, $P < 0.001$). Thus, this trial has so far demonstrated that LDG performed by the credentialed surgeons was at least as safe as ODG in terms of adverse event and short-term clinical outcomes. The primary analysis of the long-term outcomes including overall survival and relapse-free survival is planned in 2018[29].

KLASS01

The Korean Laparoscopic Gastrointestinal Surgery Study (KLASS) group 01 trial is another multicenter (13 institutions) RCT to confirm oncological safety of LDG for EGC in comparison with ODG[32,33]. The primary endpoint of this study is 5-year overall survival. Surgeons had to have performed at least 50 cases of both LDG and ODG, and their institution should have performed more than 80 cases of both LDG and ODG, respectively. Between 2006 and 2010, 1416 patients (705 LDGs and 711 ODGs) were enrolled. Regarding short-term outcomes, LDG improved the overall complication rate (LDG *vs* ODG, 13.0% *vs* 19.9%, $P = 0.001$), particularly wound complication (LDG *vs* ODG, 3.1% *vs* 7.7%, $P < 0.001$). The major intra-abdominal complication (LDG *vs* ODG, 7.6% *vs* 10.3%, $P = 0.095$) and mortality rates (LDG *vs* ODG, 0.6% *vs* 0.3%, $P = 0.687$) were similar between the groups. Thus, this trial has so far demonstrated that LDG for patients with clinical stage Ⅰ GC was sufficiently safe and has a benefit of lower occurrence of wound complication compared with conventional ODG. The long-term outcomes are being awaited.

Laparoscopic total gastrectomy for EGC

These multicenter prospective studies only cover distal gastrectomy. At this moment, both JGCA and JSES have commented that Laparoscopic total gastrectomy (LTG) should be cautiously introduced because of its technical difficulties in complicated alimentary tract reconstruction as well as the LN dissection at the splenic hilum or along the short gastric arteries[4,34]. Since techniques for laparoscopic esophagojejunostomy has recently been established among expert laparoscopic surgeons[35,36], JCOG is planning a phase Ⅱ study to determine the safety of LTG with D1+ LN dissection for clinical stage Ⅰ disease[37]. KLASS group has already been conducting a similar phase Ⅱ trial (KLASS03) to properly evaluate the perioperative morbidity and mortality of LTG for EGC since 2012[17].

LG for AGC

Application of LG for AGC remains to be debated not only because of the lack of evidence on long-term outcomes, but also because of the technical difficulty in performing complete D2 LN dissection and a concern for the innate risk of cancer cell dissemination to the peritoneal cavity[5,16,17,38]. Having said that, acceptable short- and long-term outcomes of the LG for AGC have been reported by a couple of experienced surgeons including us[38-41]. Currently, large-scale multicenter RCTs have been conducted in Japan (The Japanese Laparoscopic Surgery Study Group, JLSSG 0901[42]), Korea (KLASS02[17,43]), and China (The Chinese Laparoscopic Gastrointestinal Surgery Study, CLASS 01[17]) in order to determine the feasibility of LDG for locally AGC (Table 1).

LG for AGC at our institute

History: Laparoscopic surgery was launched in the early 90's in our country[44]. At that time, most laparoscopic surgeons applied laparoscopic surgery, using its minimally invasive nature, to less extended surgery[45]. However, we assumed from the beginning that laparoscopic surgery should be suitable for meticulous LN dissection using laparoscopically enhanced anatomy and reduced venous bleeding *via* pneumoperitoneum irrespective of the limited range of motion, poor depth perception, and limited tactile sensation[5,46]. Then, we introduced laparoscopic assistance into moderate to advanced GI surgery in combination with a caudocranial and mediolateral approach to overcome those limitations in 1995, and developed techniques for LDG and LTG with D2 dissection for AGC, which were published for the first time in the world[47,48]. Since then, we have performed

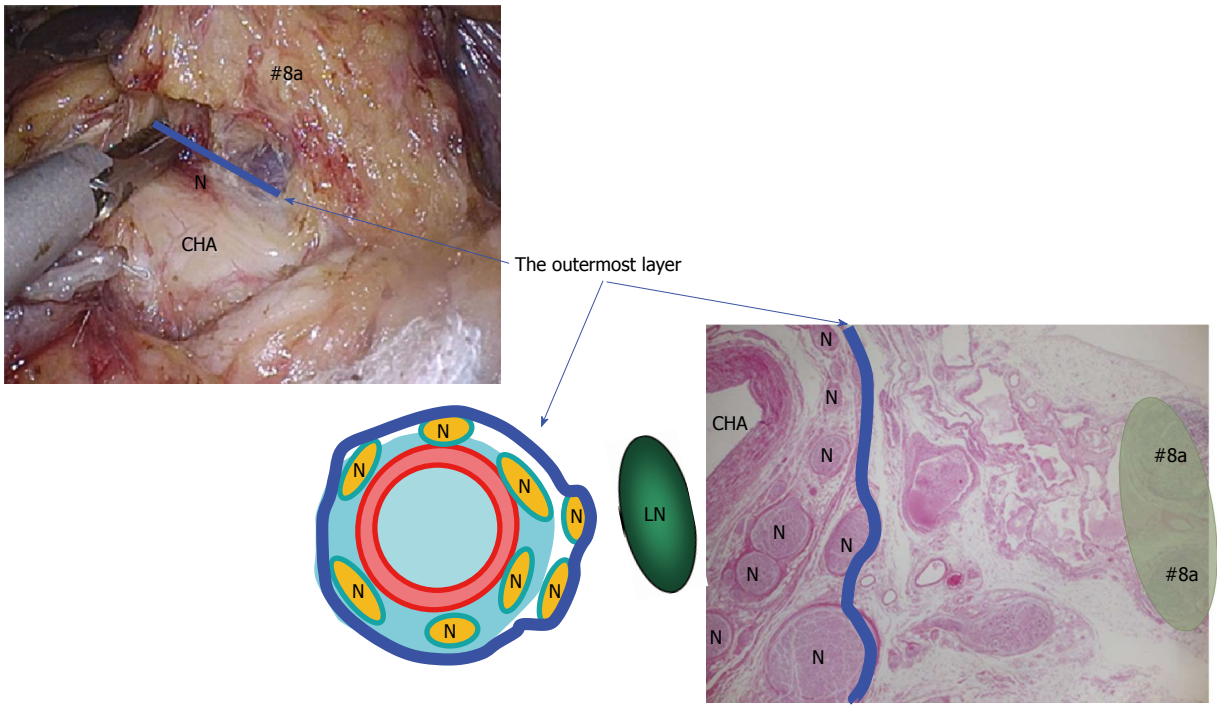

Figure 1 Outermost layer of the autonomic nerve. Shown in the blue line, lies between the vascular sheath of the major arteries and the fat tissue including lymph nodes. Appropriate tension given to this thin loose connective tissue layer generates sufficient space for safe, adequate and reproducible prophylactic lymph node dissection along the major arteries. LN: Lymph node; N: Nerve; CHA: Common hepatic artery.

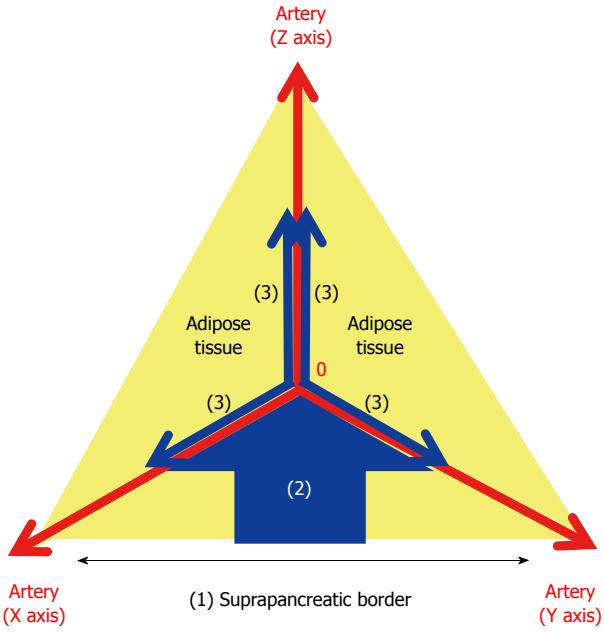

Figure 2 XYZ-axis theory. The following three steps result in effective probing of the outermost layer: (1) dissection of the serosal membrane on the suprapancreatic border; (2) dissection of the fat tissue in the caudo-cranial direction towards the zero point; and (3) dissection of the fat tissue bearing the target LNs in the medio-lateral direction along the outermost layer on the XZ and YZ axes. The outermost layer adjacent to the zero point should be exposed during the 2nd step.

more than 1500 LGs. At present, the standard type of operation for curable GC at our institute is totally laparoscopic D2 gastrectomy[5].

Suprapancreatic lymph node dissection: Outermost-layer oriented medial approach: D2 dissection entails removal of the LNs in the suprapancreatic area in distal and total gastrectomy[4]. Dissection of this area is technically demanding due to the serious risk of bleeding and/or pancreatic leakage derived from a major vessel or organ injury[49,50]. To improve the safety, efficacy, and reproducibility of suprapancreatic LN dissection, we developed our original methodology called outermost layer-oriented medial approach[46,50]. In this approach, the thin loose connective tissue layer between the autonomic nerve sheaths of the major arteries and the adipose tissue bearing lymphatic tissue is dissected[46,50]. We termed this layer as the outermost layer of the autonomic nerve (Figure 1)[46]. To identify this layer throughout the dissection process, we developed an original surgical theory, "XYZ-axis" theory (Figure 2), consisting of the following three steps: (1) cut the serosal membrane on the suprapancreatic border; (2) dissect suprapancreatic adipose tissue caudocranially towards the junction of the three arteries (zero point) to find the outermost layer; and (3) dissect the target adipose tissue mediolaterally along the outermost layer spreading on the XZ and YZ axes. Using this theory, the outermost layer could easily be found not only at the junction of left gastric, common hepatic, and splenic arteries (Figure 3A), but also at that of gastroduodenal, right gastroepiploic, and anterior superior pancreaticoduodenal arteries (Figure 3B) and that of proper hepatic and right gastric arteries (Figure 3C).

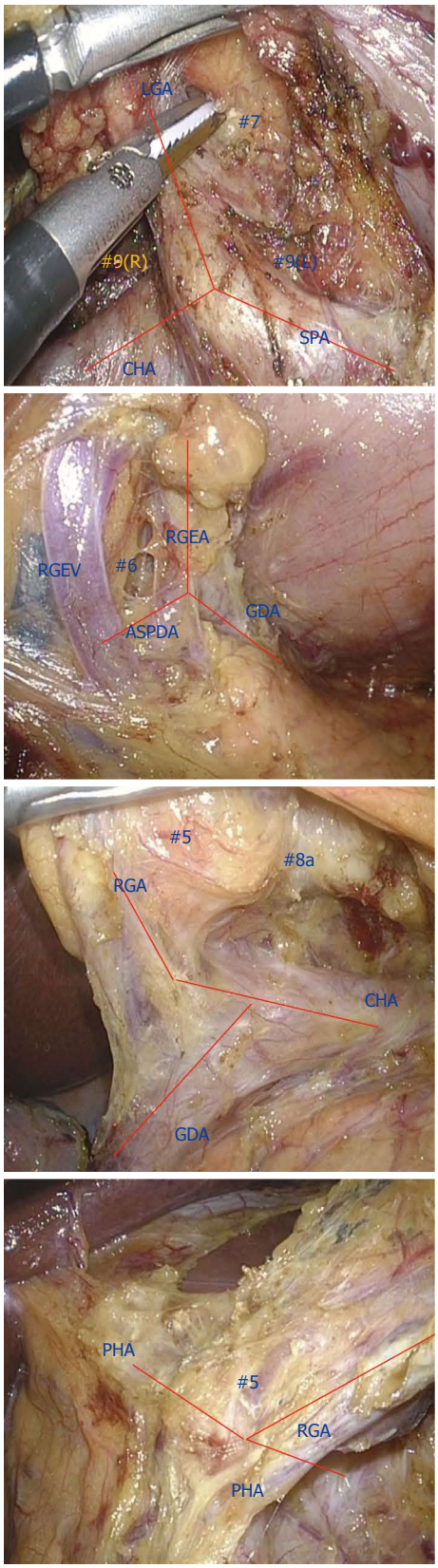

Figure 3 Lymph node dissection along the outermost layer using the XYZ-axis theory. A: No. 7 and 9 dissection, B: No. 6 dissection, C: No. 5 dissection.

LTG for AGC: Splenic hilar lymph node dissection: According to the JGCA guidelines, D2 total gastrectomy is recommended for advanced proximal GC[3,4]; however, as mentioned before, D2 lymphadenectomy combined with splenectomy or pancreaticosplenectomy has been reported to increase morbidity and mortality[5,51,52]. Therefore, the practical importance of station 10 LN dissection and splenectomy in D2 total gastrectomy is controversial[6-8].

We started totally LTG (TLTG) for AGC in 1997[47] and have established a stable and robust methodology, including splenic hilar LN (SHLN) dissection, even though LTG but not LDG has still been one of the independent risk factors for postoperative complications of LG[53,54]. Regarding the extent of SHLN dissection, D2 lymphadenectomy combined with distal pancreaticosplenectomy (D2 + PS) is performed in patients with tumors infiltrating into the pancreatic body or tail. D2 lymphadenectomy combined with splenectomy (D2 + S) is performed in patients with LN metastasis at the station 11 d or 10 or in those with greater curvature invasion. Spleen-preserving D2 lymphadenectomy (D2-S) is performed in patients with tumor depths cT $\geqslant$ 3 without LN metastasis at the station 11 d or 10, whereas D2 lymphadenectomy with preservation of station 10 LNs and the spleen (D2-10) is performed in patients without greater curvature invasion and with tumor depths cT2 (Figure 4)[55].

Regarding the operating procedures, additional care to control the extent of SHLN dissection in TLTG was given to: (1) the layer on the fusion fascia at the infrapancreatic border of the pancreatic tail; (2) the layer on the subretroperitoneal fascia on the left diaphragmatic crus around the upper pole of the spleen; and (3) the outermost layer of the splenic artery. Using these layers, the aforementioned four different types of SHLN dissection could easily be performed. Procedural details are summarized in our previous literature[55]. In this previous study, multivariate analysis revealed that operative time was the only significant factor associated with postoperative complications. Operative time, morbidity, and pancreatic fistula increased with increasing extent of SHLN dissection. Therefore, the extent of SHLN dissection should be appropriately attenuated if this is allowed by oncological factors. At present, according to the latest JGCA guidelines[4], complete clearance of station 10 nodes by splenectomy should still be considered for potentially curable T2-4 tumors invading the greater curvature of the upper stomach. However, in patients with T2-4/N0-2/M0 GC not invading the greater curvature, the JCOG0110 trial demonstrated that prophylactic splenectomy should be avoided to improve operative safety and survival[2,56].

Intracorporeal anastomosis: To fully utilize the advantages of LG, totally laparoscopic gastrectomy with intracorporeal anastomosis is promising. We have preferred intracorporeal anastomosis with linear staplers because of its handy, quick visible, and reproducible natures. In LDG, we have used delta-shaped anastomosis for Billroth-Ⅰ reconstruction[57-59],

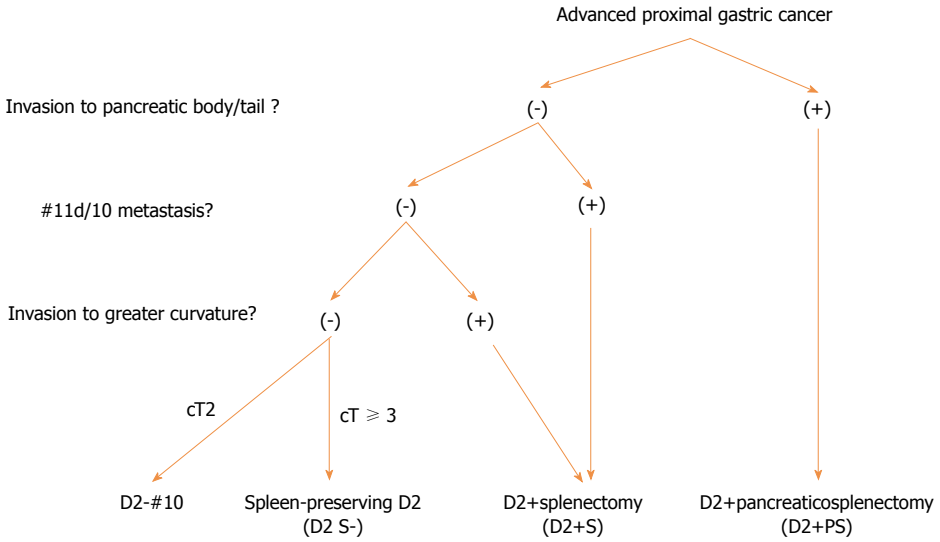

Figure 4 Indication for splenic hilar lymph node dissection at FHU.

antiperistaltic side-to-side anastomosis for Billroth-Ⅱ reconstruction, and functional end to end anastomosis for Roux-en-Y reconstruction[5]. In total gastrectomy, we have used functional end to end anastomosis[36] and overlap method[35] for intraabdominal and intrathoracic esophagojejunostomy, respectively. In proximal gastrectomy, modified overlap method with no-knife stapler has been used[60]. The details on intracorporeal anastomosis in LG are well summarized in the review article by Hosogi *et al*[60].

Outcomes: The short-term and long-term outcomes of LG for AGC at our institute have been satisfactory from both technical and oncological point of view (LG *vs* OG: mortality, 1.1% *vs* 0%, $P = 0.519$; morbidity, 24.2% *vs* 28.5%, $P = 0.402$; 5-year disease free survival, 65.8% *vs* 62.0%, $P = 0.737$; overall survival, 68.1% *vs* 63.7%, $P = 0.968$). Details are demonstrated in our previous reports[38,55].

Robotic gastrectomy

In Japan, da Vinci S HD Surgical System received approval by the Drugs, Cosmetics and Medical Instruments Act in November, 2009. We introduced da Vinci S to our institution in 2009 for the first time in our county, and have been actively using this system for operable patients with resectable upper GI cancer who agreed to uninsured use of the robot[46,53,61].

According to the latest meta-analysis of robotic gastrectomy (RG) *vs* LG, combining the findings from previous observational studies with small sample size, use of the robot significantly increased operative time and cost, whereas there were no significant differences in other short-term outcomes including blood loss, number of dissected lymph nodes, surgical margins, postoperative complications and duration of hospital stay[62]. The only large non-randomized prospective study (NCT01309256), recently reported from Korea, demonstrated similar outcomes[63]. These reports suggested that use of the robot might even deteriorate the cost-effectiveness[62,63]. In other words, the greatest issue around RG is a lack of clear benefits of the robotic system which corroborate the longer duration of operation and higher cost[63]. However, most of the patients enrolled in these previous studies had pathological stage Ⅰ diseases, and these studies failed to eliminate the learning effect or the selection bias at least partly generated by more expensive copayment in RG[62,63]. The impact of RG on long-term outcomes has largely been unclear[64,65]. Thus, the advantages of RG for AGC conducted by fully-trained robotic surgeons have never been clarified. In addition, several reports have demonstrated the short learning curve of RG[66-69].

Since 2009, we have performed RG for more than 250 patients not only with EGC but with AGC. Then, according to our retrospective analyses in comparison with LG (EGC *vs* AGC, 57% *vs* 43%), RG reduced morbidity down to one fifth including pancreatic fistula, leading to further improvement in short-term postoperative courses, although it slightly increased blood loss and operative time[53,70]. Multivariate analyses clearly demonstrated that conventional LG (non-use of the surgical robot), total gastrectomy (*vs* distal) and D2 lymphadenectomy (*vs* D1+) were the significant independent risk factors determining postoperative complications[53]. Moreover, the greater the extent of gastric resection and LN dissection, the more effective the use of the robot[53]. In terms of long-term results, 3-year overall survival did not change between RG and LG[71]. Not only oncological factors including tumor size and clinical stage but also surgical factors including pancreatic fistula were found to be associated with three-year recurrence free survival, indicating the oncological as well as surgical importance of preventing pancreatic fistula[71,72]. These

data suggest that the best indication for the use of the robot might be RG for AGC with D2 dissection[53]. Thus, multi-institutional prospective studies conducted by experienced robotic surgeons, in which considerable number of patients with AGC are enrolled, should be required to determine whether use of the robotic system for AGC truly attenuates pancreatic fistula, possibly leading to improvement in long-term outcomes[70].

Since the beginning of October, 2014, we have been conducting a multi-institutional single-arm prospective study (UMIN000015388), which Japanese Ministery of Health, Labor, and Welfare has recently approved for Advanced Medical Technology ("senshiniryo")[70]. This study was designed to determine the impact of the use of the robot, for minimally invasive radical gastrectomy to treat resectable GC, on short-term outcomes, mainly focusing on postoperative complications, as well as long-term outcomes and cost. The specific hypothesis of this study was that the use of the robot in patients with cStage Ⅰ or Ⅱ diseases reduces the morbidity (Clavien-Dindo Classification Grade ≥ Ⅲ[73]) of 6.4% in conventional LG down to 3.2%. The sufficient sample size was calculated to be 330. All the patients will be registered in 2 years after starting this study and followed up for 3 years, thus the expected study period should be 5 years in total. Interim analyses will be done once the initial 220 cases are registered. As of January 31, 2016, 122 patients from 5 institutions have been registered.

ESOPHAGEAL CANCER

History

Since 1992, when Cuschieri *et al*[74] first reported on VATS-E, many groups have described various methods[75-79]. In Japan, Akaishi *et al*[75] first reported on thoracoscopic total esophagectomy with en bloc mediastinal lymphadenectomy in 1996. Kawahara *et al*[76] demonstrated the details of VATS-E with extended lymphadenectomy in 1999, and Osugi *et al*[80] clarified the long-term outcomes of VATS-E. We performed prone VATS-E with CO2 insufflation in 2006 for the first time in our country[61].

Indication

The indication for VATS-E is relatively wider than that for LG[10]. VATS-E has currently been applied up to locally advanced EC even after neoadjuvant chemoradiotherapy[9,10]. Only some conditions including T4 tumor, severe intrathoracic adhesion, and one-lung ventilation failure are considered to be excluded from the indication for VATS-E[10,77,81].

Left lateral decubitus vs prone position

Regarding the patient positions used for VATS-E, similar to right transthoracic open esophagectomy (OE), the left lateral decubitus position had been mostly used[75,82]. However, the prone position has increasingly been used recently[74,83-85]. Prone VATS-E is characterized by operating surgeon-friendly sense of use brought by more ergonomic set up as well as a drier operative field given by gravity in combination with the positive intrathoracic pressure generated by CO_2 insufflation[61]. To enjoy these advantages of the prone position as well as those of laparoscopic horizontal magnified view with overcoming the laparoscopic limited range of motion, we fully mobilize the "meso-oesophagus"[86] from lower up to upper mediastinum prior to the LN dissection of station 106 recR, 106 recL+tbL, and 112 (Japanese Classification of Esophageal Cancer, the 11th ed[87]) using the 6-trocar system in the hemi-prone position (Figure 5).

Outcomes

To date, a number of single-institution studies have demonstrated acceptable short-term outcomes of VATS-E for thoracic EC regarding operative time, blood loss and postoperative complications, which are comparable with those of conventional OE[13,81]. According to a meta-analysis based on these small case-control studies, VATS-E reduced blood loss, total morbidity and respiratory complications, leading to shorter intensive care unit and hospital stay in comparison with OE[88-90]. In terms of long-term outcomes, a limited number of case-control studies have demonstrated the comparable results with conventional OE[80,89,91,92]. Therefore, to determine the feasibility and beneficial effects of VATS-E, multicenter prospective RCTs are warranted.

ECOG2202: The Eastern Cooperative Oncology Group (ECOG) performed the first prospective phase Ⅱ multicenter trial (ECOG2202[93]) to assess the feasibility of VATS-E. A total of 110 patients were enrolled at 17 credentialed sites. The primary endpoint was 30-d mortality. 30-d and perioperative mortality was 2.1% and 2.9%, respectively. Grade 3 or 4 (CTCAE v3.0[27]) adverse effects occurred in 49.5% of the eligible 105 patients. Estimated 3-year overall survival was 58.4% (95%CI: 47.7%-67.6%). These data suggested that VATS-E was feasible and safe with acceptable perioperative and oncological outcomes.

Traditional Invasive *vs* Minimally Invasive Esophagectomy trial: Traditional Invasive *vs* Minimally Invasive Esophagectomy (TIME) trial is the first multicenter RCT comparing short-term outcomes of prone VATS-E and those of OE, which was conducted by a study group in Europe[77,94]. In this study, 56 and 59 patients were randomly assigned to the OE and the VATS-E group, respectively. As a result, VATS-E reduced, intraoperative blood loss, postoperative pain, postoperative pulmonary infection, and vocal cord palsy, leading to reduced hospital stay and improved postoperative QoL. No siginificant difference was observed in mortality and the number of

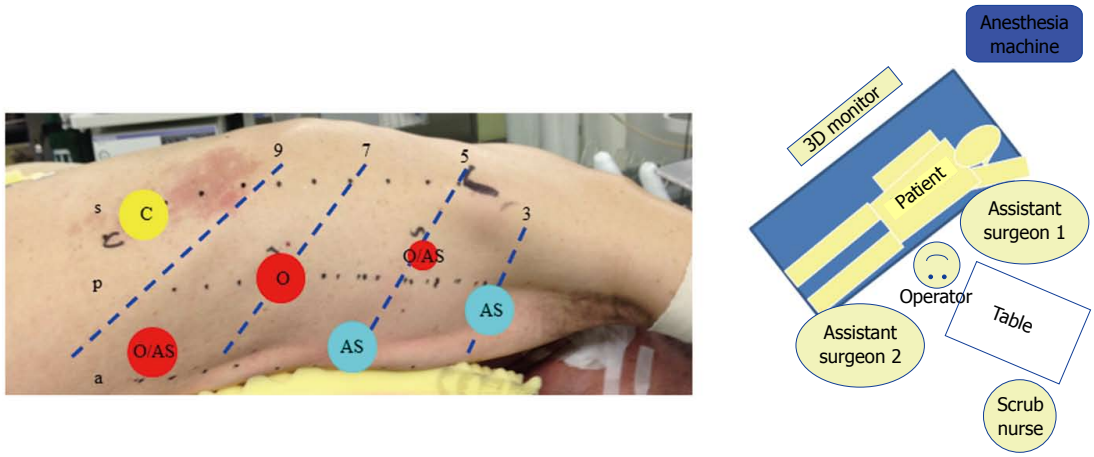

Figure 5 Setup for prone VATS-E at Fujita Health University. A: The patient in the hemi-prone position using the six-trocar system. 12 mm trocars are used except for the trocar in the 5th intercostal space (ICS) behind the posterior axillary line; B: OR setup. s: Scapula angle line; p: Posterior axillary line; a: Anterior axillary line; 3: 3rd ICS; 5: 5th ICS; 7: 7th ICS; 9: 9th ICS; O: Operating surgeon; AS: Assistant surgeon.

dissected lymph nodes. These findings suggested the advantages of VATS-E over OE in terms of short-term outcomes.

Robotic esophagectomy

The robotic esophagectomy has been less commonly performed than robotic gastrectomy. Thus, the impact of the use of D*VSS* on esophagectomy has been assessed mostly in case-series with small sample size[61,95-109]. Various groups have reported on feasibility and safety with good short-term outcomes in a wide-range of approaches to esophagectomy[110]. Van der Sluis *et al*[111] have reported sufficient oncological long-term outcomes of robotic esophagectomy for advanced esophageal cancer (5-year overall survival, 42%). Hernandez *et al*[100] demonstrated that the learning curve for a robotic-assisted procedure appears to begin near proficiency after 20 cases for surgeons experienced in conventional minimally invasive approach. Further studies are warranted to determine advantages and disadvantages of robotic esophagectomy.

Since 2009, we have performed robotic radical esophagectomy in the prone position for more than 40 patients. Then, compared to conventional MIS, robotic approach significantly reduced incidence of vocal cord palsy and hoarseness, suggesting that the use of the robot, which promotes more accurate recurrent laryngeal nerve identification and dissection[95], should reduce the chances of recurrent laryngeal nerve injury, resulting in preserved laryngopharyngeal function[61].

DISCUSSION

Although MIS for upper GI cancer has consistently appeared to improve short-term outcomes and at least preserve long-term outcomes, solid evidences that verify feasibility of MIS and even superiority to open surgery have still been lacking. Advantages and disadvantages of LDG for EGC and AGC over ODG will soon be clarified after the ongoing multicenter RCTs are concluded, however, those of LTG and VATS-E will have been unclear for the time being.

One of the principal reasons why surgeons have been attracted to MIS must be the laparoscopically enhanced anatomy provided by the magnified vivid image with high definition in combination with the horizontal view. To fully utilize these advantages of MIS, the disadvantages of MIS including limited range of motion has to be overcome. We believe one of the solutions may be the laparoscopic manipulation in the caudocranial and/or mediolateral manner, and another may be the use of the surgical robot as indicated in our previous reports[46,50,53,55,61].

We wish to further develop MIS for advanced cancer and that requiring advanced skills by actively utilizing novel technologies including the surgical robot, based on the principles and methods grown through conventional MIS and open surgeries.

In conclusion, technical advancements and development of endoscopic instruments will continue to evolve MIS for upper GI cancer. The outcomes should be validated in a scientific fashion.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Recent updates of precision therapy for gastric cancer: Towards optimal tailored management

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Abstract

Signaling pathways of gastric carcinogenesis and gastric cancer progression are being avidly studied to seek optimal treatment of gastric cancer. Among them, hepatocyte growth factor (HGF)/c-MET, phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathways have been widely investigated. Their aberrant expression or mutation has been significantly associated with advanced stage or poor prognosis of gastric cancer. Recently, aberrations of immune checkpoints including programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) have been suggested as an important step in the formation of a microenvironment favorable for gastric cancer. Accomplishments in basic research have led to the development of novel agents targeting these signaling pathways. However, phase Ⅲ studies of selective anti-HGF/c-MET antibodies and mTOR inhibitor failed to show significant benefits in terms of overall survival and progression-free survival. Few agents directly targeting STAT3 have been developed. However, this target is still critical issue in terms of chemoresistance, and SH2-containing protein tyrosine phosphatase 1 might be a significant link to effectively inhibit STAT3 activity. Inhibition of PD-1/PD-L1 showed durable efficacy in phase Ⅰ studies, and phase Ⅲ evaluation is warranted. Therapeutic strategy to concurrently inhibit multiple tyrosine kinases is a reasonable option, however, lapatinib needs to be further evaluated to identify good responders. Regorafenib has shown promising effectiveness in prolonging progression-free survival in a phase Ⅱ study. In this topic highlight, we review the biologic roles and outcomes of clinical studies targeting these signaling pathways.

Key words: Gastric cancer; Hepatocyte growth factor; Mammalian target of rapamycin; Signal transducer and activator of transcription 3; Programmed cell death ligand-1

Core tip: Among various cellular signaling pathways, hepatocyte growth factor/c-MET, phosphoinositide 3-kinase/Akt/mammalian target of rapamycin and janus kinase 2/signal transducer and activator of transcription 3 pathways are reportedly important in gastric carcinogenesis and metastasis. Aberrations of immune checkpoints have been vigorously investigated. However, clinical results of their target agents have not always matched the theoretical expectations of efficacy. In this review, we summarize the biologic impacts of the aforementioned signaling pathways, and their recent clinical outcomes including those of multiple kinase inhibitors in gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth common malignant tumor worldwide, and the second most common cause of cancer-related mortality[1]. The progress in therapeutic approaches has allowed complete remission of early gastric cancer by surgical or even endoscopic resection of tumors. However, if gastric cancer is advanced when diagnosed, the prognosis is generally poor and survival time is short even after surgical complete resection. Therefore, highly selective and effective chemotherapy remains an important issue for appropriate management of advanced gastric cancer.

A recent notable study provided a comprehensive molecular evaluation of primary gastric adenocarcinoma tissues as part of The Cancer Genome Atlas project[2]. The authors proposed four subtypes of gastric cancer according to the molecular characteristics: Epstein-Barr virus positive tumors, microsatellite instability tumors, genomically stable tumors and chromosomal unstable tumors. This study is a prime example of efforts to develop optimal classification of gastric cancer by analyzing common dysregulated pathways and to provide distinct tailored therapy for individual patients. Since the report of significant clinical benefits of trastuzumab in human epidermal growth factor receptor 2 (HER2)-positive gastric and esophagogastric junction (EGJ) adenocarcinoma[3], various targets have been investigated in the treatment of advanced gastric cancer. These targets include epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), hepatocyte growth factor(HGF)/c-MET and mammalian target of rapamycin (mTOR)[4]. However, we still have a long way to go before complete conquest of gastric cancer.

In this topic highlights, we aimed to review the biologic roles of several molecular signaling pathways on the basis of recent trials of targeted therapies in advanced gastric cancer. These include the HGF/c-MET, phosphoinositide 3-kinase (PI3K)/Akt/mTOR, janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) and programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) pathways. In the latter part, we focus on clinical outcomes of newly developed agents targeting the aforementioned pathways and summarize the findings of some clinical studies of multi-kinase inhibitors (MKIs), which can simultaneously multiple receptor tyrosine kinases (RTKs) in advanced gastric cancer.

CELLULAR SIGNALING PATHWAYS OF GASTRIC CANCER

HGF/c-MET pathway

c-MET is a heterodimeric subfamily of RTK. c-MET is composed of an α-chain, which possesses only an extracellular domain, and a β-chain composed of extracellular, transmembrane and intracellular domains[5]. The ligand of c-MET, HGF, is converted into an active form that causes dimerization and activation of the c-MET receptor. The activated HGF/c-MET signal leads to autophosphorylation of multiple tyrosine residues of the intracellular region of c-MET, such as Y1230, Y1234, Y1235, Y1349 and Y1356, which form multi-functional docking sites to recruit several intracellular adaptor proteins[6]. Among them, Grb2-associated binder 1 (GAB1) can directly bind to c-MET or forms a complex with growth factor-bound protein 2 (GRB2) to indirectly interact with c-MET. The c-MET association recruits several main adaptor proteins including STAT3 and PI3K, which in turn activate downstream biologic effects including cellular proliferation, migration/invasion and induction of epithelial-mesenchymal transition (EMT) (Figure 1)[7].

Clinical impact of the HGF/c-MET pathway in gastric cancer has been well documented. High protein expression rate of c-MET in gastric carcinoma tissue has been demonstrated by immunohistochemistry (IHC; 43%-82%)[8,9] and by gene amplification rate (2%-10%)[9-12]. In both approaches, the elevated expression of c-MET has been positively associated with advanced tumor stage and poor survival rate. Consequently, multiple agents targeting the HGF/c-MET signaling pathway are being evaluated. Tivantinib, an anti-c-MET tyrosine kinase inhibitor (TKI), used in combination with the EGFR TKI, erlotinib, has extended progression-free survival (PFS) in patients

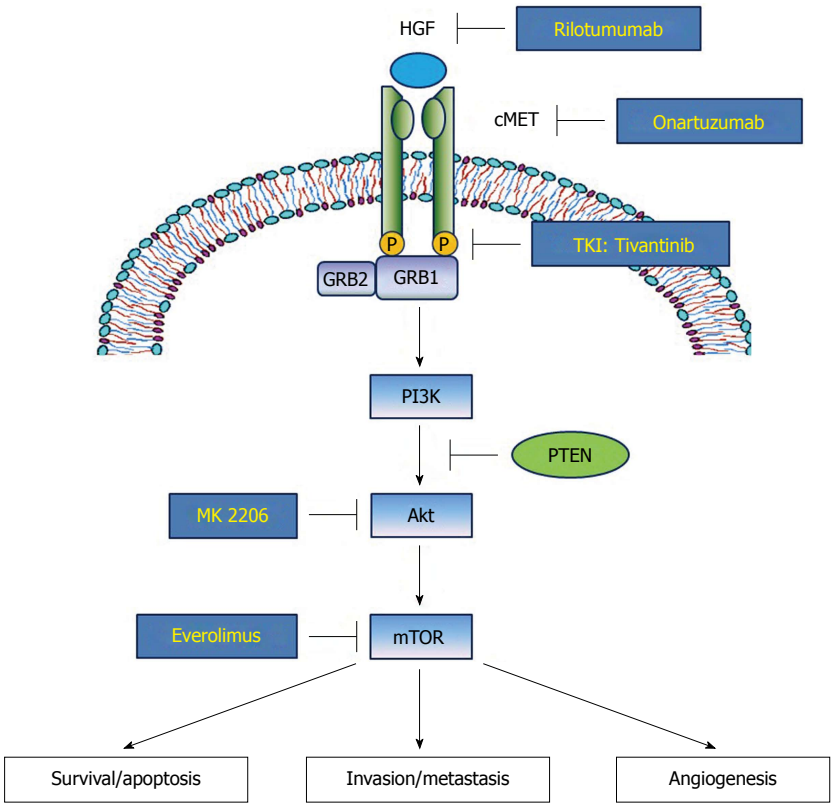

Figure 1 Hepatocyte growth factor/mesenchymal epithelial transition factor and phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway, and their inhibitors evaluated in gastric cancer patients. HGF: Hepatocyte growth factor; cMET: Mesenchymal epithelial transition factor; GAB1: Grb2-associated binder 1; GRB2: Growth factor-bound protein 2; PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase and tensin homolog; mTOR: Mammalian target of rapamycin.

with locally advanced or metastatic non-squamous non-small-cell lung cancer in a phase Ⅲ trial[13]. In gastric cancer, phase Ⅲ studies for rilotumumab, an anti-HGF monoclonal antibody, and onartuzumab, an anti-c-MET monoclonal antibody, have been completed, and clinical outcomes of tivantinib were recently reported[14].

PI3K/Akt/mTOR pathway

The PI3K-Akt-mTOR pathway plays a pivotal role in oncogenesis and progression including cell growth, survival, invasion/metastasis and angiogenesis, and gastric cancer is no exception. PI3K is usually activated through binding and stimulation of various RTKs by growth factors including HGF and c-MET. Activated PI3K subsequently phosophorylates and activates phosphatidylinositol 3,4-biphosphate (PIP2), phosphatidylinositol 3,4,5-triphosphate (PIP3), phosphoinositide-dependent protein kinase 1 and Akt[15]. Akt, which is also termed protein kinase B, is a major effector protein of the PI3K pathway. Phosphorylated Akt (p-Akt) modulates various biologic functions like cell survival, migration/invasion and angiogenesis through downstream adaptor molecules including mTOR (Figure 1)[16].

Genetic alteration of biological signals involving the PI3K/Akt/mTOR pathway has been frequently detected in gastric carcinoma. For example, a point mutation of *PIK3CA* encoding p110 (a class IA subunit of PI3K) is often observed in gastric carcinoma tissues, ranging from 4.3%-25%[17-21], with the point mutation mostly seen in exon 9 and exon 20[17]. Their mutation or gene amplification is positively associated with the T stage of gastric cancer[20,22]. In contrast, *PTEN*, which encodes phosphatase and tensin homolog and inactivates Akt by converting PIP3 to PIP2, is deleted in 4%-23% of gastric cancers[21,23,24] and loss of heterozygosity (LOH) is observed in 17%-47% cases of gastric cancer. LOH of PTEN is significantly associated with p-Akt level in gastric carcinoma tissues, TNM stage and poor prognosis of survival[25-29]. Activated Akt signaling promotes mTOR protein complexes 1 and 2 (mTORC1 and mTORC2), which can play pivotal roles in cancer cell migration and metastasis. Prevalence of mTOR expression is reported as approximately 50% in gastric cancer tissues, and is negatively associated with PTEN expression[30,31].

Clinical and laboratory evidence indicates the promising potential of targeting the PI3K-Akt-mTOR signaling pathway for efficacious treatment of gastric cancer, and various kinds of inhibitors or antibodies acting on this pathway have been developed and tried. These inhibitors are classified into several categories that include PI3K inhibitors, dual mTOR1/mTOR2 inhibitors, Akt inhibitors, mTOR1 inhibitors and dual PI3K/mTOR inhibitors[15]. Among them, a phase Ⅰ study

of isoform specific PI3K inhibitor (p110α) BYL719 is ongoing (NCT01613950)[32], and clinical outcomes of Akt inhibitor MK 2206 and mTOR1 inhibitor everolimus and rapamycin were previously reported, and are dealt with more fully in the latter part of this review.

JAK2/STAT3 pathway and inhibitory role of SH2-containing protein tyrosine phosphatase 1

The most established stimulator of STAT3 signaling pathway is the interleukin (IL)-6 family that includes IL-6, IL-11 and leukemia inhibitory factor, which bind to their receptors, and then phosphorylate and activate JAK2. Activated JAK2 recruits and activates STAT3 by phosphorylation, which can dimerize and translocate into the nucleus to act as a transcription factor and up-regulate various target genes involving cellular proliferation, migration/invasion and angiogenesis[33]. Indeed, persistent constitutive activation of JAK2/STAT3 in cancer cells is closely associated with gastric carcinogenesis and poor prognosis[34]. Besides this classic effect of JAK2/STAT3 pathway in cancer development, another pivotal role of STAT3 protein is the tumor microenvironment, where immune cells can be recruited and STAT3 can mediate various interactions with cancer cells to generate tumor progression. In gastric cancer, *Helicobacter pylori* (*H. pylori*)-induced cytotoxin-associated antigen (CagA) is closely associated with STAT3 activity in both gastric epithelial cells and mucosal immune cells. For example, *H. pylori* infection and CagA secretion can lead to IL-23 release from dendritic cells, which binds to their receptor and activates JAK2/STAT3 transmembrane signaling of naïve $CD4^+$ T-cells, and causes differentiation of T-helper (Th)-17 specific lineages to release associated cytokines including IL-17[35]. Up-regulated IL-17 can promote pro-inflammatory and oncogenic environment. Expression level of IL-17 is positively correlated with depth of tumor, lymphovascular invasion and lymph node involvement in gastric cancer tissues[36,37], and IL-17 mediates angiogenesis *via* up-regulation of VEGF *in vivo* and *in vitro*[38]. In gastric epithelial cells, CagA is translocated *via* the type-Ⅳ secretion system and releases IL-11. The released IL-11 bind to their receptor and activate the JAK2/STAT3 cascade[39]. Activated STAT3 functions as a transcription factor to induce many target genes involved in proliferation, invasion/metastasis and angiogenesis including cyclin D1, surviving, matrix metalloproteinase-9, CD44v6 and VEGF[34,40].

Thus, a therapeutic strategy to target the STAT3 signaling pathway appears to be reasonable. Routes of inhibition include blockade of JAK activation by dephosphorylation, inhibition of STAT3 phosphorylation, dimerization or gene transcription[35]. In terms of dephosphorylation, several phosphatases have been reported to be associated with STAT3 activity. Among them, SH2-containing protein tyrosine phosphatase 1 (SHP1) may be crucial in the down-regulation of the JAK2/STAT3 pathway by dephosphorylation[41-43]. Several candidate agents including natural compounds were reported to induce SHP1 and inhibit STAT3 activity. Sorafenib and its synthetic analogues also can act as a SHP1 agonist to inhibit phosphor-STAT3 activity and show various anti-cancer effects, such as promotion of apoptosis, overcoming of radio- or chemo-resistance and inhibition of EMT or fibrosis on hepatocellular carcinoma cell lines[44-51]. However, the exact inhibitory role of SHP1 in gastric cancer development and progress is unknown. We recently showed that expression of SHP1 is reduced or ameliorated in various gastric cancer cell lines due to epigenetic silencing, and that reinforced SHP1 expression significantly inhibits cellular proliferation, migration/invasion and induce apoptosis[52]. SHP1 might be a promising target to effectively inhibit JAK2/STAT3 activity in gastric cancer cells (Figure 2).

Immune checkpoints

Immune checkpoints regarding tumor infiltrating lymphocytes and immune evasion mechanism associated with carcinogenesis have been studied in the search for alternative therapeutic targets. Among them, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1, which are minimally expressed on the surface of resting T-lymphocytes but are widely expressed on activated T-lymphocytes, have been intensively studied for gastric carcinogenesis, and anti-PD-1 antibodies are already in clinical trials of gastric cancer chemotherapy[53]. Ligands for PD-1 (PD-L1) and CTLA-4 (B7-1/B7-2), which are expressed on the surface of tumor cells, bind to PD-1 and CTLA-4 respectively, inhibit pivotal function of effector T-cells for immune surveillance and consequently promote the growth of gastric cancer cells (Figure 3)[54].

PD-1 expression differs between gastric cancer tissues and non-cancerous tissues, with the significantly up-regulated PD-1 level in gastric cancer tissues being significantly correlated with poor clinical parameters including increased tumor size, advanced stage, metastasis and patient survival[55-58]. Furthermore, PD-1 expression on $CD4^+$ and $CD8^+$ T cells from gastric cancer tissues is higher than non-cancer tissues or peripheral blood mononuclear cells from normal subjects, and is significantly associated with disease progression[59]. However, a recent Korean study demonstrated that expression rate of PD-L1 on gastric cancer tissues was 43.6%, and was related to less advanced stage, intestinal type, well/moderately differentiated adenocarcinoma rather than poor differentiation and better overall survival (OS) and disease-free survival[60]. A recent Chinese study investigated PD-L1 expression level using large number of gastric cancer tissues (almost 400 specimens); PD-L1 expression was significantly associated with TIL density, and moderate to high TIL density was closely correlated with better prognosis[61]. Thus, the

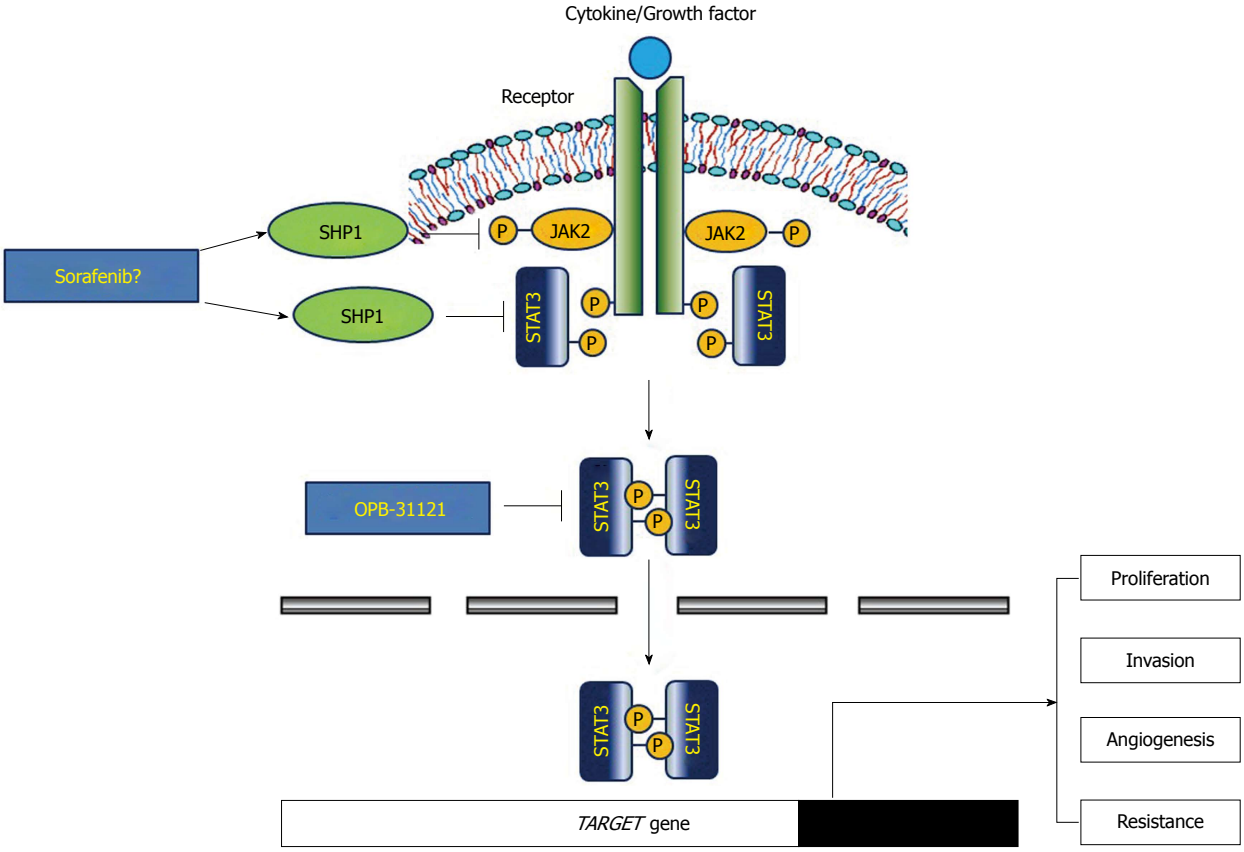

Figure 2 Janus kinase 2/signal transducer and activator of transcription 3 pathway and inhibitory role of SH2-containing protein tyrosine phosphatase 1. JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; SHP1: SH2-containing protein tyrosine phosphatase 1.

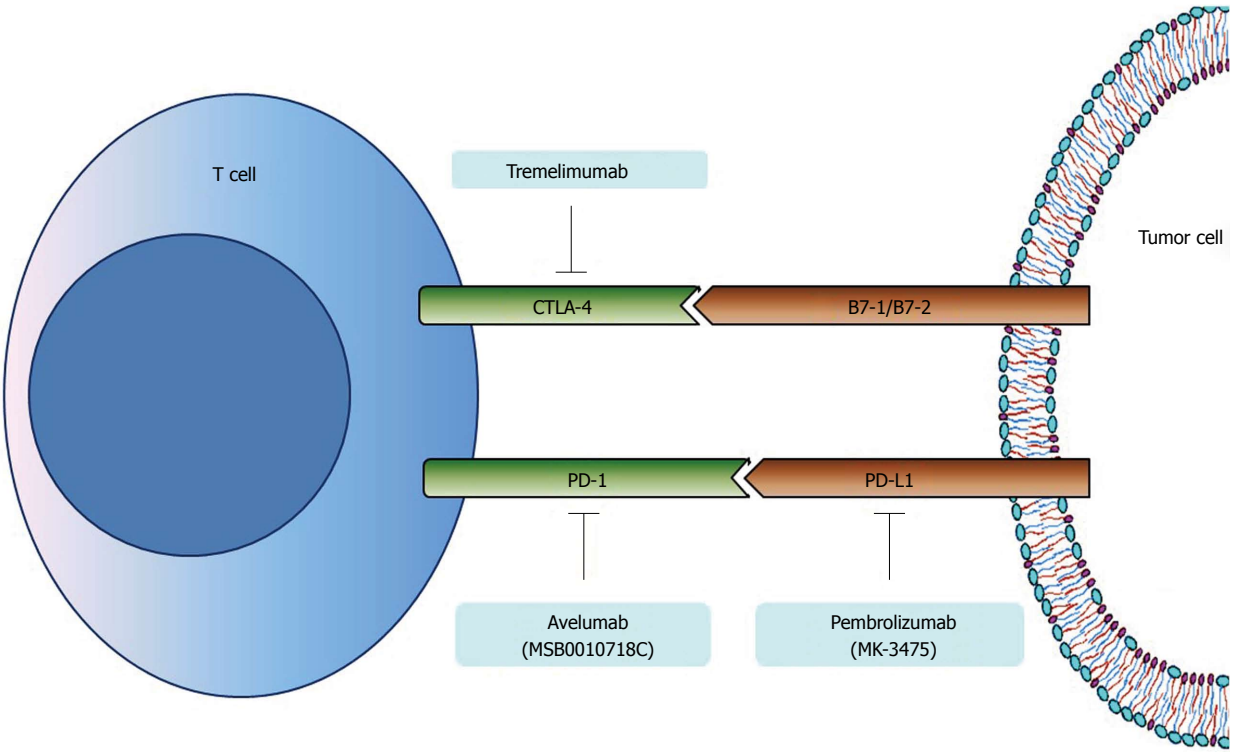

Figure 3 Immune checkpoints on tumor cell and T-cell, and their monoclonal antibodies evaluated in gastric cancer patients. CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; PD-1: Programmed cell death-1; PD-L1: Programmed cell death ligand-1.

Table 1 Clinical outcomes of recent trials of targeted therapy in advanced gastric and esophagogastric junction adenocarcinoma

Author and trial	Line of treatment	Phase of study	*n*	Treatment arms	Outcomes
Anti-HGF/cMET antibodies					
Cunningham *et al*[63], RILOMET-1 (2015)	First	Ⅲ	609	ECX + rilotumumab *vs* ECX + placebo	OS: 9.6 mo *vs* 11.5 mo (HR = 1.37, *P* = 0.016) PFS: 5.7 mo *vs* 5.7 mo (HR = 1.30, *P* = 0.016) ORR: 30% *vs* 39.2% (OR 0.67, *P* = 0.027)
Shah *et al*[65], METGastric (2015)	First	Ⅲ	562	mFOLFOX + ornatuzumab *vs* mFOLFOX + placebo	OS: 11.0 mo *vs* 11.3 mo (HR = 0.82, *P* = 0.244) PFS: 6.7 mo *vs* 6.8 mo (HR = 0.90, *P* = 0.429) ORR: 46% *vs* 41% (*P* = 0.253)
Malka *et al*[66], PRODIGE 17 ACCORD 20 MEGA (2015)	First	Ⅱ	162	mFOLFOX alone *vs* mFOLFOX + panitumumab *vs* mFOLFOX + rilobumumab	4-mo PFS rate: 71 *vs* 63 *vs* 63% PFS: 5.8 mo *vs* 5.2 mo *vs* 7.6 mo ORR: 54% *vs* 44% *vs* 50%
Akt/mTOR inhibitors					
Hudis *et al*[68] (2013)	Second/third	Ⅰ	34	Trastuzumab + Akt inhibitor (MK-2206)	RR (including stable disease): 24% Time to progression: 72 d
Ohtsu *et al*[70] GRANITE (2013)	Second/third	Ⅲ	646	Everolimus *vs* BSC	OS: 5.4 mo *vs* 4.3 mo (HR = 0.90, *P* = 0.124) PFS: 1.7 mo *vs* 1.4 mo (HR = 0.66, *P* < 0.001) ORR: 4.5% *vs* 2.1%; DCR: 43.3% *vs* 22.0%
Shen *et al*[71] (2014)	First	Ⅱ	40	Everolimus + cisplatin + HDFL	OS: 10.5 mo (95%CI: 8.6-12.3) PFS: 6.9 mo (95%CI: 4.9-8.4)
STAT3 inhibitor					
Oh *et al*[74] (2015)	Second/third	Ⅰ	25	STAT3 inhibitor (OPB-31121)	RR (including stable disease): 44.4%
Immune checkpoints inhibitors					
Ralph *et al*[89] (2010)	Second	Ⅱ	18	Tremelimumab	OS: 4.8 mo (95%CI: 4.06-5.59) 12 mo OS rate: 33% (95%CI: 14-54) RR (including stable disease): 27.8%
Bang *et al*[90], KEYNOTE-012 (2015)	Second/third	Ⅰ	39	Pembrolizumab (MK-3475)	OS: 11.4 mo; PFS 1.9 mo ORR: 22% (95%CI: 10-39)
Yamada *et al*[93] (2015)	Second/third	Ⅰ	20	Avelumab (MS0010718C)	PFS: 11.9 wk (95%CI: 6.0-12.3) ORR: 15.0% (95%CI: 3.2-37.9)
Multikinase inhibitors					
Sun *et al*[95] (2010)	First	Ⅱ	44	Sorafenib + docetaxel + cisplatin	OS: 13.6 mo (90%CI: 8.6-16.1) PFS: 5.8 mo (90%CI: 5.4-7.4) PR: 41% (90%CI: 28-54)
Martin-Richard *et al*[96], GERCAD (2013)	First	Ⅱ	40	Sorafenib + oxaliplatin	OS: 6.5 mo (95%CI: 5.2-9.6) PFS: 3 mo (95%CI: 2.3-4.1) RR (including stable disease): 50.0%
Hecht *et al*[100], LOGiC (2015)	First	Ⅲ	487	CapeOx + lapatinib *vs* CapeOx + placebo	OS: 12.2 mo *vs* 10.5 mo (HR = 0.91, *P* = 0.349) PFS: 6.0 mo *vs* 5.4 mo (HR = 0.82, *P* = 0.0381) ORR: 53% *vs* 39% (*P* = 0.0031)
Satoh *et al*[101], TyTAN (2014)	Second	Ⅲ	261	Lapatinib + paclitaxel *vs* Paclitaxel alone	OS: 11.0 mo *vs* 8.9 mo (HR = 0.84, *P* = 0.1044) PFS: 5.4 mo *vs* 4.4 mo (HR = 0.85, *P* = 0.2441) ORR: 27% *vs* 9% (*P* < 0.001)
Pavlakis *et al*[103], INTEGRATE (2015)	Second/third	Ⅱ	147	Regorafenib *vs* placebo	OS: 5.8 mo *vs* 4.5 mo (HR = 0.74, *P* = 0.11) PFS: 2.6 mo *vs* 0.9 mo (HR = 0.40, *P* < 0.0001) RR (including stable disease): 44% *vs* 16%
Lee *et al*[106] (2015)	First	Ⅱ	66	CapeOx + pazopanib	PFS: 6.5 mo; OS: 10.5 mo; ORR: 57.6%

ECX: Epirubicin/Cisplatin/Capecitabine; mFOLFOX: 5-fluorouracil/leukovorin/oxaliplatin; OS: Overall survival; HR: Hazard ratio; PFS: Progression free survival; ORR: Objective response rate; BSC: Best supportive care; DCR: Disease control rate; HDFL: High-dose 5-fluorouracil/leucovorin; CapeOx: Capecitabine/oxaliplatin.

exact relationship between PD-1/PD-L1 expression and clinical parameters needs to be further evaluated.

RECENT TRIALS OF TARGET THERAPY FOR GASTRIC CANCER

The pivotal ToGA study of targeted therapy for the treatment of unresectable gastric/EGJ cancer investigated the synergistic effects of trastuzumab, a monoclonal anti-HER2 antibody, with capecitabine/cisplatin or fluorouracil/cisplatin regimen. OS and PFS were significantly prolonged[3]. Since then, various target agents have been tried for the optimal treatment of advanced gastric cancer. Among them, newly developed drugs targeting the HGF/c-MET, PI3K/Akt/mTOR, JAK2/STAT3 and PD-1/PD-L1 pathways are dealt with here, and MKIs that simultaneously target multiple tyrosine kinases is also introduced in this section. A portion of these studies were presented at the 2015 annual meeting of the American Society of Clinical Oncology (ASCO); their outcomes are summarized in Table 1.

Phase Ⅲ studies of anti-HGF/c-MET antibodies

Rilotumumab, an anti-HGF monoclonal antibody, and onartuzumab, an anti-c-MET monoclonal antibody,

have been tried as first-line treatments for gastric or EGJ adenocarcinoma in phase Ⅲ studies. Their clinical outcomes were presented at the ASCO 2015 meeting. Rilotumumab significantly increased PFS when combined with ECX (epirubicin/cisplatin/capecitabine) regimen in a phase Ⅰb/Ⅱ study[62]. From this background, the phase Ⅲ RILOMET-1 study of first-line therapy of MET-positive, HER2-negative gastric/EGJ cancer compared rilotumumab (15 mg/kg) plus ECX with placebo plus ECX was performed. OS (9.6 mo *vs* 11.5 mo, HR = 1.37, *P* = 0.021) and objective response rate (ORR: 30.0% *vs* 39.2%, OR = 0.67, *P* = 0.027) were significantly inferior in the rilotumumab group, and subgroup analysis also showed that no subgroups appeared to be benefit with rilotumumab arm regardless of the degree of MET positivity[63]. This result is contrary to that of a phase Ⅰ/Ⅱa study, which contained a larger number of Asian patients (18%) than the RILOMET-1 population (1%). The different racial distribution may have contributed to the opposite outcomes between the two studies. Thus, the phase Ⅲ RILOMET-2 study has been performed to investigate the efficacy of rilotumumab in combination with cisplatin/capecitabine regimen as the first line chemotherapy among Asian patients with unresectable gastric/EGJ cancer[64].

The METGastric study of onartuzumab (10 mg/kg) for the treatment of HER2-negative, MET-positive metastatic gastric or EGJ adenocarcinoma without prior treatment, used onartuzumab in combination with the 5-fluorouracil (5-FU)/leukovorin/oxaliplatin (FOLFOX) regimen and compared outcomes with FOLFOX alone[65]. The addition of onartuzumab to FOLFOX was ineffective in the intention-to-treat analysis and OS, PFS and ORR were not significantly different between the two groups. However, addition of onartuzumab showed a marginal effect in OS for the moderate-to-strong MET positive subgroup (9.7 mo *vs* 11.0 mo, HR = 0.64, *P* = 0.062). Grade 3 or 4 adverse events were more common in the onartuzumab arm. Furthermore, a French phase Ⅱ study that compared FOLFOX plus rilotumumab or panitumumab, an anti-EGFR antibody, with FOLFOX alone for first-line treatment of metastatic, HER2-negative gastric or EGJ adenocarcinoma showed that adding panitumumab or rilotumumab seemed more toxic and was not more effective than mFOLFOX6 alone[66]. Considering recent outcomes of phase Ⅱ/Ⅲ studies of rilotumumab and ornatuzumab, targeting HGF/c-MET in gastric cancer has little rationale for further evaluation. However, cMET still has potential for promising biomarkers considering that c-MET-positive gastric/EGJ cancers have strong association with shorter OS and poor prognosis[67], and future research needs to search for other significant predictive factors for response to anti-HGF/c-MET therapy.

Akt and mTOR inhibitors

A phase Ⅰ study evaluated the combinatory effect of MK-2206, a potent pan-Akt inhibitor, with trastuzumab for treatment of HER2-positive, refractory gastric carcinoma. The rational was that the PI3K/Akt pathway is a main downstream signaling pathway of HER2 and is closely related with trastuzumab resistance. Oral MKN-2206 was given either 135 mg every week or 60 mg every other day with trastuzumab 8 mg/kg intravenously on day 1 every 3 wk. Clinical benefit response rate including stable disease more than 4 mo was 24%, and median time to progression was 72 d[68].

The PI3K/Akt pathway might be successfully inhibited by targeting mTORC1 kinase, and the development of rapamycin analogs (*e.g*., everolimus, temsirolimus) have been promoted[15]. A multicenter phase Ⅱ study of everolimus, an oral inhibitor of mTOR, in patients with refractory metastatic gastric cancer showed a disease control rate of 56.0%, PFS of 2.7 mo (95%CI: 1.6-3.0 mo) and OS of 10.1 mo (95%CI: 6.5-12.1 mo), which warrant further phase Ⅲ evaluation[69]. However, results of the phase Ⅲ GRANITE-1 study comparing everolimus with best supportive care for previously treated advanced gastric cancer were disappointing, and researchers failed to demonstrated significant benefit in OS (5.4 mo *vs* 4.3 mo, HR = 0.90, *P* = 0.124); indeed, PFS was significantly increased in the everolimus arm (1.7 mo *vs* 1.4 mo, HR = 0.66, *P* < 0.001)[70]. A phase Ⅱ multicenter study of low dose everolimus (10 mg on days 1, 8 and 15) plus cisplatin and a weekly 24-h infusion of high-dose 5-FU and leucovorin (cisplatin 35 mg/m^2 intravenous infusion for 24 h on days 1 and 8, 5-FU 2000 mg/m^2 and leucovorin 300 mg/m^2 intravenous infusion for 24 h on days 1, 8 and 15) for treatment-naïve gastric cancer was conducted but failed to increase ORR as in a preplanned statistical assumption (52.5%)[71]. However, in one case everolimus was tried after failure of 1st and 2nd line chemotherapy for a young male metastatic gastric cancer patient with multiple liver metastases. A subsequent mutational analysis revealed a PIK3CA hotspot mutation and pS6 overexpression in the primary tumor. The patient achieved stable disease for 1 year and pS6 expression was nearly abolished after two cycles of everolimus treatment[72]. Furthermore, a phase Ⅱ study of everolimus for refractory metastatic gastric and EGJ adenocarcinoma showed that a subgroup with strong pS6 expression (≥ 2 + IHC staining) was significantly correlated with better PFS and disease control rate[73]. Therefore, subgroup analysis for finding of positive predictive biomarkers in patients treated with everolimus needs to be performed.

STAT3 inhibitors and effect of SHP1 inducers

Few agents capable of directly targeting STAT3 have been developed, and clinical trials of STAT3 inhibitors in the treatment of gastric cancer are lacking. A recent phase Ⅰ study reported that OPB-31121, an oral STAT3 inhibitor, showed an overall response rate

Table 2 Ongoing clinical trials of target therapy in advanced gastric and esophagogastric junction adenocarcinoma

Trial identifier	Line of treatment	Phase of study	Treatment arms	Primary endpoint
Akt/mTOR inhibitors				
NCT01613950[32]	Second/third	Ib	AUY922/BYL719	MTD
Immune checkpoints inhibitors				
NCT02335411 (KEYNOTE-059)[91]	Third	Ⅱ	Cohort 1: pembrolizumab monotherapy Cohort 2: pembrolizumab + 5-FU/cisplatin or capecitabine/cisplatin	ORR
NCT02370498 (KEYNOTE-061)[92]	Second	Ⅲ	Pembrolizumab *vs* paclitaxel	PFS, OS
Multikinase inhibitors				
NCT02015169[107]	Neoadjuvant	Ⅱ	XELOX + lapatinib	R0 resection rate
NCT01913639[108]	First	Ⅱ	FOLFOX + regorafenib	PFS

PFS: Progression free survival; OS: Overall survival; MTD: Maximum tolerated dose; 5-FU: 5-fluorouracin; ORR: Objective response rate; XELOX: Capecitabine/oxaliplatin; FOLFOX: 5-fluorouracil/leucovorin/oxaliplatin; mTOR: Mammalian target of rapamycin.

of 44.4% assessed as stable disease in advanced solid tumors including gastric cancer[74]. However, STAT3 not only up-regulates various target oncogenes associated with gastric carcinogenesis and metastasis, but is closely related with drug resistance of standard chemotherapeutic agents including 5-FU, cisplatin and adriamycin in gastric cancer[75-77]. In this regard, targeting of STAT3 remains a critical issue in the treatment of gastric cancer, and development of specific and effective inhibitors of STAT3 should be further investigated. Several natural compounds[76,78-80] and pharmacologic medicines, such as proton pump inhibitors[75,81], inhibit STAT3 activity in *in vitro* and *in vivo* studies of gastric cancer. These agents are expected to show a synergetic effect or enhance chemosensitivity when combined with standard chemotherapy agents.

Several natural compounds inhibit the STAT3 activation pathway through induction of SHP1 in hematopoietic cancer cell lines[42,43,82-85] and hepatoceullar carcinoma (HCC) cell lines[86,87]. We recently showed that plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinonoid constituent extracted from the roots of the medical plant *Plumbago zeylanica* L, suppresses STAT3 activity and consequently targets gene expression *via* induction of SHP1 in gastric cancer cells[88]. Because most gastric cancer cells showed reduced or lack of expression of SHP1, a therapeutic strategy to indirectly inhibit STAT3 pathway might be an alternative option in pharmacologic treatment of gastric cancer and SHP1 may play pivotal roles in this signaling pathway. As mentioned above, several MKIs, such as sorafenib and sunitinib, show a significant link between SHP1 and suppression of STAT3 activity in HCC cells.

Immune checkpoint inhibitors: anti-PD-1/PD-L1 antibodies

The anti-CTLA-4 monoclonal antibody tremelimumab was developed and a phase Ⅱ study was performed to evaluate its use in second-line chemotherapy in advanced gastric and esophageal adenocarcinoma. However, the results were disappointing and only one patient achieved partial response among 18 enrolled patients, and stable disease was observed only in four patients[89]. Concerning the PD-1/PD-L1 pathway, pembrolizumab (MK-3475), an anti-PD-1 monoclonal antibody, and avelumab (MS0010718C), an anti-PD-L1 monoclonal antibody, have been developed. Pembrolizumab was tried for rescue therapy of recurrent or metastatic gastric or EGJ adenocarcinoma, which were positive for PD-L1, in the KEYNOTE-012 study[90]. ORR by central review was 22.2%, PFS 1.9 mo and OS 11.4 mo, and pembrolizumab showed durable efficacy and manageable safety profile for the heavily pre-treated, PD-L1 positive population. Further studies to support the efficacy of pembrolizumab in advanced gastric cancer are now in progress. For example, KEYNOTE-059 (NCT02335411) is a phase Ⅱ study of pembrolizumab monotherapy or in combination with standard chemotherapy[91] and KEYNOTE-061 (NCT02370498) is a phase Ⅲ study to compare pembrolizumab monotherapy with paclitaxel as the second-line therapy[92] (Table 2).

In Japan, avelumab was tried for refractory stage Ⅳ gastric and EGJ adenocarcinoma. A dose of 10 mg/kg was administered intravenously every 2 wk until progression. Most of adverse events were grade 1 or 2, ORR was 15.0% and PFS was 11.9 wk. Additional studies to evaluate the efficacy of avelumab and biomarkers from tumor tissue and blood samples including PD-L1 expression need to be evaluated[93].

MKIs

RTKs play crucial roles in the development of proliferation, differentiation, migration/invasion and apoptosis in gastric cancer. Currently, various inhibitors targeting the tyrosine kinase motif have been developed, and some display concurrent inhibitory effects of multiple tyrosine kinases. One of the first generation MKIs was sorafenib, which can inhibit BRAF, VEGF receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR)[94]. A phase Ⅱ study investigated the efficacy of sorafenib in combination with docetaxel and cisplatin as the first-line chemotherapy in metastatic gastric or EGJ adenocarcinoma. Partial response was achieved in

41% (90%CI: 28%-54%), and the median PFS was 5.8 mo (90%CI: 5.4-7.4 mo) and median OS was 13.6 mo (90%CI: 8.6-16.1 mo). No additional toxicities were observed by adding sorafenib to docetaxel/cisplatin regimen. The results of this study warranted further evaluation of sorafenib in chemotherapy of gastric cancer[95]. However, another multicenter phase Ⅱ study of oxaliplatin and sorafenib as the second-line chemotherapy after failure of cisplatin/fluoropyrimidine regimen in advanced gastric adenocarcinoma revealed a median PFS of 3.0 mo (95%CI: 2.3-4.1 mo) and median OS of 6.5 mo (95%CI: 5.2-9.7 mo), which failed to support the implementation of a phase Ⅲ study[96]. Sorafenib was also evaluated for combination therapy with oral fluoropyrimidine and cisplatin, such as S-1/cisplatin[97] and capecitabine/cisplatin[98], in phase Ⅰ studies. Both studies showed tolerable safety profile and acceptable efficacy.

Lapatinib is a MKI that competitively inhibits ATP binding of tyrosine kinase in both HER2 and EGFR, and which is approved for the treatment of HER2-positive breast cancer[99]. Two large-scale, randomized, phase Ⅲ trials were recently reported. The researchers evaluated the efficacy and safety of lapatinib in HER2-positive, advanced or metastatic gastric and EGJ adenocarcinoma. The LOGiC study addressed lapatinib as the first-line chemotherapy in combination with capecitabine/oxaliplatin, and lapatinib arm was compared with capecitabine/oxaliplatin alone[100]. Median OS was not significant between both arms (12.2 mo *vs* 10.5 mo, P = 0.349), while PFS was significantly longer (6.0 mo *vs* 5.3 mo, P = 0.0381) and ORR was higher (53% *vs* 39%, P = 0.0031) in the lapatinib arm. Subgroup analysis for OS revealed that Asians and younger patients (< 60 years) showed significant benefit. The TyTAN study compared lapatinib plus paclitaxel with paclitaxel alone in the second-line treatment of gastric cancer in an Asian population[101]. This study showed no significant difference of median OS and PFS between both arms (11.0 mo *vs* 8.9 mo, P = 0.1044; 5.4 mo *vs* 4.4 mo, P = 0.2441; respectively). However, better efficacy was observed in the lapatinib arm in HER2-3+ subgroup. Further studies are warranted to examine the factors predicting good responders to lapatinib therapy.

Several novel MKIs have been investigated for the treatment of refractory gastric cancer. Findings were presented at the ASCO 2015 meeting. Among them, regorafenib, which inhibits multiple tyrosine kinases related to angiogenesis (VEGFR1-3), tumor microenvironment [PDGFR-β, fibroblast growth factor receptor (FGFR)] and oncogenesis (KIT), was previously developed and reported as effective in colon cancer and gastrointestinal stromal tumors (GISTs)[102]. The phase Ⅱ INTEGRATE study was designed and performed to investigate the efficacy of regorafenib in refractory, metastatic gastric and EGJ adenocarcinoma by comparing regorafenib 160 mg/d with placebo[103]. PFS was significantly increased in regrafenib group (2.6 mo *vs* 0.9 mo, HR = 0.40, P < 0.0001), however, OS was not significantly different between two groups (5.8 mo *vs* 4.5 mo, HR = 0.74, P = 0.11). An interesting thing is that HR = for PFS was significantly lower in Korean patients than in Western patients from Canada and Australia, which indicates that regorafenib might be more effective in Asian patients. Pazopanib is another potent MKI of VEGFR1-3, PDGFRα/β and FGFR1/3, and was previously approved by the United States Food and Drug Administration for the treatment of patients with advanced renal cell carcinoma[104]. A phase Ⅱ study was performed and reported the combinatory effect of pazopanib with capecitabine/oxaliplatin regimen as the first-line chemotherapy in metastatic gastric and EGJ cancer. ORR was 57.6% and adverse events of grade 3-4 were neutropenia (15.1%), anemia and thrombocytopenia (both 10.6%)[105].

CONCLUSION

Many studies have focused on revealing biologic relevant mechanism of development and progression of gastric cancer, and many medical agents targeting these pathways have been validated in clinical trials. However, most of them failed to reach significant benefits in phase Ⅲ trials, and novel therapeutic strategies are necessary in the future. To achieve this goal, individualized and precise target therapy should be planned on the basis of exploration of biologic characteristics of individual gastric cancer patients. In addition, targeting multiple RTKs rather than focusing on single pathway and attempts to overcome chemoresistance and enhance synergism with standard chemotherapeutic agents are expected to be prevalent. These approaches will hopefully lead to a more effective treatment, perhaps even conquest, of gastric cancer.

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REVIEW

Auto immune hepatitis

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Abstract

To provide an update of the latest trends in epidemiology, clinical course, diagnostics, complications and treatment of auto immune hepatitis (AIH). A search of the MEDLINE database was performed using the search terms: "auto immune hepatitis", "clinical presentation", "symptoms", "signs", "diagnosis", "auto antibodies", "laboratory values", "serology", "histopathology", "histology", "genetics", "*HLA* genes", "*non-HLA* genes", "environment", "epidemiology", "prevalence", "incidence", "demographics", "complications", "HCC", "PBC", "PSC", "corticosteroid", "therapy", "treatment", "alternative treatment". English-language full-text articles and abstracts were considered. Articles included reviews, meta-analysis, prospective retrospective studies. No publication date restrictions were applied. AIH is an immune meditated progressive inflammatory liver disease that predominantly affects middle-aged females but may affect people of all ages. The clinical spectrum of AIH is wide, ranging from absent or mild symptoms to fulminant hepatic failure. The aetiology of AIH is still unknown, but is believed to occur as the consequence of an aberrant immune response towards an un-known trigger in a genetically susceptible host. In the absence of a gold standard, diagnosis is based on the combination of clinical, biochemical and histopathological criteria. Immunosuppressive treatment has been the cornerstone of treatment since the earliest description of the disease in 1950 by Waldenström. Such treatment is often successful at inducing remission and generally leads to normal life expectancy. Nevertheless, there remain significant areas of unmet aetiological a clinical needs including fundamental insight in disease pathogenesis, optimal therapy, duration of treatment and treatment alternatives in those patients unresponsive to standard treatment regimens.

Key words: Auto immune hepatitis; Diagnosis; Liver; Epidemiology; Treatment

Core tip: Autoimmune hepatitis (AIH) is a chronic inflammatory liver disorder of unknown aetiology, which can lead to hepatic failure and premature

death when untreated. In AIH there is no existence of a pathognomonic feature and therefore the diagnosis rests on a combination of immunological, biochemical, and histological features together with exclusion of other liver diseases. Due to large heterogeneity of the disease, AIH might be unrecognised. Immunosuppressive treatment has been the cornerstone of treatment. Such treatment is often successful at inducing remission. For most patients life long treatment is indicated. In patients in whom all treatments fail, liver transplantation remains a final option.

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INTRODUCTION

The first to describe a chronic form of hepatitis in young women was Jan Waldenström in 1950[1]. Later, the disease was associated with other autoimmune diseases and was termed "lupoid hepatitis" because of the presence of antinuclear antibodies and lupus erythematosus cells[2]. These observations led to the idea that the foundation of this disease was a loss of immunological tolerance. The term Auto Immune Hepatitis (AIH) in its current meaning was introduced by Mackay and colleagues in 1965 when the concept of autoimmunity was acknowledged at an international meeting[3].

AIH is now recognized as a relatively rare chronic inflammatory liver disease predominantly affecting females in which a loss of tolerance against hepatic tissue is assumed. Based on the type of serum auto-antibodies, AIH can be subdivided into two types: type 1 AIH, identifiable by antinuclear antibodies (ANA) and/or anti-smooth muscle antibodies (SMA), and type 2 AIH, predominantly found in children and defined by antibodies against liver kidney microsomes type 1 (anti-LKM-1) or for anti-liver cytosol type 1 antibodies[4,5].

EPIDEMIOLOGY

There are few studies that have investigated the epidemiology of AIH. The majority of these studies are hampered by the fact that no predefined criteria for disease diagnosis were applied. In some older studies there has been admixture of patients with chronic hepatitis C and finally some of the studies may have been subject to tertiary referral bias (Table 1).

Nevertheless, incidence data are more or less comparable in Western Europe, ranging from 0.8 to 3 per 100000 with a prevalence ranging from 11 to 24 per 100000[5-9]. In Asia AIH seems to be less frequent, with incidence numbers ranging between 0.08 and 0.15 in Japan[10].

Substantially higher prevalence data of 42.9 cases per 100000 were found in a well defined native Alaskan population, although it should be noted that this study involved a small catchment area and a very limited number of patients[11]. Based on the available studies it is estimated that 11%-20% of all cases of chronic hepatitis in Western countries is caused by AIH[12]. The prevalence of AIH is still gradually increasing. Whether or not this reflects a true rise in incidence, as seen in other immune-mediated diseases like Crohn's disease, increased awareness of the disease or different diagnostic criteria is unknown.

Women are affected more frequently than men with a sex ratio of around 4:1[8]. In women a bimodal age pattern is usually seen, one in the late teens and one around the menopause but it should be stressed that disease can develop in all age groups and both genders[4,13].

PATHOGENESIS

The etiology of AIH remains unknown and fundamental questions regarding disease pathogenesis remain to be resolved. It is generally believed that AIH occurs in a genetically susceptible host as the consequence of an exaggerated immune reaction towards hepatic tissue[14]. Such a response can occur when effector lymphocyte responses are abundant and inappropriate leading to tissue damage, or, alternatively, when there is a numerical and/or functional defect in regulatory T cells (Treg) controlling such responses. This defect is more obvious at disease presentation than during treatment induced remission, where a partial recovery is observed.

Whilst abundant pro-inflammatory responses have been identified in most, if not all immune-mediated diseases, it has been very difficult to gain evidence for a primary defect in regulatory T cells in the majority of these diseases. Tregs isolated from children and adults with AIH were profoundly dysfunctional, suggesting that an underlying Treg deficiency plays a permissive role in the pathogenesis of AIH[15-17].

More recent studies omitted to find either functional or numerical Treg impairments in AIH patients and thus the question as to whether AIH is the result of defective immunoregulation warrants further investigation.

A third, not mutually exclusive mechanism may relate to molecular mimicry, which has been proposed as a mechanism by which exogenous substances may trigger an immune response against autoantigens. Such a response may spark an inflammatory reaction and the resulting hepatocellular injury may give rise to the release of other previously hidden antigens that may further fuel the inflammatory reaction. Exogenous pathogens implicated in this process include,

Table 1 Studies of incidence and prevalence of autoimmune hepatitis

Ref.	Year	Cases	Incidence/100000	Prevalence/100000
Toda *et al*[10]	1997	496	0.8	-
Whalley *et al*[125]	2007	200	3.0	-
Werner *et al*[9]	2008	473	0.85	10.7
Grønbæk *et al*[7]	2014	1721	1.68	23.9
Gerven *et al*[8]	2014	1313	1.1	18.3
Ngu *et al*[39]	2010	138	2.0	24.5
Delgado *et al*[126]	2013	100	0.67	11.0
Primo *et al*[127]	2004	13	1.37	11.61
Hurlburt *et al*[11]	2002	77	-	42.9

amongst others, the hepatitis C virus. A sequence homology between HCV polyprotein and cytochrome P4502D6 (CYP2D6) was previously reported, which was identified as anti-LKM-1 autoantibodies[18,19]. Indeed, anti-LKM-1 is seropositive in up to 10% of HCV patients. Other proposed triggers include other hepatotrophic viruses, as well as drug induced liver injury caused by antibiotics (including nitrofurantoin and minocycline), statins and anti-TNF agents[20-25].

GENETIC FACTORS

Genetic factors have long been implicated in disease pathogenesis yet systematic studies addressing the genetic epidemiology of AIH including familial occurrence, disease concordance in twins or ethnic differences in disease prevalence are lacking. Nevertheless, there are several observations that support a genetic basis for AIH. These include the association with other autoimmune diseases with a known genetic basis in up to a quarter of patients[8]. Additionally, associations with alleles of the Major Histocompatibility Complex (MHC) that encode the Human Leucocyte Antigens (HLA) were already described in the late seventies and confirmed and refined thereafter in numerous studies in different ethnic groups[26-28]. Such associations are found with most autoimmune diseases, most likely because they contribute to the specificity of immune reactions. HLA typing of patients with AIH reveals strong association with the HLA-DRB1 locus, with the haplotypes DRB1*0301 (HLA-DR3) and DRB1*0401 (HLA-DR4) as the main susceptibility factor in white Northern Europeans and North Americans[27,29-31]. Intriguingly there is evidence for substantial genetic heterogeneity in AIH with different MHC associations in different ethnic populations. Thus, in Japanese patients HLA-DRB1*0405 is the most important susceptibility allele[32,33] whereas primary associations with DRB1*0404 were found in Mexican patients[34].

The HLA alleles not only determine overall disease susceptibility but appear also to act as modifiers of the clinical phenotype. For instance, HLA-DR4 was found to be associated with female gender, less severe disease, more common autoimmune disease, and older age of onset[35-40].

Despite the fact that the MHC loci confer a 6 to 7 fold increased disease risk, these variants alone cannot explain the genetic predisposition for AIH. Genes outside the MHC have only been studied in candidate gene approaches involving limited numbers, making them prone to overestimation of significance. Most extensively studied is the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene[41,42]. A recent study in the Netherlands involving a substantial number of patients however observed no significant differences in allele and genotype frequencies of the *CTLA-4* gene between AIH patients and controls[43].

More recently, genome-wide association studies have emerged as a powerful and unbiased approach for the identification of new genetic susceptibility loci in autoimmune diseases. Very recently this methodology was applied in a multicentre cohort of type 1 AIH patients. This study confirmed the involvement of the MHC region and identified *SH2B3* as the first genetic risk factor outside the MHC region. In addition, several other loci were identified supporting the thesis that AIH has a complex genetic basis[27].

CLINICAL FEATURES

The clinical manifestation of AIH can range from mild or severe symptoms to fulminant hepatic failure[44]. In all patients with liver disease AIH should be considered, so that that appropriate treatment can be instituted without delay. Up to 40 percent of patients presents with acute hepatitis, characterizes by right upper-quadrant abdominal pain, fatigue, jaundice and arthralgia[45]. However a fulminant manifestation or a long sub clinical course with only minimal increase of liver enzymes and non specific symptoms, such as arthralgia or fatigue, may be seen[12,46-49] (Table 2).

Clinical manifestations of AIH may vary among ethnic groups. Thus, non-Caucasian patients (the majority being from African-American descent) had more aggressive disease at initial presentation, lower reaction to immunosuppressive therapy, and worse outcomes when compared to Caucasian patients[44]. Higher rates of cirrhosis were found in Hispanic *vs* Caucasian patients, and a trend towards worse survival among Asians[50].

Other autoimmune diseases are common in up to

Table 2 Presentation and symptoms in auto immune hepatitis

Acute hepatitis	
Chronic hepatitis	
Hepatomegaly	
Splenomegaly	
Spider naevi	
Palmar erythema	
Non specific symptoms:	
Tiredness	
Fever	
Loss of appetite	
Upper abdominal pain	
Arthralgia	
Extrahepatic autoimmune disease (most common mentioned):	
Thyroiditis	10%-23%
Primary biliary cirrhosis	10%-20%
Diabetes	7%-9%
Primary sclerosing cholangitis	2%-8%
Rheumatoid arthritis	2%-5%
Celiac disease	1%-2%

40% of AIH patients. They included, among others thyroid disease, diabetes, inflammatory bowel disease and rheumatoid arthritis. A recent study demonstrates that celiac disease is more prevalent among AIH patients compared to the general population[51]. In addition AIH may have cholestatic features that can resemble primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) and overlap with these diseases have been described in 10%-20% and 2%-8% of cases, respectively[9,14,52-55] (Table 2). So far, there have not been uniform definitions or diagnostic criteria for the overlap of AIH with PBC or PSC. It is still under debate as to whether these overlap syndromes represent variants of the main autoimmune liver diseases or hallmarks of a separate entity[56]. The presence of features of different diseases can occur simultaneously as well as sequentially in each form of overlap syndromes. AIH and PBC are the most frequently described autoimmune liver diseases. The pattern of abnormalities in laboratory tests can help determine the origin of the disease. In AIH a hepatic pattern is found, and a primarily cholestatic pattern in PBC; in addition, elevation of IgG is characteristic of AIH, an increase in IgM is commonly found in PBC patients.

Due to an absence of a well validated scoring system for the diagnosis of PBC-AIH overlap, the criteria developed by Chazouillères *et al*[57] are commonly applied.

In various reports AIH-PSC overlap syndrome has been described and is characterised by ANA and/or SMA seropositivity, hypergammaglobulinaemia and interface hepatitis - all features typical of "classical" AIH - in conjunction with cholestatic biochemical changes, frequently associated with inflammatory bowel disease, and histological evolution to fibrous obliterative cholangitis, ductopenia, portal tract oedema and/or bile stasis[58].

DIAGNOSIS

The diagnosis is based on the combination of clinical and laboratory features and histological changes after exclusion of other causes of hepatitis[59].

Laboratory abnormalities

AIH is suggested by a patient with elevated Alanine-aminotransferase (ALT) and Aspartate transaminase (AST) activity, raised Immunoglobulin G (IgG), high titres of circulating antibodies, negative serum tests and exclusion of toxic hepatitis. However not all these laboratory findings need to be present in an individual patient.

Elevation of serum IgG is a common finding in AIH[60], but normal IgG levels may be found in up to 30% of patients[61,62].

Auto antibodies are the hallmark of AIH and can constitute an important part of the diagnostic work up. The classic antibodies associated with AIH are Antinuclear antibodies (ANA), anti-smooth-muscle antibodies (ASMA) and Anti Liver kidney microsomal (LKM-1). About 70%-80% of AIH patients have significant titres ($\geqslant$ 1:40) of ANA or ASMA and overall 3%-4% have anti LKM-1, while up to 20% are seronegative for these antibodies[60].

ANA are the most commonly found auto antibodies in AIH, yet are rather non-specific since they can be found in a large variety of diseases as well as in healthy individuals[63]. ANA may be the only antibody present or may occur in conjunction with ASMA. ASMA are the second major class of antibodies which have proved useful in the diagnosis of AIH. Although less prevalent than ANA they are more specific[64].

Autoantibody detection not only supports in the diagnosis but also classifies between type 1 and type 2 AIH. Type 1 AIH is associated with the presence of ANA and/or SMA and type 2 with the presence of anti-LKM-1 and/or anti-liver cytosolic-1 (LC-1). In Northern Europe and North America type 2 AIH accounts for less than 10% of all patients[55].

Antibodies to soluble liver antigen (SLA) or liver pancreas antigen (LP) are found in 10%-30% of patients with AIH. These antibodies are specific for AIH and may proof useful in the diagnosis[65]. Antibodies to actin and atypical peripheral anti-neutrophilic cytoplasm are also commonly seen in type 1 AIH, however their applicability is limited due to lack in specificity[59].

Liver histology

A liver biopsy is usually necessary to confirm the diagnosis, provide histological assessment of disease severity and exclude other causes of hepatitis. There are no individual histological criteria that prove the diagnosis of AIH[66]. Interface hepatitis (or piecemeal necrose) is the histological hallmark of AIH and is a process of inflammatory infiltration and erosion of the hepatic parenchyma at the junction of the portal

Table 3 Simplified diagnostic criteria for auto immune hepatitis[75]

Variable	Cutoff	Points
ANA or ASMA	⩾ 1:40	1
ANA or ASMA	⩾ 1:80	
or LKM-1	⩾ 1:40	2
or SLA	Positive	
IgG	> Upper normal limit	1
	> 1.10 times upper normal limit	2
Liver histology (evidence of hepatitis is a necessary condition)	Compatible with AIH	1
	Typical AIH	2
Absence of viral hepatitis	Yes	2
		⩾ 6: probable AIH
		⩾ 7: definite AIH

ANA: Antinuclear antibodies; ASMA: Anti-smooth-muscle antibodies; LKM-1: Anti Liver kidney microsomal; IgG: Immunoglobulin G.

tract[67]. It is found in 84%-98% of patients[13,45,68], but can also be seen in patients with drug-induced and viral hepatitis[68]. The infiltrates consist of hepatic mesenchymal cells containing lymphocytes, plasma cells and histiocytes that typically accompany these cells. Patients presenting with chronic AIH typically have plasma cells infiltrated at the interface and throughout the lobule. Plasma cells are not invariability present and paucity of plasma cells does not therefore exclude a diagnosis of AIH. They may be absent in up to one third of the patients[68,69].

In a recent study, emperipolesis and rosette formation appear superior histological predictors of AIH when compared to the typical histological features of interface hepatitis and plasma cells[70].

Diagnosis scoring system

Because there is no golden standard for the diagnosis of AIH, diagnostic scoring systems have been established that support the diagnosis in most of patients. The IAIHG scoring system, originally published in 1993[71] and revised in 1999[60], was developed as a search tool to ensure comparability of study populations. Despite a high degree of sensitivity (100%) and specificity (90%)[72-74], these criteria have been proven impractical in the day to day clinical practice.

In 2008 the IAIHG produced a simplified system for the diagnosis of AIH which is less complex and enhances applicability in clinical practice[75]. This system is based on four variables: presence and level of anti bodies, IgG concentration, typical histological features and absence of viral markers (Table 3). Recently three studies report that the simplified scoring system performs with high specificity (97%-99%) and lower sensitivity (81%-88%) when compared to the original diagnostic criteria yet requires further prospective validation[72,76,77].

TREATMENT

Indication of treatment

The short and long term efficacious of immune suppression in patients with AIH has been described unequivocally. When left untreated, an estimated 40% of patients will die within six months of diagnosis[78]. When treated adequately, the 20-year survival rate for all treated patients exceeds 80%, and life expectancy is similar to that of age and sex matched normal subjects from the same geographical area[79].

Updated treatment guidelines have recently been emerged by the European Association for the Study of the Liver (EASL) in 2015, the British Society of Gastroenterology in 2011 and the American Association for the Study of Liver Diseases (AASLD) in 2010[4,80,81]. Patients with AST levels 10-fold the upper normal limit, or fivefold the upper normal limit in concurrence with IgG levels at least twice the upper normal limit, or histological features of bridging necrosis or multiacinar necrosis, should be offered immunosuppressive treatment because of clear survival benefit (Table 4). Patients not satisfying these criteria must be personalized and treatment should be based on clinical judgement[4].

Standard treatment

Current therapeutic strategies for AIH consist of an induction with prednisone and frequently include subsequent addition of azathioprine (AZA) as steroid-sparing maintenance therapy[80]. Prednisone is introduced at a dose of 1 mg/kg with a maximum of 60 mg/d in monotherapy or a maximum of 30 mg/d in combination treatment[4,12]. After AST and ALT normalize, prednisone alone can be reduced by 10 mg/wk until a dose of 20 mg.

Patients treated with combination therapy can reduce prednisone by 5 mg/wk until 15 mg. A slower reduction is advised after this point[4,82]. For maintenance treatment AZA can be used at a dose 1-2 mg/kg per day either alone or in combination with low dose prednisone[4,83]. A recent review based on available randomised controlled trials found that prednisone monotherapy and prednisone in combination wit AZA are both feasible induction therapies for AIH, while maintenance therapy prednisone and AZA and Monotherapy AZA are superior to prednisone monotherapy[84]. AIH patients

Table 4 Indication for treatment of auto immune hepatitis (adapted from Manns *et al*[4])

Absolute	Relative
Serum AST ⩾ 10 fold ULN	Symptoms (fatigue, arthralgia, jaundice)
Serum AST ⩾ 5 fold ULN and IgG level ⩾ twice normal	Serum AST and/or IgG less than absolute criteria
Bridging necrosis or multiacinar necrosis on histological examination	Interface hepatitis

AST: Aspartate transaminase; ULN: Upper limit normal; IgG: Immunoglobulin G.

treated with corticosteroids and/or AZA have the risk of many side effects on both drugs. The side effects of long term treatment with corticosteroids are well known; acne, moon shape face, striae, weight gain and loss of bone density. Adverse effects of thiopurines are common and generally occur shortly after the start of therapy. They include allergic reactions, flu-like illness, nausea fever, malaise, rash, abdominal pain, hepatotoxicity, pancreatitis and myelosuppression[83,85-87]. The principal side effects of AZA are cytopenia and liver test abnormalities, which may be difficult to distinguish from inherent AIH disease activity.

Remission and relapse

Remission of previously symptomatic patients is defined as a complete normalisation of all inflammatory parameters, including AST, ALT, bilirubine, IgG, recovery from symptoms and inactive liver histology[4,9,82]. In 80%-90% of patients with moderate/severe AIH, serum ALT decreases after starting treatment. Usually a decrease is seen within two weeks. As transaminase decrease, clinical symptoms revolve and liver functions shows marked improvement within 3-6 mo after starting prednisone treatment either with or without AZA[81].

There is no prescribed duration of the length of treatment. Because histological restore lags behind clinical and biochemical improvement by 3-8 mo, treatment should be continued for at least this period[88,89]. Proper patient selection including sustained remission on immunosuppressive Monotherapy for a minimum of 2 years can markedly improve the success rate of treatment withdrawal[90]. The AASLD and EASL guidelines recommend treatment withdrawal, when serum liver and immunoglobulin levels have been repeatedly normal for a period of at least two years. Liver biopsy prior to termination of treatment is preferred[4,80]. Relapse is characterized by an increase in ALT levels (three times upper normal limit) and/or increase of serum IgG level to more than 2 g/L following tapering of steroid doses or after complete withdrawal of immunosuppression[4]. Literature from the 1970s showed a high risk of relapse after drug withdrawal[88,91], but this was later disputed and it was recommended that drugs withdrawal should be attempted[92]. A more recent retrospective analysis found that relapse occurred in almost all patients with AIH when immunosuppressive medication was discontinued or tapered[4,92,93]. Relapse occurred despite prior attainment of complete remission, including a histological inactive follow up biopsy prior to tapering in a subgroup of patients. In patients who have relapsed once, a subsequent attempt to withdrawal therapy was invariably associated with the re-occurrence of a relapse[93]. Since repeated relapses were associated with a poorer long term prognosis patients should receive life long treatment[94,95]. A lifelong follow up should occur in patients who successfully stopped immunosuppression, while a relapse can occur 10 years later[93].

Alternative treatment

In up to 10% of AIH patients, the therapeutic strategy of prednisone and AZA is unsuccessful, due to intolerable side effects or lack of clinical response[4,81]. In patients who fail on standard therapy, alternative immunosuppressive treatments have been tried with encouraging results. Cyclosporine[96-98], tacrolimus[99,100], methotrexate[101], cyclophosphamide[102] and mycophenolate mofetil[103-105] have been tried, with varying degrees of success, as a replacement for AZA.

In a small recent study allopurinol was added to the AZA or mercaptopurine treatment in patients who fail treatment due to ineffectiveness or intolerance, due to skewed thiopurine metabolism. The combination of low dose thioprines and allopurinol proved an effective and well-tolerated alternative in the treatment of AIH. Larger and controlled studies are needed to confirm these outcomes[106]. As an alternative for prednisone, budesonide is receiving considerable attention.

In two recent studies in patients with noncirrhotic AIH oral budesonide, in combination with azathioprine, induces and maintains remission. This treatment causes fewer steroid-specific side effects[107,108]. Routine use is not currently recommended, while the trial duration is short and the fact that no follow up date were presented[81]. AZA is the prodrug of 6-mercaptopurin (6-MP) and is converted into 6-MP in a nonenzymatic manner before exhibiting its antiproliferative and immunosuppressive properties. In patients with ulcerative colitis and Crohn's disease 6-MP has a beneficial role in AZA-intolerant patients[109]. In patients with AIH and AZA intolerance, 6-MP seems to be an effective and well-tolerated second line treatment[110]. The use of 6-thioguanine 6-TG), an agent more directly leading to down-stream active metabolites of AZA, showed clinical improvement in

three AIH patients intolerant to AZA. A prospective evaluation of 6-TG as possible immunosuppressive drug in AIH patients is warranted[111].

COMPLICATIONS AND PROGNOSIS

Complications in AIH are comparable to those seen in other liver diseases and in rare cases AIH presents by the occurrence of hepatic encephalopathy[112,113].

Liver fibrosis is often presence at diagnosis and a subgroup of patients have already cirrhosis at presentation[4,68] suggesting that the disease has gone unrecognized for a significant period prior to diagnosis. When left untreated, an etsimated 40% of patients will die within 6 mo of diagnosis[88,91,114]. In some patients without proper treatment, AIH progresses to cirrhosis and eventually Hepatocellular carcinoma (HCC). The presence of cirrhosis at diagnosis or during treatment and the need for long-term immunosuppressive therapy have been observed as risk factors for malignant transformation[115]. In addition risk factors for HCC furthermore include male gender, advanced stage disease, portal hypertension as ascites and esophageal varices[116]. HCC occurs in 1%-9% of AIH patients[116-118], which is less frequently compared to patients with chronic viral hepatitis[119]. Imaging with ultrasonography or computed tomography should be conducted every 6-12 mo. In patients who develop liver failure, liver transplantation needs to be considered[48,120]. When AIH is indicated for transplantation, transplanted patients, practically compared to other chronic liver diseases, have an excellent 5 year survival of between 78%-91%[121-123]. The recurrence rate of AIH after initial successful transplantation is problematic and occurs in around 30% of patients[124].

CONCLUSION

AIH is a relatively rare disease of unknown aetiology. Many factors contribute to the diagnosis, which is characterized by a female predominance, histologically evidence of periportal hepatitis in the absence of viral markers, hypergammaglobulinaemia, the presence of auto antibodies in serum, plasmacellular infiltrates and an optimal response to steroids in most patients. In AIH there is no existence of a pathognomonic feature and therefore the diagnosis rests on a combination of immunological, biochemical, and histological features together with exclusion of other liver diseases. Due to large heterogeneity of the disease, AIH might be unrecognised. The clinical manifestation of AIH can range from mild or severe symptoms to fulminant hepatic failure. AIH generally responds to immunosuppressive treatment and treatment is required as soon as the diagnosis is made. For most patients lifelong treatment is indicated. In patients in whom all treatment attempts fail liver transplantation needs to be considered.

AIH remains a major diagnostic and therapeutic challenge. Growing insights into the clinical presentation of AIH highlights the importance of evaluation of the current diagnostic criteria, role of genetic and environmental factors, as well as the development of new treatment strategies.

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ORIGINAL ARTICLE

Basic Study

Altered tryptophan hydroxylase 2 expression in enteric serotonergic nerves in Hirschsprung's-associated enterocolitis

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Author contributions: Coyle D performed the experimental procedures and is the primary author of the final manuscript; Murphy JM and Doyle B carried out experimental procedures; O'Donnell AM, Gillick J and Puri P designed the study and co-authored and revised the final manuscript.

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Informed consent statement: Informed written consent was obtained from the parents/legal guardians of all children enrolled in this study.

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Abstract

AIM: To determine if expression of colonic tryptophan hydroxylase-2 (TPH2), a surrogate marker of neuronal 5-hydroxytryptamine, is altered in Hirschsprung's-associated enterocolitis.

METHODS: Entire resected colonic specimens were collected at the time of pull-through operation in children with Hirschsprung's disease (HSCR, n = 12). Five of these patients had a history of pre-operative Hirschsprung's-associated enterocolitis (HAEC). Controls were collected at colostomy closure in children with anorectal malformation (n = 10). The distribution of expression of TPH2 was evaluated using immunofluorescence and confocal microscopy. Protein expression of TPH2 was quantified using western blot analysis in the deep smooth muscle layers.

RESULTS: TPH2 was co-expressed in nitrergic and cholinergic ganglia in the myenteric and submucosal plexuses in ganglionic colon in HSCR and healthy controls. Co-expression was also seen in submucosal interstitial cells of Cajal and PDGFRα^+ cells. The density of TPH2 immuno-positive fibers decreased incrementally from ganglionic bowel to transition zone

bowel to aganglionic bowel in the myenteric plexus. Expression of TPH2 was reduced in ganglionic bowel in those affected by pre-operative HAEC compared to those without HAEC and healthy controls. However, expression of TPH2 was similar or high compared to controls in the colons of children who had undergone diverting colostomy for medically refractory HAEC.

CONCLUSION: Altered TPH2 expression in colonic serotonergic nerves of patients with HSCR complicated by HAEC may contribute to intestinal secretory and motor disturbances, including recurrent HAEC.

Key words: Serotonin; Tryptophan hydroxylase 2; Hirschsprung; Enterocolitis; Ganglionic

Core tip: Despite optimal surgery, children with a history of Hirschsprung's disease (HSCR) complicated by Hirschsprung's-associated enterocolitis (HAEC) are at higher risk of long-term colonic dysfunction. Tryptophan hydroxylase-2 (TPH2) is a surrogate marker for neuronal 5-hydroxytryptamine (5-HT). We hypothesized that expression of TPH2 is altered in the colon of children with HAEC. We found the density of serotonergic nerves to be differentially reduced in the ganglionic colon of children with HSCR and HAEC compared to those without HAEC compared to controls, although expression is normalized in those with diverting colostomy. Abnormal neuronal 5-HT expression may contribute to post-operative colonic dysfunction in HSCR.

Coyle D, Murphy JM, Doyle B, O'Donnell AM, Gillick J, Puri P. Altered tryptophan hydroxylase 2 expression in enteric serotonergic nerves in Hirschsprung's-associated enterocolitis. *World J Gastroenterol* 2016; 22(19): 4662-4672 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4662.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4662

INTRODUCTION

Hirschsprung's-associated enterocolitis (HAEC) is the most serious complication of Hirschsprung's disease (HSCR) and is the leading cause of disease-related mortality. It occurs in 17%-50% of patients with HSCR and may occur before or after a pull-through operation[1,2]. Although a scoring system for HAEC exists, there is no agreed definition of this condition. It is typically described as an inflammatory disease of the colon leading to a spectrum of symptoms ranging from abdominal distension and loose stools to life-threatening toxic megacolon[3,4]. The etiology and pathogenesis of HAEC are still incompletely understood. It has been proposed that intestinal barrier dysfunction, abnormal innate immunity and the presence of a disturbed microbiome are all potential contributors to its etiology[1]. However, given that the primary abnormality in HSCR is the absence of enteric ganglia in the distal colon, it follows that the enteric nervous system may have a role in the pathogenesis.

5-Hydroxytryptamine (5-HT), also commonly known as serotonin, is a major neuroendocrine signaling molecule. While the gut is the single largest reservoir of 5-HT, the wide range of its functions therein have only been elucidated relatively recently. The majority of enteric serotonin is stored in the mucosa in the enterochromaffin (EC) cells. Approximately 1%-5% of enteric serotonin is stored in the serotonergic enteric nerves, where it acts as a neurotransmitter[5,6]. The rate limiting step in the synthesis of 5-HT is the conversion of L-tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase (TPH). The conversion of 5-hydroxytryptophan to 5-HT then occurs rapidly through the actions of L-amino acid decarboxylase[7]. However, the synthesis pathway of enteric 5-HT differs depending on whether 5-HT is being synthesized in EC cells, where the TPH1 isozyme of TPH predominates, or in serotonergic neurons, where TPH2 predominates. In this way, it is possible to indirectly evaluate the expression of neuronal 5-HT separately from that produced in EC cells[5,7].

5-HT has many roles, including activation of intrinsic reflexes such as peristalsis, vasodilation, and secretion. When released from EC cells, mucosal 5-HT has been demonstrated to promote inflammation - an activity which is counterbalanced by its re-uptake *via* the serotonin transporter (SERT)[5,8]. Abnormal mucosal 5-HT activity has been demonstrated in inflammatory and functional bowel disorders such as ulcerative colitis and irritable bowel syndrome[9]. Conversely, enteric neuronal 5-HT is anti-inflammatory and neuroprotective, an activity which has obvious importance in the setting of inflammation and enterocolitis, as neuronal damage can frequently result[8]. It has previously been reported that populations of mucosal EC cells are deficient in the ganglionic bowel of children with HSCR who have previously had HAEC. It is unclear if this is a facilitator or an effect of HAEC[10].

We hypothesized that, in children who had previously been treated for HAEC, neuronal 5-HT expression is altered compared to those who did not require treatment for HAEC. In this study we aimed to investigate the distribution of 5-HT in the aganglionic and ganglionic colon of children with HSCR and in healthy controls and to quantify expression of TPH2 in serotonergic enteric nerves of these patients.

MATERIALS AND METHODS

Specimen collection

The study was approved by the ethics committees of both centers (Our Lady's Children's Hospital Ethics Committee, GEN292.12; Temple Street Children's

University Hospital Research and Ethics Committee, 13.003). Informed written consent was obtained from parents/legal guardians prior to specimen collection. The procedures carried out during the study were in conformance with the principles expressed in the Declaration of Helsinki.

Full-length colonic specimens resected during pull-through operations for HSCR were obtained fresh from 12 patients, incorporating aganglionic, transition zone and ganglionic bowel (age range 3 mo-14 mo). Colonic control specimens were similarly obtained from the proximal colostomy limb of 10 patients at the time of descending/sigmoid colostomy closure in children following surgical correction of anorectal malformation (age range 7 mo-21 mo). The level of the most proximal extent of the transition zone was routinely confirmed by 3,3'-diaminobenzidine (DAB) immunohistochemistry probing for protein gene product 9.5 (PGP 9.5), which stains nerve cells. All experiments incorporated comparison of ganglionic bowel in HSCR with transition zone and aganglionic bowel as well as healthy controls.

Double-label immunofluorescence

Colonic sections were embedded in OCT compound [VWR, Ireland (361603E)] and snap frozen in liquid nitrogen. Twenty micron sections were cut and were fixed in 10% neutral buffered formalin (Sigma-Aldrich, Ireland [HT501128-4L]). Cell membranes were permeabilized by rinsing in 1% w/v PBS with 1% Triton X-100. Sections were blocked in 10% bovine serum albumin [BSA, Sigma-Aldrich, Ireland (A2153-50G)] diluted in 1% w/v PBS with 0.05% Tween® [Sigma-Aldrich, Ireland (P1379)] (PBST) for 90 min at room temperature to prevent non-specific antibody binding. Samples were incubated simultaneously in both primary antibodies of interest, diluted in 10% BSA, at 4 ℃ overnight. Antibodies to the following antigens were used to label specific cell types in the colonic wall: HuD (PGP 9.5) was used to label nerve cells; TMEM16A [anoctamin-1 (ANO1)])was used to label interstitial cells of Cajal (ICCs); platelet derived growth factor receptor-α (PDGFRα) was used to label PDGFRα$^+$ cells, neuronal nitric oxide synthase (nNOS) was used to label nitrergic neurons, vasoactive intestinal peptide (VIP) was used to label peptidergic neurons, and choline acetyltransferase was used to label cholinergic neurons. A detailed description of the primary antibodies used in the study is seen in Table 1. Following incubation in primary antibody solution, samples were rinsed intensively in 1% PBST, following which they were incubated in a solution containing both secondary antibodies specific to the host species of each primary antibody (Table 1), diluted in 10% BSA, for 90 min at room temperature. After intensive rinsing in 1% PBST, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain [Thermo Scientific, Ireland (EN62248)]. Sections were mounted with glass coverslips using Mowiol® 4-88 fluorescence mounting medium [Sigma Aldrich, Ireland (81381-50G)], which was constituted according to manufacturer's specifications. Specimens were visualized using laser scanning confocal microscopy (LSM700 Confocal Microscope, Carl Zeiss MicroImaging GmbH, Jena, Germany). Resulting images were processed, including calculation of TPH2 immuno-positive cell counts, using ImageJ - an open-access software available from http://imagej.nih.gov/ij/.

Protein extraction and Western blot analysis

The mucosa was dissected from the deep smooth muscle layers at the time of specimen collection. Protein was extracted from the tunica muscularis layers to limit the probability of unwanted antibody binding to tryptophan hydroxylase 1 (TPH1), of which the mucosa is a substantial reservoir. Bowel tissue fragments were homogenised using a tissue homogeniser in radioimmunoprecipitation (RIPA) buffer containing 1% protease inhibitor cocktail [Sigma Aldrich, Ireland (P2714)]. Soluble and insoluble fractions were then separated by centrifugation at 4 ℃ at 3000 *g* over 30 min. The concentration of the supernatant was determined by means of a Bradford assay [Sigma-Aldrich Ltd., Arklow, Ireland (B6916)] using a standard curve generated from known concentrations of BSA. Novex® Bolt® LDS sample buffer and reducing agent [Biosciences, Dublin, Ireland (B0007 and B0009 respectively)] were added to each protein aliquot according to manufacturer's protocols. The protein concentration of each sample was then equilibrated with the addition of deionised water. Samples were denatured at 70 ℃ for 10 min and were then loaded onto an SDS polyacrylamide gel [Bolt® Novex 4%-12% Bis-Tris gel: Biosciences, Dublin, Ireland (NW04120BOX)] in NuPAGE® MES SDS running buffer [Biosciences, Dublin, Ireland (NP0002)] and separated by electrophoresis at 150 V. Proteins were then transferred from the gel to a 0.45 μm PVDF membrane at 30 V for 90 min.

Membranes were blocked in 3% dried skimmed milk dissolved in 1% PBST for 1 h to prevent non-specific antibody binding and were then incubated in primary antibody (Table 1) diluted in 10% BSA overnight at 4 ℃. Membranes were rinsed in 1% PBST for 4 h and were then incubated in species-specific secondary antibody (Table 1) for 90 min at room temperature. Following further rinses with 1% PBST for a minimum of 1 h, membranes were incubated in chemiluminescent substrate [SuperSignal™ West Pico Chemiluminescent Substrate, Thermo-Fischer, Ireland (34079)] for 5 min at room temperature before transfer to a chemiluminescence cassette for blot visualization. Protein expression levels were semi-quantitatively evaluated by densitometric analysis using the open-access image processing software

Table 1 Details pertaining to primary and secondary antibodies used in immunofluorescence and Western blot

Host species	Monoclonal/Polyclonal	Antigen	Product code	Manufacturer	Dilution
Rabbit	Polyclonal	Tryptophan hydroxylase 2	NB-100-74555	Novus biologicals (Cambridge, United Kingdom)	1:100
Mouse	Monoclonal	TMEM-16A (ANO1)	Ab190721	Abcam (Cambridge, United Kingdom)	1:200
Mouse	Monoclonal	HuD	sc-48421	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Mouse	Monoclonal	Platelet-derived growth factor alpha	sc-21789	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Goat	Polyclonal	Neuronal nitric oxide synthase	ab1376	Abcam (Cambridge, United Kingdom)	1:300
Mouse	Monoclonal	Vasoactive intestinal peptide	sc-25347	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Mouse	Monoclonal	Choline acetyltransferase	ab49382	Abcam (Cambridge, United Kingdom)	1:100
Mouse	Monoclonal	GAPDH	ab9484	Abcam (Cambridge, United Kingdom)	1:2000
Rabbit	Polyclonal	Mouse (secondary)	ab6728	Abcam (Cambridge, United Kingdom)	1:10000
Donkey	Polyclonal	Rabbit (secondary)	ab6802	Abcam (Cambridge, United Kingdom)	1:10000
Donkey	Polyclonal	Rabbit (Alexa Fluor® 488)	ab150073	Abcam (Cambridge, United Kingdom)	1:500
Goat	Polyclonal	Mouse (Alexa Fluor® 594)	ab150116	Abcam (Cambridge, United Kingdom)	1:500
Donkey	Polyclonal	Goat (Alexa Fluor® 555)	ab150134	Abcam (Cambridge, United Kingdom)	1:500

Table 2 Clinical details of patients with Hirschsprung's disease included in the study

Characteristic	*n* = 12	
Gender	Male (*n* = 11)	Female (*n* = 1)
Median age at pull-through operation	5 mo	3-14 mo
Associated syndromes	Trisomy 21	*n* = 5 (2 with HAEC)
Pre-operative HAEC	Yes (*n* = 5)	No (*n* = 7)
Diverting/levelling stoma	Yes (*n* = 3)	No (*n* = 9)

HAEC: Hirschsprung's-associated enterocolitis.

ImageJ. Statistical analysis was performed using a statistical software package (SPSS v20.0). Non-parametric analysis including Mann-Whitney *U*-test was utilized in testing for differences in TPH2 protein expression between ganglionic and aganglionic colon in HSCR and healthy controls.

RESULTS

Double-labelled immunofluorescence

Basic clinical details regarding patients whose specimens were used in this study can be seen in Table 2. The specificity of our antibody to detect neuronal TPH2 was confirmed by the presence of TPH2 co-expression with HuD, a specific marker of nerve cell bodies, seen in the myenteric plexus of normally ganglionated bowel in HSCR and in healthy controls, while no evidence of expression of either was seen in the myenteric plexus of aganglionic bowel in HSCR (Figure 1A and B). There was a dense network of ANO1-immuno-positive ICC fibers seen in the myenteric plexus. There was no co-expression of TPH2 with ANO1 (Figure 1C), although the processes of ICCs did form dense network around ganglia immuno-positive for TPH2. Of interest, there was partial co-expression of TPH2 with PDGFRα in the cell bodies of PDGFRα$^+$ fibroblast-like cells in the myenteric plexus (Figure 1D).

In the submucosa of aganglionic bowel, HuD-positive nerve cell bodies were absent, although TPH2-positive fibers were still present in reduced density (Figure 2A). Confirmation of the presence of TPH2-immuno-positive nerve cell bodies in the submucosa of ganglionated bowel is seen in Figure 2B. TPH2 was co-expressed with ANO1 in the cell processes of submucosal ICCs (Figure 2C) and with PDGFRα in the cell bodies of PDGFRα$^+$ cells (Figure 2D).

The density of TPH2-immuno-positive cells reduced incrementally from colonic controls (Figure 3A) to ganglionic bowel in HSCR (Figure 3B), transition zone (Figure 3C) and aganglionic bowel (Figure 3D), where expression of TPH2 appeared markedly reduced in the myenteric and submucosal plexuses (Figure 3E). TPH2 was found to be co-expressed with ChAT-positive cholinergic nerve cell bodies in the myenteric plexus (Figure 4A), as well as with nNOS in nitrergic ganglia (Figure 4B). VIP-immuno-positive neurons did not co-express TPH2 in the myenteric plexus (Figure 4C) or in the submucosa (data not shown). Cholinergic nerve fibers in the circular muscle co-expressed TPH2 (Figure 4D).

Western blot analysis

A specific band was detected at approximately 60 kDa, consistent with the molecular weight of TPH2. No doublet bands were observed, confirming specific antibody binding to the TPH2 isozyme, as occurred when Western blot analysis was performed using whole tissue protein extract, which contains significant quantities of TPH1. In patients who had HAEC, TPH2 expression was reduced in the aganglionic bowel, transition zone and ganglionic bowel in HSCR compared to controls ($P = 0.044$) while there was a trend towards lower expression of TPH2 patients with HSCR who had never been treated for HAEC ($n = 7$) in aganglionic bowel compared to healthy controls ($P = 0.056$) (Figure

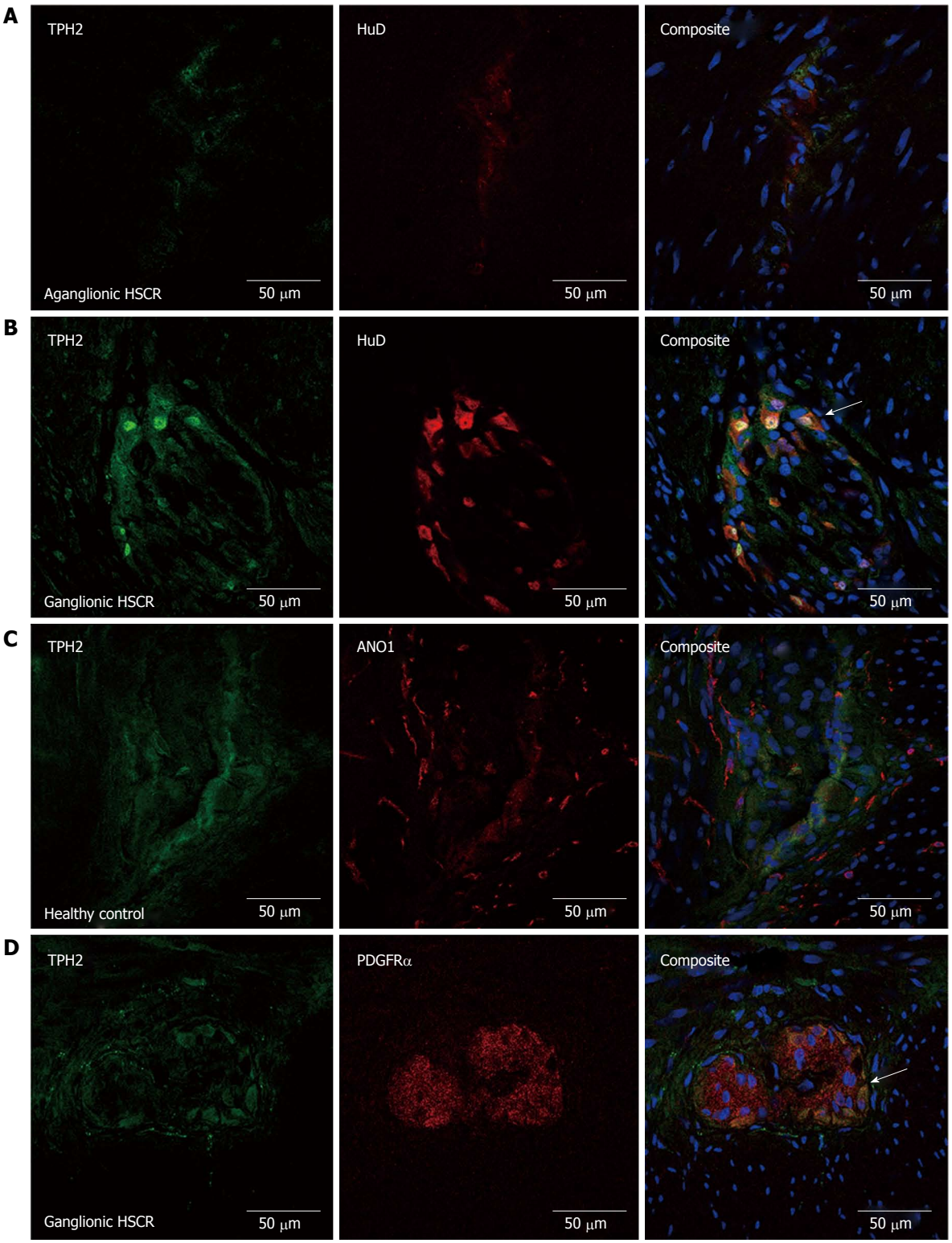

Figure 1 Confocal micrograph series demonstrating pattern of tryptophan hydroxylase-2 expression in the myenteric plexus of the colon. There is almost no HuD or TPH2 immunofluorescence seen in aganglionic colon in HSCR in (A), while there is clear co-expression (arrow head) in the nerve cell bodies in (B). While TPH2 and anoctamin-1 (ANO1) are not co-expressed in (C), the interstitial cells of Cajal (ICC) fibers form a dense network around TPH2-immuno-positive ganglia. TPH2 was co-expressed with PDGFRα cell bodies in the myenteric plexus, seen in (D). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease.

5A). However, consistent marked recovery of TPH2 expression levels was noted in the colon of patients with HSCR, who had been treated for HAEC, but who had undergone formation of a diverting stoma due to failure of medical management (P = 0.002, n = 3). Such was the recovery that expression levels were even higher than in patients who had HSCR but who did not have a history of pre-operative HAEC (Figure 5B).

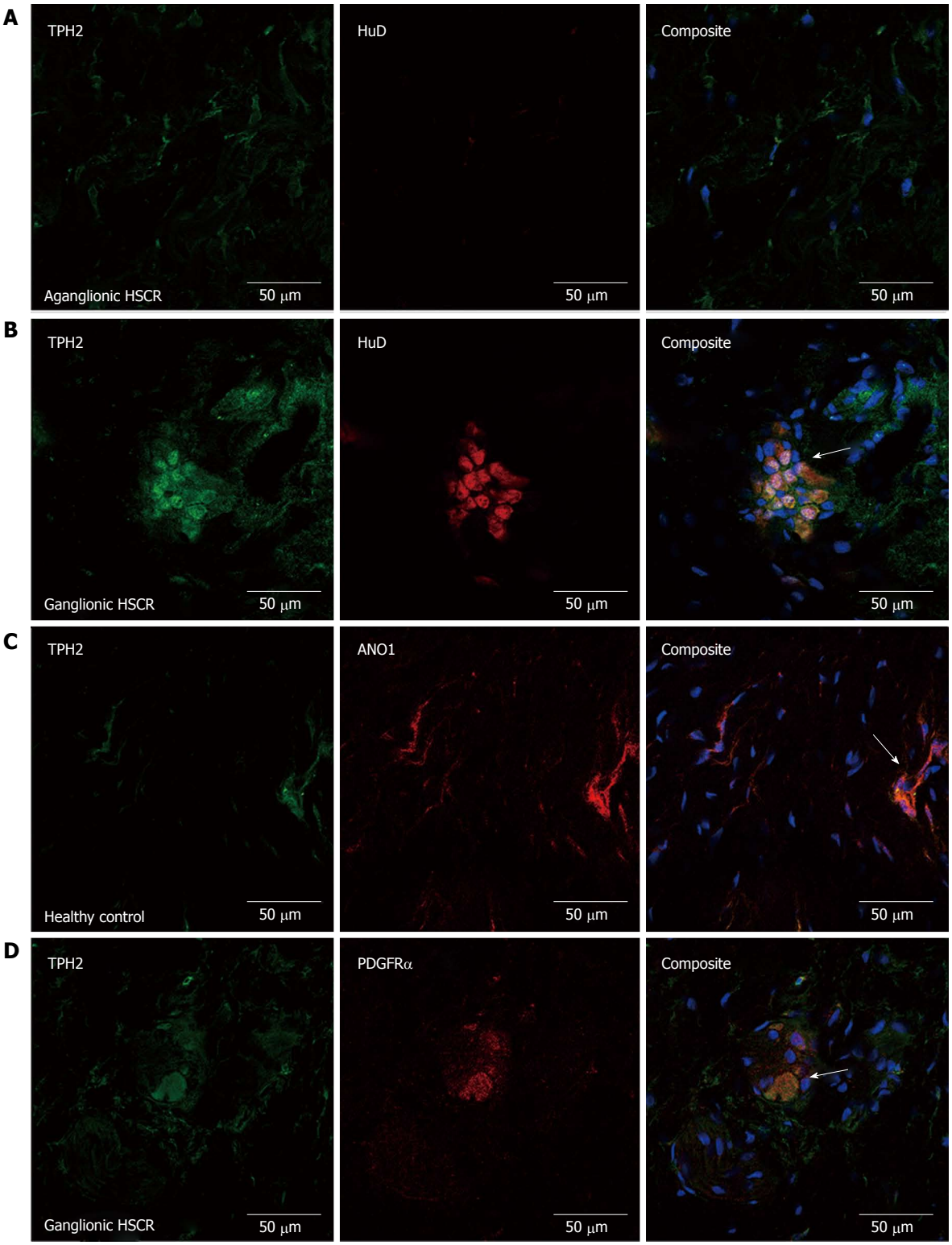

Figure 2 Confocal micrograph series demonstrating tryptophan hydroxylase-2 expression pattern in the submucosal plexus. Again, no HuD expression is seen in the aganglionic bowel, although there are some TPH2 immuno-positive fibres seen in (A). In ganglionic bowel in HSCR (B), TPH2 co-expressed with HuD (white arrow) in ganglion cells. There was co-expression of TPH2 with anoctamin (ANO)-positive submucosal interstitial cells of Cajals (ICCs) and PDGFRα^+ cells, seen in (C) and (D) respectively (white arrow). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease.

DISCUSSION

Enterocolitis is reported as the presenting feature of HSCR in between 12.5% and 40.5% of cases, with many of these cases occurring in the neonatal period[2,11]. The histopathological changes seen in HAEC range from cryptitis to mucosal ulceration, transmural necrosis and colonic perforation[1]. Only recently has an understanding of the processes contributing to the pathogenesis of enterocolitis been developed. In the

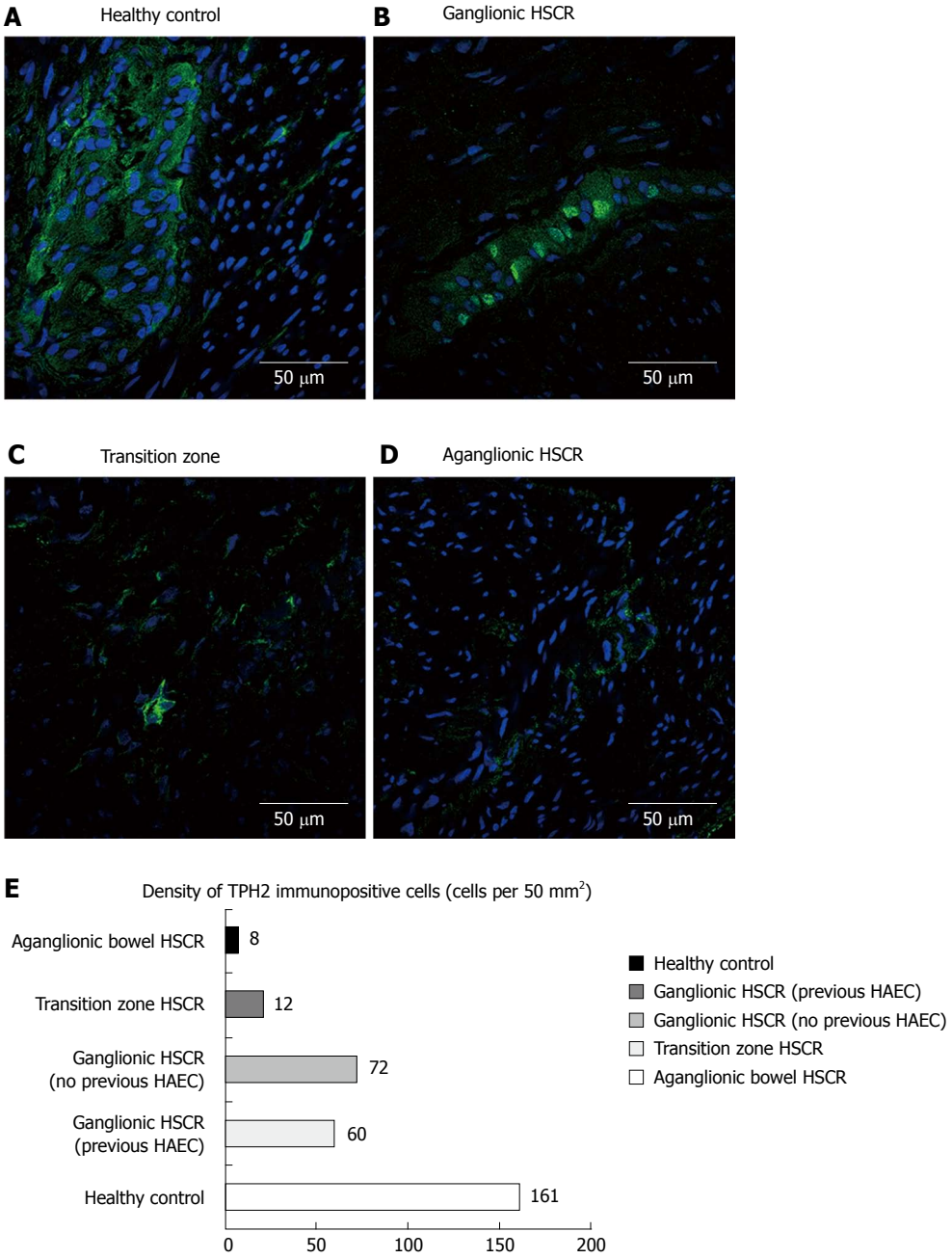

Figure 3 Double-labelled immunofluorescence. Series (A) to (D) demonstrate the incremental reduction in tryptophan hydroxylase-2 immuno-positive cells in the myenteric plexus from healthy control, to ganglionic bowel in HSCR through to aganglionic bowel. The bar graph in (E) demonstrates the mean cell counts of TPH2 immuno-positive cells, with a reduction seen particularly in transition zone and aganglionic bowel, and slight variation seen in the ganglionic bowel of those with pre-operative HAEC *vs* those who did not. TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease; HAEC: Hirschsprung's-associated enterocolitis.

colon, the enteric nervous system (ENS) is arranged into two plexuses: a submucosal plexus and a plexus that lies between the two deep smooth muscle layers, known as the myenteric plexus. The role of the myenteric plexus primarily concerns intestinal motility while the submucosal plexus regulates a myriad of epithelial functions such as enteric blood flow, immunity, release of enteric peptides from enteroendocrine cells, and epithelial transport[1,12]. It follows that the absence of a functioning ENS in the colon of those with HSCR will lead to dysregulation of these processes and, consequently, enterocolitis.

Although only 5% of 5-HT in the colon is neuronal in origin, it is notable that, in TPH1 knockout mice, gastrointestinal motility is preserved, even with stripping of the mucosa, indicating that only neuronal 5-HT is involved in gastrointestinal motility[5]. Additionally, animal models have demonstrated that neuronal 5-HT acts as a growth factor during enteric neurogenesis, promoting the development and survival of dopaminergic and GABAergic neurons[5,13]. It is thought that serotonergic neurons are large descending interneurons which, as well as being calbindin-positive, are cholinergic[14]. Our

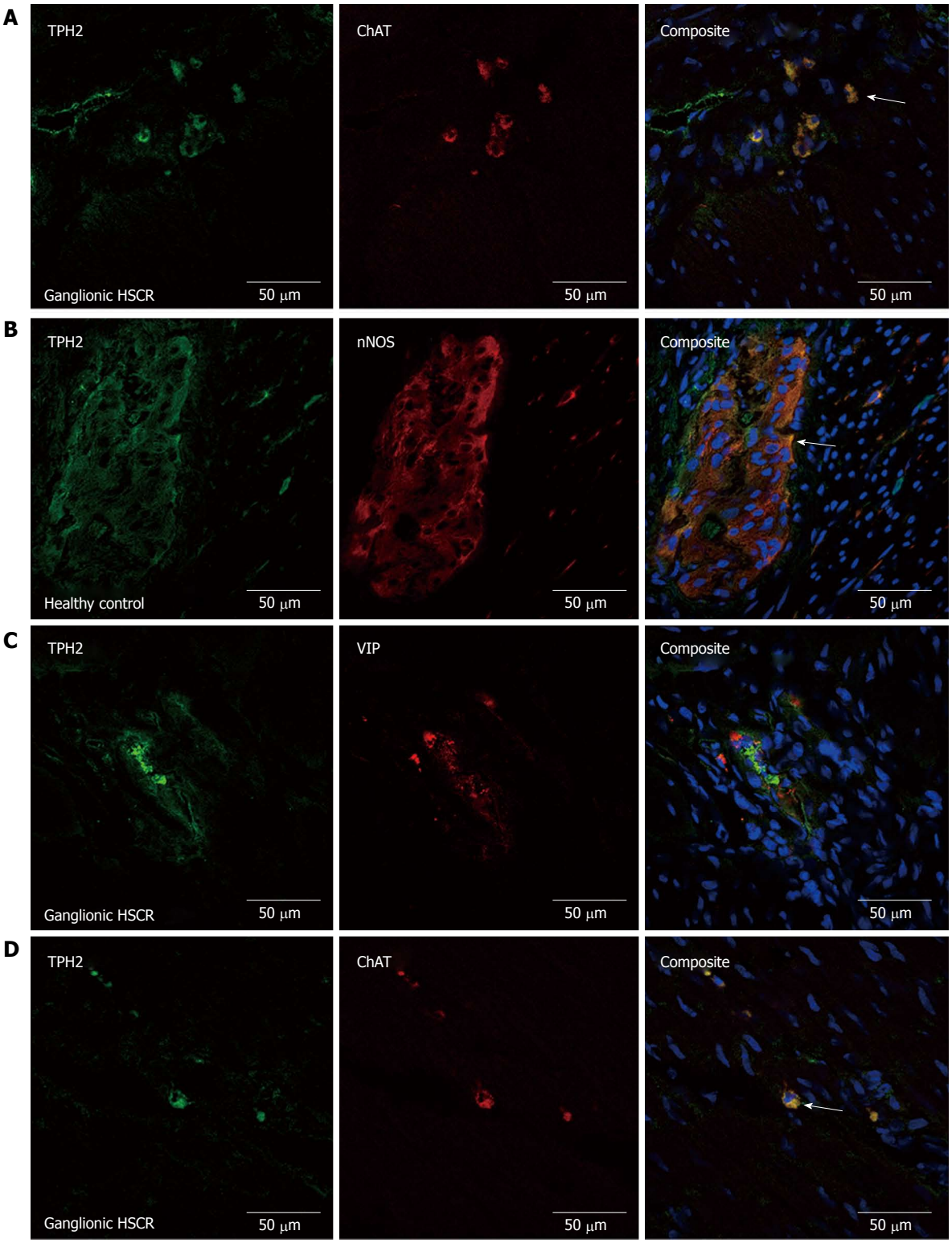

Figure 4 Confocal micrograph series. Confocal micrograph series demonstrating the cholinergic nature of TPH2 immuno-positive neurons in the myenteric plexus (A), with ChAT-TPH2 co-expression (white arrow). There was also co-expression of nNOS and TPH2 in the myenteric plexus (B) (white arrow) and submucosal plexus (not shown). VIPergic neurons did not express TPH2 in the myenteric plexus (C). Image (D) shows ChAT immune-positive cholinergic nerve fibres in the circular muscle layer, co-expressing TPH2. TPH2: Tryptophan hydroxylase-2.

findings concur with this observation, as we observed consistent co-expression of TPH2 with ChAT, a key enzyme in acetyl choline synthesis, in circular muscle nerve fibers.

Serotonergic nerves have previously been shown to make extensive synapses with myenteric ICCs, as well as nitrergic neurons. This indicates a functional role for serotonergic neurons in modulating nitrergic neurotransmission and pacemaker activity. Okamoto *et al*[15] has also observed that most colonic nitrergic

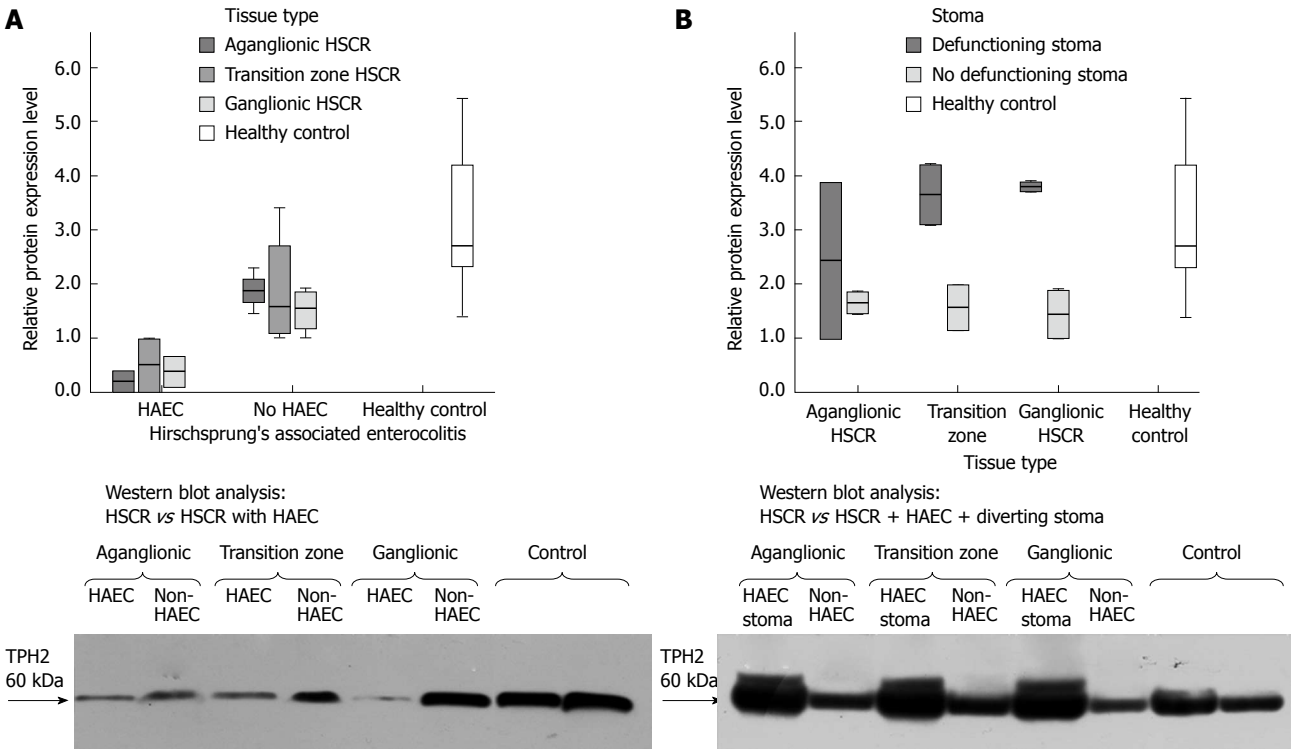

Figure 5 Western blot analysis. Western blot analysis is seen in (A) showing reduced expression of TPH2 in the colon of children with HSCR complicated by HAEC, who were managed non-operatively prior to pull-through surgery. The reduction in expression is seen across the aganglionic and ganglionic bowel of these patients. Image (B) shows the impact of diverting stoma formation on TPH2 expression in children with HSCR and pre-operative HAEC, with increased expression seen when compared to children without a history of HAEC and no stoma. In both (A) and (B), protein expression has been normalized against the loading control, GAPDH (36kDa). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease; HAEC: Hirschsprung's-associated enterocolitis.

neurons and submucosal ICCs were richly supplied by serotonergic varicosities in murine colon and suggested a potential role for neuronal 5-HT in the regulation of slow wave electrical activity. Liu *et al*[16] demonstrated that 5-HT acts on the 5-HT$_3$ receptor on ICCs, wherein the receptor functions as a Ca^{2+}-influx mechanism to augment pacemaker activity in ICCs. The intimate spatial arrangement of serotonergic nerves with nNOS-positive nerves is also important as it is thought that, through the intercession of descending inhibitory serotonergic neuronal activity, the release of nitric oxide from nNOS-positive neurons suppresses excitatory cholinergic activity and limits the rate at which colonic migrating motor complexes (CMMC) are propagated in mice[14]. The equivalent peristaltic activity to the CMMC in humans is the high-amplitude propagating contraction.

A close spatial arrangement of myenteric ICC fibers with TPH2-positive ganglia was demonstrated in our study, with co-expression of TPH2 and nNOS in myenteric and submucosal nitrergic ganglia and submucosal ANO-1 positive ICCs. In addition, we have demonstrated co-expression of TPH2 in the relatively recently described PDGFRα^+ cells. These fibroblast-like cells share morphological similarities with ICCs but are c-kit negative[17]. It is thought that they play a role in transducing purinergic neurotransmission through the activity of apamin-sensitive small-conductance Ca^{2+}-activated K^+ (SK3) channels[18]. Our immunofluorescence findings suggest a possible functional role for serotonergic neurons in modulating PDGFRα^+ cell function. It is inferable from the current evidence in the literature, that reduced density of serotonergic nerves in aganglionic and transition zone bowel and, in some patients, the ganglionic bowel, in HSCR, would disturb the inhibitory mechanisms required to maintain normal colonic motility. This is of particular relevance in patients with HAEC.

Mucosal 5-HT, secreted by EC cells, has been demonstrated in animal models to be pro-inflammatory, probably due to its activation of 5-HT receptors on dendritic cells in the lamina propria[8]. In other inflammatory conditions of the bowel, such as ulcerative colitis, reduced levels of the SERT have been reported, leading to increased 5-HT availability[8]. Conversely, neuronal 5-HT has been shown to be neuroprotective. *In vitro* and *in vivo* studies have demonstrated that neuronal 5-HT acts *via* 5-HT$_{2B}$ and 5-HT$_{4A}$ receptors respectively to promote survival of ICCs and enteric neurons respectively[7,8].

It has long been recognized that significant enteric neural damage occurs in severe inflammatory conditions of the colon, particularly in the setting of necrotizing enterocolitis[19-21]. Our finding of reduced expression of TPH2 in the aganglionic, transition zone and ganglionic bowel in HSCR complicated by

HAEC, compared with patients unaffected by HAEC and controls, is probably reflective of enterocolitis-mediated neuronal damage. The vicious circle of enterocolitis and loss of neuroprotective serotonergic neurons may thus occur. We have previously reported on the outcomes of children with HSCR complicated by HAEC. It was found that approximately one third of patients in the series experienced enterocolitis both pre- and post-operatively[2]. Disturbances in colonic function were also more common in these patients at long-term follow-up[2]. That our findings represent effect rather than cause is supported by the finding that levels of TPH2 greatly recover in the defunctioned colon of children with HSCR complicated by HAEC who were treated with diverting colostomy due to failure of medical management.

One striking characteristic of the population from whom pull-through specimens were collected for this study is the high proportion of children with trisomy 21 at 41.2%. The incidence of trisomy 21 in Ireland, at 1 in 546 live births, is the highest in Europe[22]. Children with trisomy 21 are recognized to be at a considerably higher risk of developing HAEC (approximately 51%) compared to those without trisomy 21[23]. In our study 2 of the 5 patients who developed pre-operative HAEC had trisomy 21, both of whom were treated with a diverting stoma, indicating the severe nature of their enterocolitis.

In conclusion, we have demonstrated that serotonergic neurons co-express TPH2 with ANO1-immuno-positive ICCs in the submucosa, even in aganglionic bowel. We have also shown, for the first time, co-expression of TPH2 in PDGFRα^+ cells, suggesting a possible role for serotonergic modulation of their function. TPH2 expression was reduced in the myenteric plexus and deep smooth muscle layers of aganglionic colon in children with HSCR unaffected by pre-operative HAEC. However, in children with HSCR complicated by HAEC, TPH2 expression was reduced in both aganglionic and ganglionic bowel - a finding that was reversed in the colon of children treated with diverting stoma formation due to HAEC refractory to non-operative strategies. HAEC-mediated serotonergic neuronal damage may contribute to ongoing problems with colonic function and recurrent enterocolitis despite properly performed corrective pull-through surgery.

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COMMENTS

Background

Hirschsprung's disease (HSCR) is the most common congenital gut motility disorder. It may be complicated by a severe pancolitis known as Hirschsprung's-associated enterocolitis (HAEC). Despite constituting only approximately 1%-5% of total intestinal serotonin (5-HT), neuronal 5-HT plays a key role in modulating gut motility and is thought to have an anti-inflammatory, neuroprotective role, in contrast to mucosal 5-HT which is pro-inflammatory.

Research frontiers

Dysregulation of mucosal 5-HT transport has already been implicated in ulcerative colitis and other inflammatory disorders of the human colon. While a previous study has described a reduction of enterochromaffin cells, which produce mucosal 5-HT, in the ganglionic colon of children with a history of HAEC, neuronal 5-HT expression has yet to be evaluated in HSCR. In mice, knockout of tryptophan hydroxylase 2 (TPH2), the key enzyme in the synthesis pathway of neuronal 5-HT, leads to slow gastrointestinal transit and severe intestinal inflammation. Conversely, knockout of TPH1, the key enzyme in the synthesis of mucosal 5-HT, has no effect on gastrointestinal motility.

Innovations and breakthroughs

It is known that patients with HSCR who experience pre-operative HAEC are at an increased risk of poor functional outcome despite optimal surgical treatment, with some patients continuing to experience severe constipation and recurrent enterocolitis even in the absence of a mechanical obstruction. The causes for this have yet to be fully elucidated. Current findings by the authors suggest that neuronal 5-HT is deficient in the ganglionic colon of children with a history of pre-operative HAEC. Given its anti-inflammatory, neuroprotective roles, our findings suggest a mechanism by which post-operative enterocolitis may occur.

Applications

5-HT and its receptors are some of the most important current pharmacological targets in the alteration of gut motility. While the authors have indirectly shown a reduction in neuronal 5-HT in the healthy ganglionic colon in HSCR after HAEC, it is unclear if this finding would persist at follow-up after a pull-through operation, as expression of TPH2 appeared to have recovered in patients who underwent levelling or defunctioning colostomy. These findings lay the foundation for future work examining whether the abnormalities detected in this study persist at follow-up, as well an empiric evaluation of the functional outcomes of patients in this study.

Terminology

Tryptophan hydroxylase 2 (TPH2) is an enzyme which catalyzes the conversion of L-tryptophan to 5-hydroxytryptophan, which is the critical rate-limiting step of neuronal 5-HT synthesis. It is an isozyme of TPH1, which catalyzes the same reaction in the synthesis of mucosal 5-HT in enterochromaffin cells.

Peer-review

This is a clearly presented study, addressing an important aspect of the role of neuronal 5-HT in Hirschprung's disease.

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ORIGINAL ARTICLE

Basic Study

Steatotic livers are susceptible to normothermic ischemia-reperfusion injury from mitochondrial Complex-Ⅰ dysfunction

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Author contributions: Chu MJJ, Premkumar R and Hickey AJR equally contributed to this paper; Chu MJJ designed the study, carried out experiments, performed data analysis, drafted manuscript, and revised the manuscript; Premkumar R designed the study, carried out animal surgery, and revised the manuscript; Hickey AJR designed the study, analyzed the data, and revised the manuscript; Jiang Y performed statistical analysis and revised the manuscript; Delahunt B performed histological analysis and revised the manuscript; Phillips ARJ designed the study, analyzed the data, and revised the manuscript; and Bartlett ASJR designed the study and revised the manuscript.

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Abstract

AIM: To assess the effects of ischemic preconditioning (IPC, 10-min ischemia/10-min reperfusion) on steatotic liver mitochondrial function after normothermic ischemia-reperfusion injury (IRI).

METHODS: Sixty male Sprague-Dawley rats were fed

8-wk with either control chow or high-fat/high-sucrose diet inducing > 60% mixed steatosis. Three groups (n = 10/group) for each dietary state were tested: (1) the IRI group underwent 60 min partial hepatic ischemia and 4 h reperfusion; (2) the IPC group underwent IPC prior to same standard IRI; and (3) sham underwent the same surgery without IRI or IPC. Hepatic mitochondrial function was analyzed by oxygraphs. Mitochondrial Complex-Ⅰ, Complex-Ⅱ enzyme activity, serum alanine aminotransferase (ALT), and histological injury were measured.

RESULTS: Steatotic-IRI livers had a greater increase in ALT (2476 ± 166 *vs* 1457 ± 103 IU/L, P < 0.01) and histological injury following IRI compared to the lean liver group. Steatotic-IRI demonstrated lower Complex-Ⅰ activity at baseline [78.4 ± 2.5 *vs* 116.4 ± 6.0 nmol/(min·mg protein), P < 0.001] and following IRI [28.0 ± 6.2 *vs* 104.3 ± 12.6 nmol/(min·mg protein), P < 0.001]. Steatotic-IRI also demonstrated impaired Complex-Ⅰ function post-IRI compared to the lean liver IRI group. Complex-Ⅱ activity was unaffected by hepatic steatosis or IRI. Lean liver mitochondrial function was unchanged following IRI. IPC normalized ALT and histological injury in steatotic livers but had no effect on overall steatotic liver mitochondrial function or individual mitochondrial complex enzyme activities.

CONCLUSION: Warm IRI impairs steatotic liver Complex-Ⅰ activity and function. The protective effects of IPC in steatotic livers may not be mediated through mitochondria.

Key words: Mitochondrial respiration; Fatty liver; Liver ischemia; Oxidative phosphorylation; Liver injury; Hepatic steatosis; Ischemic preconditioning

Core tip: We report a detailed mitochondrial function analysis of dietary-induced hepatic steatosis, which was not choline-deficient, during warm ischemia and after ischemia-reperfusion injury. We evaluated mitochondrial complex Ⅰ and Ⅱ activities as well as the impact of ischemic preconditioning on mitochondrial function. This study demonstrates that steatotic livers have decreased Complex-Ⅰ activity at baseline and that Complex-Ⅰ function is further impaired after warm ischemia-reperfusion injury. Ischemic preconditioning was unable to attenuate the harmful effect of ischemia-reperfusion on mitochondrial function.

Chu MJJ, Premkumar R, Hickey AJR, Jiang Y, Delahunt B, Phillips ARJ, Bartlett ASJR. Steatotic livers are susceptible to normothermic ischemia-reperfusion injury from mitochondrial Complex-Ⅰ dysfunction. *World J Gastroenterol* 2016; 22(19): 4673-4684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4673.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4673

INTRODUCTION

Hepatic steatosis is the most common liver disease found in clinical liver biopsies[1], and autopsy-based studies estimate that the prevalence of hepatic steatosis is 15%-30% in the Western world[1]. Consequently, the number of patients with hepatic steatosis encountered during liver surgery is increasing. Hepatic steatosis has been associated with a 2-3 fold increase in post-operative complication rates following liver resection[2,3]. It has been proposed that steatotic livers are more susceptible to ischemia-reperfusion injury (IRI), which impairs liver regeneration and is a major cause of liver damage, leading to worse outcomes[3].

The exact mechanism for the increased susceptibility of steatotic livers to IRI is not fully understood. Steatotic livers have been shown to have decreased recovery of adenosine triphosphate (ATP) concentrations following IRI[4]. One of the proposed underlying mechanisms behind the decreased ATP recovery and increased steatotic liver susceptibility to IRI is mitochondrial dysfunction[5]. Mitochondria are responsible for producing the bulk of cellular ATP and are, therefore, fundamental for cellular viability[6]. Impaired mitochondrial function (MF) disrupts normal cellular bioenergetics, which leads to cell death[7].

To attenuate the deleterious effect of IRI, ischemic preconditioning (IPC) of the liver has been used[8]. IPC involves a brief period of ischemia followed by reperfusion (generally, 10 min ischemia and 10 min reperfusion) prior to a period of sustained ischemic insult[9,10]. IPC has been reported to improve post-IRI liver injury in experimental[9] and clinical[10] steatotic livers. IPC has also been reported to improve ATP levels in steatotic livers[11]. While the mechanism by which IPC improves hepatic outcome following IRI is unknown, it has been postulated that IPC modulates and somehow preserves MF[9]. The aim of this study was to evaluate the impact of IPC on the MF of rat livers with steatosis after being subjected to warm (normothermic) IRI. Mitochondrial bioenergetics and liver injury markers were evaluated.

MATERIALS AND METHODS

Animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise specified. All experiments were performed in 11-wk old male Sprague-Dawley rats. The animal protocol was designed to minimize discomfort to the animals. The animals were enrolled when 3-wk old and removed from the mother at this age. They were then randomized to receive standard chow (lean animals; Teklad TB 2018; Harlan, Madison, WI, United States; 18% kcal fat, 58% kcal carbohydrate) or a high-fat/high-sucrose diet (steatotic animals; Rodent Diet D03021303; Research Diets, Inc., New Brunswick, NJ, United States; 45 kcal% fat, 25% kcal sucrose; Table

Table 1 Content of high-fat/high-sucrose diet (D03021303; Research Diets, Inc., NJ, United States)

	Gram%	Kcal%
Protein	23.7	20
Carbohydrate	41.4	35
Fat	23.6	45
Total		100
Kcal/g	4.73	
Ingredient		
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	50	200
Maltodextrin 10	45.6	182
Sucrose	250	1000
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	177.5	1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate. 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD and C Blue Dye #1	0.05	0
Total	858.15	4057

1)[12]. The animals were kept under a 12-h light/dark cycle (50%-70% humidity, 22 ± 2 ℃) with *ad libitum* access to food and water. Bodyweight and blood glucose were measured weekly. Rats were fasted for 6 h prior to surgery to mimic pre-operative fasting. All surgical procedures were started between 12:00-1:00 pm. All experiments were approved by the University of Auckland Animal Ethics Committee (R965).

Experimental design and surgical procedures

Sixty animals were randomized into one of six groups (n = 10 each): (1) Lean + Sham (Lean-Sham); (2) Lean + IRI (Lean-IRI); (3) Lean + IRI + IPC (Lean-IPC); (4) Steatotic + Sham (Steatotic-Sham); (5) Steatotic+IRI (Steatotic-IRI); and (6) Steatotic + IRI + IPC (Steatotic-IPC).

A model of partial (70%) hepatic ischemia was used that prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes[9]. Rats were anesthetized with isofluorane inhalation. Following tracheostomy, anesthesia was maintained (1%-2% isofluorane) through a pressure-controlled ventilator (Kent Scientific Corporation, Torrington, CT, United States). Core body temperature was maintained (37-38 ℃) by a thermostatically-controlled warming plate. Fluid administration *via* the right femoral vein and mean arterial pressure monitoring *via* the right carotid artery were undertaken with a radio-opaque 22G catheter and 2F solid-state pressure transducer (SPR-320 pressure catheter; Millar Instruments Inc., Houston, TX, United States), respectively.

Following a transverse laparotomy, the hepatic artery and portal vein to the left and median lobes were occluded for 60 min with a microvascular clip. Removal of the clip initiated the 240 min of reperfusion. Rats receiving IPC received 10 min of ischemia and 10 min of reperfusion prior to 60 min of ischemia. In the sham group, the rats were anesthetized, and a laparotomy was performed for 5.5 h without induction of ischemia.

The placement of a tracheostomy (reflecting endotracheal intubation) and prolonged anesthesia mimic clinical liver resection whereby patients are anesthetized continuously. We did not perform short intervals of anesthesia and repeated mini-laparotomies for our protocol, as it does not reflect clinical practice.

Tissue collections

Liver samples were obtained by "cheese-wire" ligating the liver with 4-0 silk tie. This technique caused minimal bleeding from the cut surface of the liver and allowed repeated sampling from each rat. Liver samples were obtained at various time-points: "A": baseline (immediately following laparotomy), "B": 10 min (after 10 min ischemia), "C": 20 min (end of IPC), "D": 80 min (after 60 min ischemia), and "E": 320 min (end of 240 min reperfusion phase). Liver samples were obtained for histology, MF, and enzymatic analysis. The amount of liver tissue removed did not exceed 20% of total hepatic mass. At the end of the procedure, serum (5 mL) was collected from the inferior vena cava.

Histology

Histology was performed on time-points A and E to assess severity of the hepatic steatosis and IRI, respectively. Formalin-fixed and paraffin-embedded liver samples were stained with hematoxylin and eosin (HE). A consultant specialist histopathologist (BD) blinded to the group assessed the severity of steatosis with a published clinical grading system[13]. Severity of the IRI was assessed using a 4-point grading system previously described[14].

Assessment of hepatocyte injury

The severity of hepatic injury was assessed by serum levels of alanine aminotransferase (ALT) and was analyzed using a Roche Cobas 8000 modular analyzer (c702 module, Basel, Switzerland).

Homogenized tissue preparation for mitochondrial function analysis

Liver samples were immediately placed in ice-cold (about 4 ℃) mitochondrial respiration media (Table 2). The samples were then removed from the media, blotted, weighed (20-30 mg), and placed in a 2-mL flat-bottom scintillation vial with 500 μL of mitochondrial respiration media. The sample was homogenized for 5 s with an Omni TH homogenizer (Omni International, Kennesaw, GA, United States) before analysis. Homogenates were utilized as they

Table 2 Mitochondrial respiration media

Chemical	Final concentration (mmol/L)
EGTA	0.5
$MgCl_2$	3
K-Lactobionate	60
Taurine	20
KH_2PO_4	10
Sucrose	110
Bovine serum albumin	1 mg/mL
HEPES	20 (pH 7.0 at 37 ℃)

are more physiological and decrease the risk of any organelle selection bias inherent to the process of mitochondrial isolation, allowing for the preferential selection of more healthy organelles during isolation[15]. Our protocol also permits shorter processing time and rapid measurement of the function of the entire mitochondrial population within the tissue to provide an immediate measure of mass specific flux.

Mitochondrial respiration assays

Mitochondrial respiration of liver homogenate was measured at each time-point. Respiration was measured in 2-mL chambers using an OROBOROS Oxygraph 2K (Anton Paar, Graz, Austria) at 37 ℃ in mitochondrial respiration media, with a calculated saturated oxygen concentration of 190 nmol O_2 per milliliter at 100 kPa barometric pressure, and oxygen flux calculated using the DatLab 5 analysis software. Liver homogenate (50 μL) was added to each chamber, and the remainder was stored at -80 ℃ for later analysis. To account for potential variations in mitochondrial mass, citrate synthase (CS)-normalized[16] oxygen flux (pmol $O_2.s^{-1}.U\ CS^{-1}$) was calculated.

A multiple substrate-inhibitor titration protocol was used to explore relative contributions of complex Ⅰ (CI), complex Ⅱ (CⅡ), and combined CI+CⅡ in the electron transport system (ETS). Respiration states were defined according to Gnaiger[17], where leak respiration and oxidative phosphorylation (OXPHOS) were the flux measured before and after addition of adenosine diphosphate (ADP), respectively. The assay protocol steps are described in Table 3. The integrity of tissue preparations and comparison of coupling efficiencies were made from the respiratory control ratio (RCR).

Citrate synthase and total protein measurement

Liver homogenate, as used in mitochondrial respiration assay, was analysed for CS activity and total protein content. CS activity was measured as a surrogate for mitochondrial mass[16]. Frozen (-80 ℃) liver homogenate was thawed, and CS activities were determined following the method of Srere and modified to microtitre-plate[18]. Protein content was determined by the Biuret test with bovine serum albumin as standard.

Complex Ⅰ and Complex Ⅱ activities

Liver homogenate from time-points A, D, and E were used to measure individual CI and CⅡ enzyme activities using the NADH-oxidation and dichlorophenolindophenol-oxidation method, respectively[19]. Frozen liver homogenate was thawed and centrifuged for 10 min at 600 *g* (4 ℃), and the supernatant used for analysis. Assays were conducted using a 96-well format, and results were normalized to total protein content, as determined by the Biuret method. In this report, "CI function" refers to assessments undertaken in the oxygraph, and "CI activity" refers to the isolated activity studies just described.

Statistical and data analysis

All study data were recorded in an EXCEL database. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, United States) and SAS version 9.2 (SAS institute, Cary, NC, United States). Statistical tests were set at a 5% significance level (two-sided). Student's *t*-tests were conducted on body weight and random blood glucose level to compare obese and lean rats. Difference in outcome measure between the groups of interest was tested using the analysis of covariance regression, adjusting for baseline value, bodyweight, and blood glucose measured before the procedures as appropriate. Repeated measures mixed model was used to evaluate the treatment differences at different time-points, controlling for the correlation of data collected on the same animal. The results are presented as mean ± SE of mean, with associated *P*-value.

RESULTS

Rats fed high-fat/high-sucrose diet were obese

Sprague-Dawley rats fed high-fat/high-sucrose diet for 8 weeks showed increased body weight (507 ± 10 *vs* 437 ± 6 g, $P < 0.0001$; Figure 1A) and increased random blood glucose level (6.1 ± 0.1 *vs* 4.6 ± 0.1 mmol/L, $P < 0.0001$; Figure 1B) relative to age-matched lean rats.

Obese rat livers had severe mixed steatosis

Obese rat livers had gross macroscopic fat accumulation. All lean rat livers had normal baseline underlying tissue architecture with only some mild (8% ± 1%) microvesicular steatosis when evaluated by H&E staining (Figure 1C). Obese rat livers had severe (65% ± 3%) baseline mixed steatosis with prominent macrovesicular steatosis features evident (Figure 1D). There were no signs of fibrosis or inflammation in any of the groups consistent with hepatic steatosis.

Steatotic livers had increased liver injury following ischemia-reperfusion

Effect of steatosis, IRI, and IPC on liver injury biomarkers,

Table 3 Mitochondrial respiration assay protocol

Reagents added	Final concentration in oxygraph chamber (mmol/L)	Action of reagent	Measurement output
Step 1			
Glutamate	10	CI substrates	CI leak respiration
Malate	5		(CI_{Leak})
Pyruvate	10		
Step 2			
ADP	1.25	Substrate for ATP generation	CI oxidative phosphorylation
Step 3			
Succinate	10	CⅡ substrate	CI+CⅡ oxidative phosphorylation[1]
Step 4			
Rotenone	0.001	CI inhibitor	Isolate flux to CⅡ [CⅡ(rot)]
Step 5			
Oligomycin	0.0025	ATP-Synthase inhibitor	CI+CⅡ leak respiration ($CI,_{ⅡLeak}$)
Step 6			
FCCP	0.0015	Mitochondrial uncoupler	ETS capacity
Step 7			
Antimycin A	0.0050	CⅢ inhibitor	Residual oxidase consumption

[1]The individual contribution of CⅡ to oxidative phosphorylation (CⅡ-OXPHOS) can also be derived (CI+Ⅱ-OXPHOS minus CI-OXPHOS). ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CI: Complex Ⅰ; CI-OXPHOS: Complex Ⅰ oxidative phosphorylation; CI+Ⅱ-OXPHOS: Complex Ⅰ + Complex Ⅱ oxidative phosphorylation; $CI,_{ⅡLeak}$: Complex Ⅰ + Complex Ⅱ leak respiration; CI_{Leak}: Complex Ⅰ leak respiration; CⅡ: Complex Ⅱ; CⅢ: Complex Ⅲ; ETS: Electron transfer system; FCCP: Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone; Rot: Rotenone.

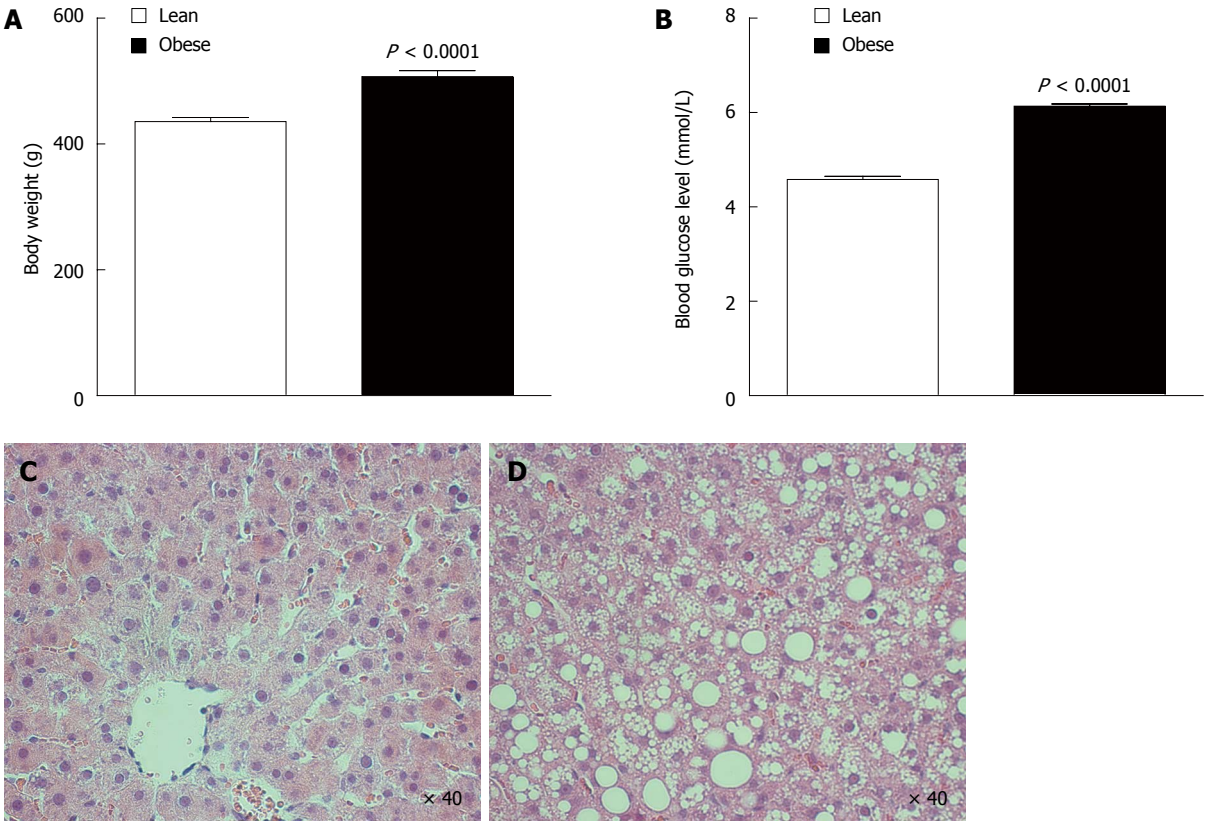

Figure 1 Bodyweight, random blood glucose, and baseline histology of high-fat/high-sucrose-fed (obese) and lean Sprague-Dawley rats. Obese Sprague-Dawley rats were significantly heavier (A) with higher random blood glucose (B) than age-matched lean rats. Baseline liver tissue sections were stained for hemotoxylin and eosin (x 40 magnification). Representative slides are displayed, and obese rats (D) showed severe mixed hepatic steatosis while lean rat livers (C) showed mild microvesicular steatosis. Data are shown as mean ± SE (n = 30 rat/group). Statistical analyses were performed using Students t-tests for body weight and random blood glucose.

tissue injury scores, and histology are shown in Figure 2 and 3, respectively. Both Lean-Sham and Steatotic-Sham livers did not have any biochemical (Figure 2A) or histological evidence of injury induced by the sham surgery (Figures 2B, 3A and 3B). Conversely, IRI was associated with increased serum ALT (Figure 2A) and worse liver histology injury scores in both Steatotic-IRI and Lean-IRI rats compared to Steatotic-Sham and

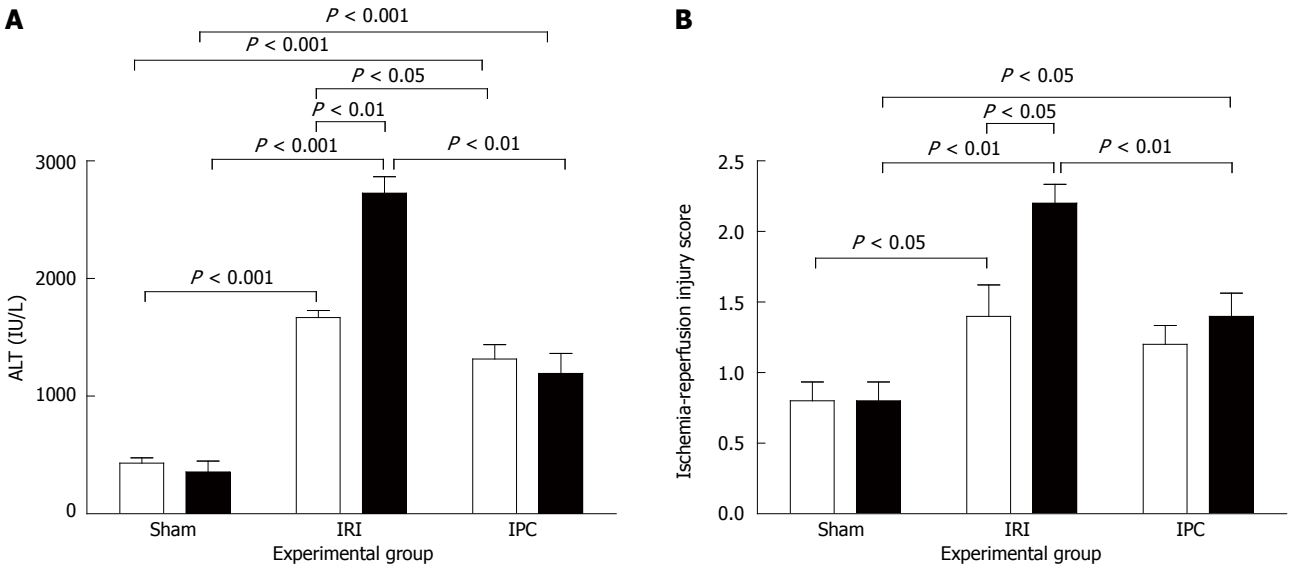

Figure 2 Serum alanine aminotransferase levels and histology injury score following ischemia-reperfusion. Serum alanine aminotransferase (ALT) (A) and histology injury score (B) following reperfusion were significantly higher in rats subjected to IRI compared to sham rats. Both injury markers were significantly higher in obese rats compared to lean rats. Compared to corresponding IRI groups, IPC decreased ALT levels in both lean and obese rats and decreased injury score in obese rats. Data are shown as mean ± SEM (n = 10 rat/group; lean rats, open bar; obese rats, closed bar). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

Lean-Sham rats, respectively (Figure 2B). These same injury markers were also found to be significantly higher in Steatotic-IRI rats compared to Lean-IRI rats (Figure 2). IPC led to improvement in the serum ALT in Steatotic-IPC and Lean-IPC rats compared to Steatotic-IRI and Lean-IRI rats, respectively (Figure 2A). IPC also decreased injury score in Steatotic-IPC rats compared to Steatotic-IRI rats (Figure 2B). These results indicate that IPC was able to attenuate liver injury in steatotic livers.

Baseline mitochondrial function in steatotic livers were similar to lean livers

The baseline mitochondrial functions were similar between steatotic and lean rat livers. Baseline (T = 0) samples from steatotic rat livers (n = 30) had similar MF to lean rat livers (n = 30) (Figure 4A-F). These results indicate that steatotic liver mitochondria were initially functioning adequately *in vivo*.

Sham-operated rat liver mitochondrial function remained stable

There were no changes in MF in both Lean-Sham and Steatotic-Sham livers with a stable CI-OXPHOS, CⅡ-OXPHOS, and RCR throughout all time-points (Figure 4A-F). These data indicate that the act of repeated liver sampling from each rat did not in itself significantly influence the underlying MF.

Prolonged ischemia led to impaired mitochondrial function

At the end of 60 min of ischemia, both lean and steatotic livers demonstrated impaired MF with significantly lower CI-OXPHOS (about 30%-40%), CⅡ-OXPHOS (45%-60%), and RCR (about 60%-80%) compared to pre-ischemic levels or corresponding Sham livers (Figure 4A-F). There was no observable difference in MF between Lean-IRI and Steatotic-IRI livers at the end of ischemia. These findings indicate impaired MF occurs to the same extent in all groups immediately following 60 min of ischemic insult.

Reperfusion injury led to decreased Complex Ⅰ mediated respiration in steatotic livers

After 60 min of ischemia and 240 min of reperfusion, MF in Lean-IRI returned to pre-ischemic levels and was comparable to Lean-Sham livers (Figure 4A, C, E). In Steatotic-IRI livers, CI-OXPHOS flux and RCR were significantly lower compared to baseline levels or Steatotic-Sham livers (Figure 4B, F) whereas CⅡ-OXPHOS flux rates returned to pre-ischemic levels and was comparable to Steatotic-Sham livers (Figure 4D). CI-OXPHOS flux rates and RCR in Steatotic-IRI livers were observed to be 57% and 54% relative to Lean-IRI livers post-reperfusion (P < 0.01). These results indicate that, unlike lean livers, steatotic liver CI function was impaired by IRI, leading to decreased RCR.

Ischemic preconditioning had no significant effect on hepatic MF

MF in both types of livers subjected to IPC was similar to livers subjected to IRI only (Figure 4A-F). Lean-IPC and Steatotic-IPC livers demonstrated a similar pattern of CI-OXPHOS, CⅡ-OXPHOS, and RCR at each of the sampling time-points as Lean-IRI and Steatotic-IRI livers, respectively (Figure 4A-F). Post-reperfusion, CI-OXPHOS, and RCR in Steatotic-IPC livers remained impaired, while CⅡ-OXPHOS was comparable to pre-ischemic levels. These results indicate that despite the improvement in injury markers (ALT and histology)

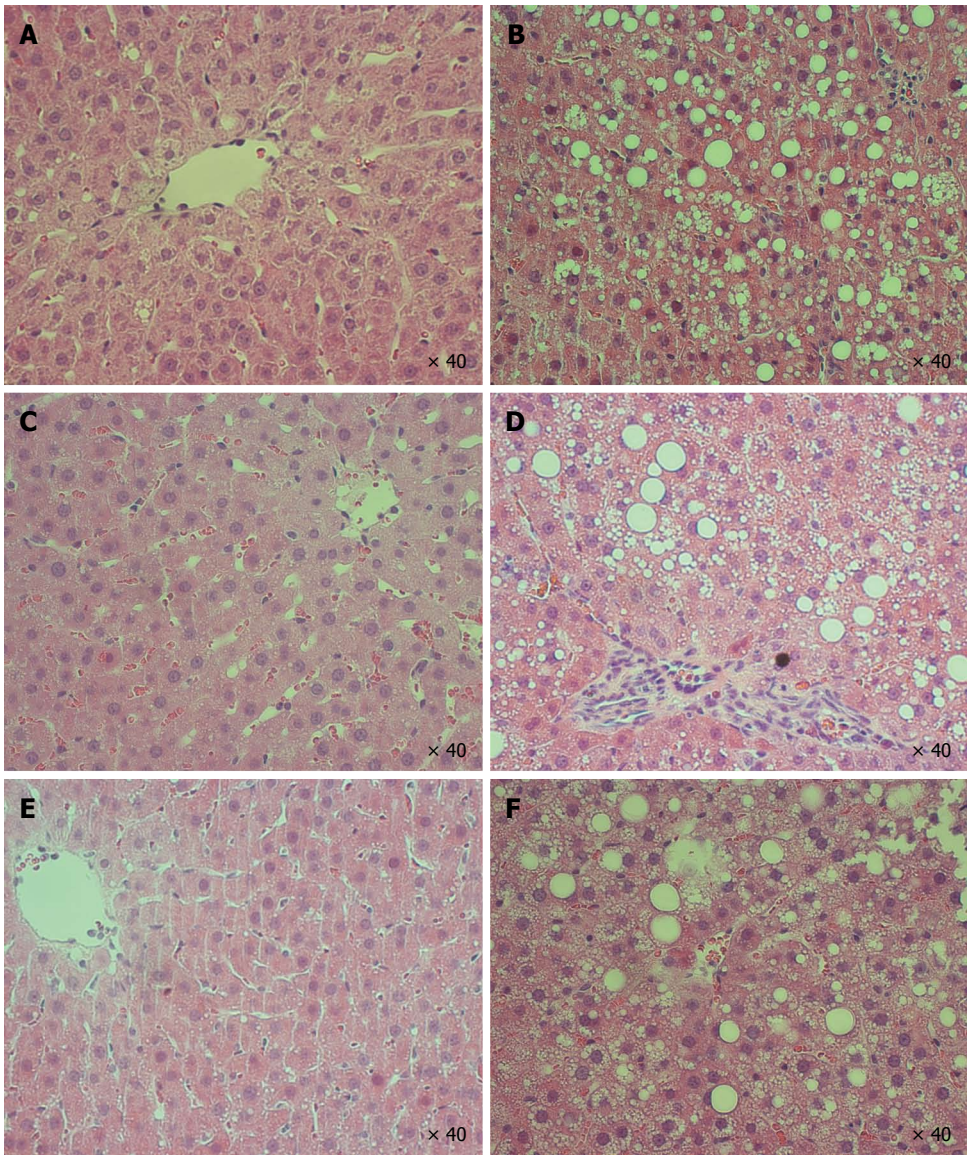

Figure 3 Liver histology following ischemia-reperfusion. No evidence of injury was observed in Lean-Sham (A) and Steatotic-Sham (B) livers. Lean-IRI livers (C) had mild injury while Steatotic-IRI livers (D) had moderate-severe injury. Both Lean-IPC (E) and Steatotic-IPC livers (F) were observed to have mild injury following reperfusion. Representative slides are shown (n = 10 rat/group). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

in IPC livers (Figures 2 and 3), IPC did not influence underlying MF over this timeframe.

Citrate synthase activity was unaffected by ischemia-reperfusion injury

Citrate synthase (CS) activity in lean and steatotic livers was stable throughout the experiment and was not affected by IRI or IPC (Figure 5A and B). There was also no difference in CS activity between lean and steatotic livers at all time-points measured.

Decreased Complex Ⅰ but not Complex Ⅱ enzymatic activity following reperfusion in steatotic livers

Baseline CI activity was significantly lower in steatotic livers compared to lean livers [78.4 ± 2.5 *vs* 116.4 ± 6.0 nmol/(min·mg protein), $P < 0.001$], while baseline CⅡ activity was similar between the two groups [104.9 ± 3.3 *vs* 116.8 ± 6.1 nmol/(min·mg protein), $P = 0.08$; Figure 5C and D]. Following 60 min of ischemia, both types of liver demonstrated significantly lower CI activity (Figure 5C and D) compared to pre-ischemic or sham livers. Steatotic liver CI activity was also observed to be lower post-ischemia compared to lean livers. Following reperfusion, CI activity returned to pre-ischemic levels in lean livers but remained significantly lower in steatotic livers by approximately 65% (Figure 5D). CⅡ activity (Figure 5E and F) was stable throughout the procedure, and there was no difference in CⅡ activity between steatotic and lean livers. IPC did not have any significant beneficial effect on CI and CⅡ activity in both types of livers. These activity results showed that IRI led to decreased CI activity in steatotic livers and also that IPC was not able to influence CI or CⅡ activities; all of which was

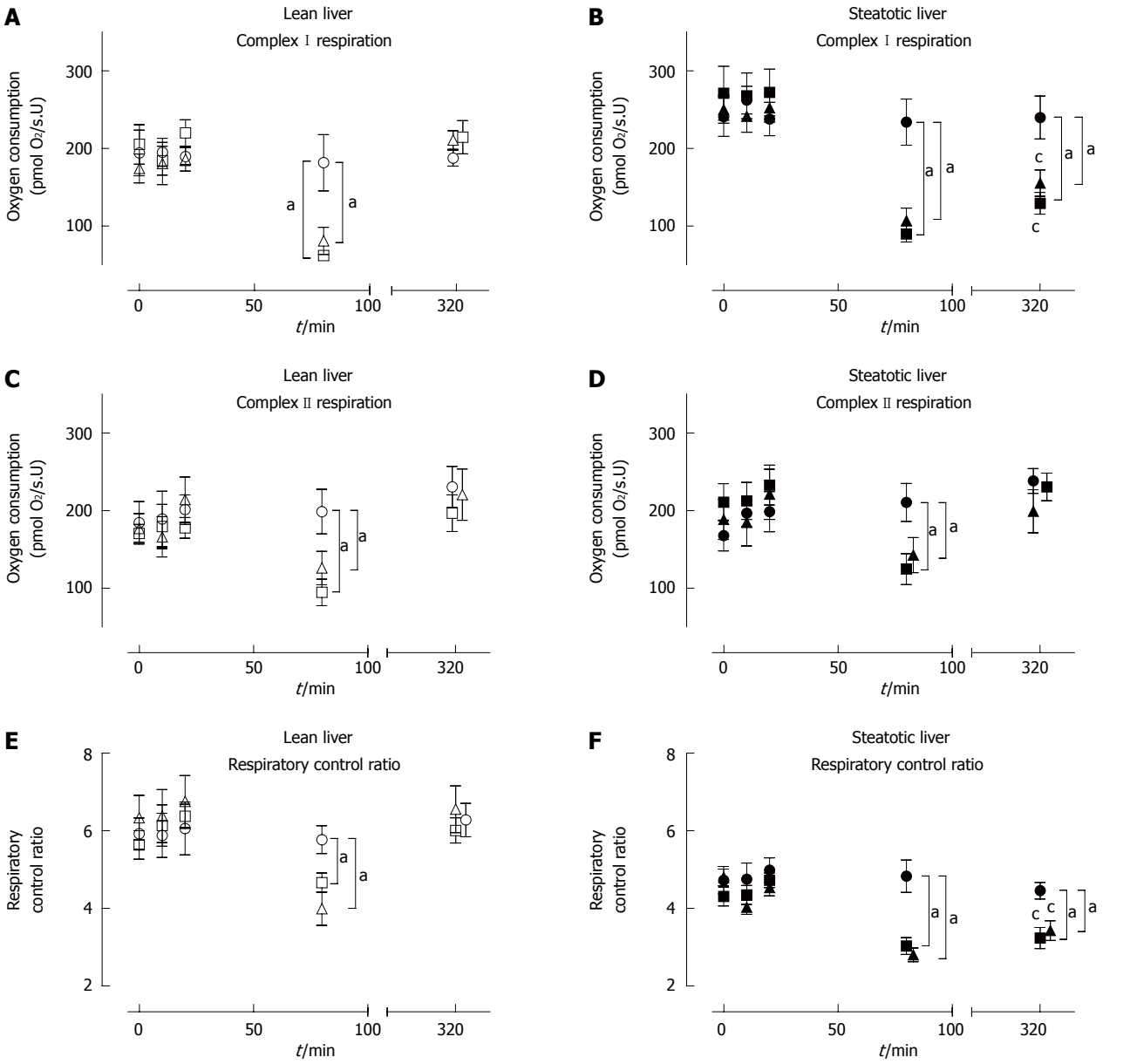

Figure 4 Mitochondrial function of lean and steatotic livers subjected to sham or ischemia-reperfusion injury with or without ischemic preconditioning. Baseline MF was similar between lean and steatotic livers in all outcome measures. Lean-Sham and Steatotic-Sham had stable CI-OXPHOS (A, B), C Ⅱ-OXPHOS (C, D), and RCR (E, F) throughout the procedure. CI-OXPHOS (A, B), C Ⅱ-OXPHOS (C, D), and RCR (E, F) were significantly lower following 60 min of ischemia in Lean-IRI, Lean-IPC, Steatotic- IRI, and Steatotic-IPC livers compared to the corresponding sham group. Following reperfusion, CI-OXPHOS (B) and RCR (F) were significantly lower in Steatotic-IRI and Steatotic-IPC livers compared to Steatotic-Sham or lean livers, while C Ⅱ-OXPHOS (D) returned to pre-ischemic levels comparable to Steatotic-Sham or lean livers (D). Data are shown as mean ± SE (n = 10 rat/group; Lean-Sham, open circle; Lean-IRI, open square; Lean-IPC, open triangle; Steatotic-Sham, closed circle; Steatotic-IRI, closed square; Steatotic-IPC, closed triangle). [a]$P < 0.05$ *vs* Lean-IRI; [c]$P < 0.05$ *vs* Lean-IPC (end of reperfusion). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

consistent with the earlier oxygraph functional analysis (above).

DISCUSSION

In this study, we used Sprague-Dawley rats with diet-induced hepatic steatosis. Compared to normal lean livers, steatotic livers demonstrated increased parenchymal injury following IRI, as indicated by their raised serum ALT and histology injury scores. Steatotic livers had lower baseline CI activity but similar baseline CS and C Ⅱ activity compared to lean livers. The steatotic livers were also observed to have decreased CI activity and function following IRI, which unlike lean livers, showed no recovery of either activity or function even after prolonged reperfusion times. This finding indicated that CI is a particularly vulnerable site of IRI-induced damage in steatotic livers. Our results demonstrated that IPC was effective in decreasing liver injury in both lean and steatotic livers according to ALT levels. However, this protective effect was not translated to measures of CI function or CI activity. In summary, these data demonstrate that steatotic livers developed significant underlying mitochondrial

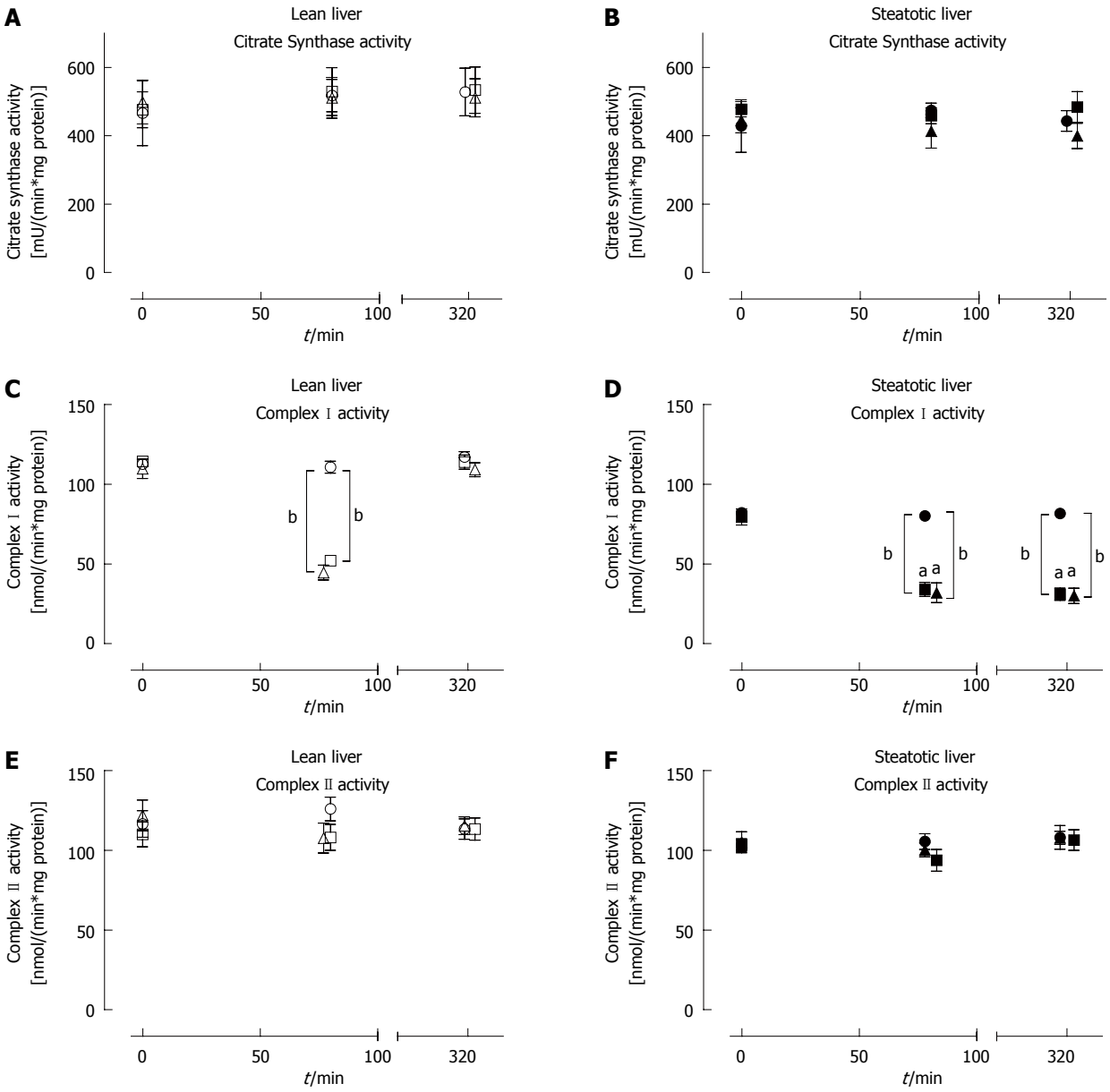

Figure 5 Citrate synthase, Complex Ⅰ and Complex Ⅱ activity at baseline, following ischemia and post-reperfusion in lean and steatotic livers. CS activity was similar between lean (A) and steatotic livers (B) throughout the procedure and was not affected by IRI or IPC. Baseline CI enzyme activity in steatotic livers were lower than lean livers (C, D). After ischemia, CI enzyme activity was significantly lower in lean and steatotic livers compared to sham livers. Additionally, CI enzyme activity (D) was lower in steatotic livers post-ischemia compared to lean livers. After reperfusion, CI activity remained lower in Steatotic-IRI and Steatotic-IPC livers (D) compared to Steatotic-Sham or lean livers. CⅡ activity (E, F) remained stable throughout the procedure and was similar between both types of livers. IPC did not have a significant effect on CI or CⅡ activity in both types of livers. Data are expressed as mean ± SE (n = 10 rat/group; Lean-Sham, open circle; Lean-IRI, open square; Lean-IPC, open triangle; Steatotic-Sham, closed circle; Steatotic-IRI, closed square; Steatotic-IPC, closed triangle). $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* time- and group-matched lean livers. CS: Citrate synthase; CI: Complex Ⅰ; CⅡ: Complex Ⅱ; IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

impairment that was worsened by IRI and not able to be recovered by IPC.

In this study we developed a novel methodology for the repeated procurement of liver samples from the same animal over time, which has not been published before. This technique not only decreased the number of animals used but also presents statistical advantages by enabling repeated measures analyses to identify study effects. The theoretical disadvantage of the progressive hepatectomy samples altering the status of the subsequent samples did no eventuate. In particular, when the Sham groups were examined, there were no significant changes found in serum ALT, histological scores, or MF using the progressive sampling approach.

MF and complex enzyme activities

Steatotic livers had impaired MF, which is thought to contribute to the increased steatotic liver susceptibility to IRI[5]. CS activity was similar across groups and time-points, indicating that MF differences in this study are due to alterations in mitochondria activity and not

a difference in mitochondrial mass.

A key dysfunction was found in CI, which is a large protein (about 1 MDa) comprising 45-47 subunits. It is embedded in the mitochondrial inner membrane to form an essential component of the mitochondrial ETS[20]. Impaired CI function has a substantial effect on ATP generation and contributes to a wide range of pathologies[21]. Our MF findings are consistent with a previous study that reported similar baseline CI function between steatotic and control livers and lower post-reperfusion CI function in isolated mitochondria from steatotic livers[5]. We have now extended this finding to show that the individual CI activity in tissue homogenates was also affected in steatotic livers post-IRI. Steatotic liver CI has previously been shown to be susceptible to oxidative damage from decreased mitochondrial antioxidants[5]. Further exacerbating this effect, CI can be a major site of reactive oxygen species production[22], and steatotic livers produce more reactive oxygen species *in vivo* than lean livers[5]. Oxygen reperfusion post-ischemia also leads to greater superoxide generation in steatotic livers relative to lean livers[23]. The lower CI activity observed in steatotic livers may be due to damage from IRI or may be a physiological response of steatotic livers to limit oxidative mitochondrial damage; which may be contributing to the decrease in tolerance of steatotic livers to IRI.

Complex Ⅱ is the only mitochondrial membrane-bound enzyme that is also involved in the citric acid cycle, as it oxidizes succinate and transfers electrons to co-enzyme Q[24]. CⅡ function has been reported to be similar at baseline and post-IRI in steatotic and lean rat livers[5], and our results corroborate this finding. CⅡ abnormalities are infrequently reported in the literature[19], and its function in steatotic livers are seldom reported. We observed that CⅡ activity post-ischemia and post-reperfusion was similar between steatotic and lean livers, which had not been previously described in this context. CⅡ activity and function appears to be unaffected by IRI, suggesting that CⅡ is more resistant to damage than CI. CⅡ may even contribute to superoxide production through an apparent reverse electron flow to CI instead[24], although this scenario is somewhat controversial, as it appears to defy thermodynamics and redox potentials[21]. Despite intact CⅡ function, CⅡ-driven flux is less effective in ATP synthesis (coupled to OXPHOS at Complex Ⅲ and Complex Ⅳ) compared to CI-driven flux (coupled to OXPHOS at CI, Complex Ⅲ and Complex Ⅳ), and this reduction in CCI-driven flux should impair reconstitution of ATP pools on reperfusion.

Effect of ischemic preconditioning

MF recovery following IRI is thought to be essential, as it generates the majority of cellular ATP[6]. Inadequate MF post-IRI would lead to decreased or delayed ATP generation during the critical period of reperfusion and could impair liver recovery. In this study, we hypothesized that IPC protects the steatotic liver from IRI-induced damage by protecting MF. We showed that IPC was partially protective against normothermic IRI (biochemical and histological indices) in both lean and steatotic livers, consistent with other studies[9,11]. In our study, liver transaminase levels were improved in preconditioned steatotic livers, which had not been previously investigated in diet-based models of obesity. These results were consistent with the limited clinical data on the effect of IPC on biochemical markers from steatotic livers subjected to normothermic IRI during liver resection[10,25].

Our results also indicated that despite some improvement in conventional liver injury markers, IPC did not improve MF or key enzyme activities over the duration of the study. This finding was in contrast to results from a previous study in choline-deficient rats that showed improved conventional liver injury markers and MF in preconditioned steatotic livers post-IRI[9]. Importantly, there were substantial differences in our study design to that of the only other previous experimental study. In that study, Rolo *et al*[9] performed MF analysis on isolated mitochondria at 25 ℃ and demonstrated that RCR was lower in both lean and steatotic livers post-IRI, and these effects were normalized by IPC in both groups of livers. However, mitochondrial respiration and particularly State 4 respiration are sensitive to assay temperature[26], and the results may not be truly representative of physiological MF at 37 ℃. Furthermore, the process of mitochondrial isolation results in the loss of fragile and/or damaged mitochondrial sub-populations. Here, we decided to use tissue homogenates as a means to lessen the potential for any mitochondrial selection bias[15]. Additionally, animals fed the choline-deficient diet used by Rolo *et al* showed weight loss, which is in contrast to the obesity often seen clinically in patients with hepatic steatosis[27]. In this study, the combination of a dietary model, tissue homogenates, and undertaking MF analysis at 37 ℃ represented the first advancement to a more physiological and clinically relevant MF analysis of the interaction of steatosis, IRI, and IPC. It is of note that most other previous studies on IPC in steatotic livers were performed in genetic models of hepatic steatosis[28]. However, the underlying mutations in these models are not prevalent in clinical hepatic steatosis pathophysiology, and the high-fat/high-carbohydrate used in our study more closely resembles the clinical setting[27].

The mechanism of IPC has been extensively reviewed elsewhere[29], but the direct impact of IPC on mitochondria is not as well characterized. In experimental lean rat livers subjected to IRI, ATP recovery was unaffected by IRI; but in steatotic livers, ATP recovery was found to be impaired post-IRI compared to lean livers[30]. In other studies, IPC was reported to preserve ATP recovery in lean livers post-IRI[29], while other studies have suggested that IPC was

also effective in improving ATP recovery in steatotic livers post-IRI[11]. For our study, we investigated MF and complex activities. We chose a 10′ + 10′ IPC protocol, as this was similar to the first clinical protocol described by Clavien *et al*[10]. In their study, IPC led to an improvement in biochemical and histological markers of injuries. This finding was then replicated in a further prospective trial[25], which demonstrated that IPC improved ATP levels in younger patient's post-reperfusion. In older patients, however, IPC decreased ATP levels post-reperfusion when compared to control livers. We found no improvement in MF with IPC, and although we were not able to measure ATP in this study, our findings suggest that the protective effect of IPC in this model was not likely to be mediated through increased mitochondrial ATP production. Therefore, there may be other mechanisms underlying the effect of IPC on hepatic ATP recovery. These mechanisms may include decreased cellular metabolism in preconditioned livers leading to conservation of ATP or reduced microcirculatory dysfunction. Alternatively, some of the IPC benefit may be due to increased production of nitric oxide and to opening of ATP-dependent potassium channels in preconditioned livers with a subsequent decrease in energy consumption[29].

The lack of full protection and liver function recovery from IPC observed by us and others in clinical and animal studies may reflect persistent underlying mitochondrial dysfunction, as demonstrated in our present study[3]. This observation supports future investigation of other IPC protocols or combinatorial use with mitochondrial-targeted therapies, as these may provide further clinical improvements. However, it could also potentially be influenced by our animal model, as our model differs from those published in the literature[9]. For completeness, liver weights should have been measured, but samples were obtained in "piece-meal" fashion and its was not possible.

As the prevalence of metabolic syndrome continues to rise in the population, hepatic steatosis has become the most common hepatic abnormality[1]. Therefore, it is important to identify new ways to improve outcomes from steatotic liver surgery. In this study, we investigated the impact of IPC on MF in a steatosis setting. We demonstrated that IRI was associated with increased liver injury in steatotic livers. Although the precise mechanisms underlying the increased susceptibility of steatotic liver to IRI remain unclear, we have shown for the first time, using a clinically relevant diet model and MF analysis at physiological temperatures, that there was an inherent decreased in CI activity in steatotic livers, which worsened following IRI. Our results also showed that the IPC protocol used in our study, while improving liver biomarkers and histology, did not influence MF directly. If we are to improve further the clinical benefit of IPC on livers with steatosis, then testing alternative IPC protocols or adjunct mitochondrial therapies will also be needed.

COMMENTS

Background

Steatotic livers are encountered with increasing frequency in liver surgery. It has been associated with poor outcome following warm ischemia-reperfusion injury (IRI). One possible proposed mechanism was mitochondrial dysfunction. However, the relationship between hepatic steatosis and mitochondrial dysfunction in warm IRI has not been clearly defined. Ischemic preconditioning has been touted as a possible therapeutic option for attenuating the harmful effect of IRI.

Research frontiers

Mechanisms of injury in steatotic livers are poorly understood, and further understanding will improve patient outcome.

Innovations and breakthroughs

This study is the first to investigate mitochondrial function, mitochondrial Complex Ⅰ and Complex Ⅱ activities; and the impact of ischemic preconditioning on mitochondrial function in warm IRI, in a dietary-induced model of hepatic steatosis.

Applications

Steatotic livers have decreased baseline Complex Ⅰ activity. After reperfusion injury, Complex Ⅰ function and activity were impaired in steatotic livers compared to lean livers. Ischemic preconditioning did not influence mitochondrial function in this setting.

Terminology

Mitochondrial Complex Ⅰ is paramount for intact mitochondrial function, and impairment of Complex Ⅰ leads to impaired ATP (cellular energy currency) production and consequently cell death.

Peer-review

It is well known that hepatic steatosis increases susceptibility to IRI and that this effect is linked to mitochondrial dysfunction. This study explored alterations of mitochondrial Complexes Ⅰ and Ⅱ in lean and high-fat, high sucrose diet–induced steatotic rat livers after 1 h-warm ischemia plus 4 h reperfusion. The authors showed that there was a significant decrease in Complex Ⅰ in steatotic livers compared to lean livers but there was no difference in Complex Ⅱ between these two groups. IPC decreased alanine aminotransferase release and histological changes after IRI but did not blunt decreases in Complex Ⅰ in steatotic livers. This study obtained some interesting data.

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ORIGINAL ARTICLE

Basic Study

Contribution of mammalian target of rapamycin in the pathophysiology of cirrhotic cardiomyopathy

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at dehpour@yahoo.com. No additional data are available.

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Abstract

AIM: To explore the role of mammalian target of rapamycin (mTOR) in the pathogenesis of cirrhotic cardiomyopathy and the potential of rapamycin to improve this pathologic condition.

METHODS: Male albino Wistar rats weighing 100-120 g were treated with tetrachloride carbon (CCl_4) for 8 wk to induce cirrhosis. Subsequently, animals were administered rapamycin (2 mg/kg per day). The QT_c intervals were calculated in a 5-min electrocardiogram. Then, the left ventricular papillary muscles were

isolated to examine inotropic responsiveness to β-adrenergic stimulation using a standard organ bath equipped by Powerlab system. Phosphorylated-mTOR localization in left ventricles was immunohistochemically assessed, and ventricular tumor necrosis factor (TNF)-α was measured. Western blot was used to measure levels of ventricular phosphorylated-mTOR protein.

RESULTS: Cirrhosis was confirmed by hematoxylin and eosin staining of liver tissues, visual observation of lethargy, weight loss, jaundice, brown urine, ascites, liver stiffness, and a significant increase of spleen weight ($P < 0.001$). A significant prolongation in QT_c intervals occurred in cirrhotic rats exposed to CCl_4 ($P < 0.001$), while this prolongation was decreased with rapamycin treatment ($P < 0.01$). CCl_4-induced cirrhosis caused a significant decrease of contractile responsiveness to isoproterenol stimulation and a significant increase in cardiac TNF-α. These findings were correlated with data from western blot and immunohistochemical studies on phosphorylated-mTOR expression in left ventricles. Phosphorylated-mTOR was significantly enhanced in cirrhotic rats, especially in the endothelium, compared to controls. Rapamycin treatment significantly increased contractile force and myocardial localization of phosphorylated-mTOR and decreased cardiac TNF-α concentration compared to cirrhotic rats with no treatment.

CONCLUSION: In this study, we demonstrated a potential role for cardiac mTOR in the pathophysiology of cirrhotic cardiomyopathy. Rapamycin normalized the inotropic effect and altered phosphorylated-mTOR expression and myocardial localization in cirrhotic rats.

Key words: Cirrhotic cardiomyopathy; Rat; Mammalian target of rapamycin; Rapamycin; Inotropic effect

Core tip: Enhanced levels of cardiac phosphorylated mammalian target of rapamycin (mTOR) contribute to impairment of electrophysiological and mechanical function induced by cirrhosis, called "cirrhotic cardiomyopathy". Here, we find that the mTOR inhibitor rapamycin normalized the impaired inotropic responsiveness to β-adrenergic stimulation and prolonged Q-T interval in tetrachloride carbon (CCl_4)-induced cirrhotic rats. Cardiac ventricular expression of phosphorylated-mTOR (p-mTOR) was increased in rats with cirrhosis, and this effect was ameliorated by rapamycin. CCl_4-induced cirrhosis was associated with an increase in cardiac proinflammatory cytokine tumor necrosis factor-α, and this increase was reversed by rapamycin as well.

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INTRODUCTION

For a long time, cardiac dysfunction in liver cirrhosis, termed "cirrhotic cardiomyopathy", was thought to be a common occurrence in patients suffering from alcoholic cirrhosis[1,2]. During the last decade, however, non-alcoholic cirrhotic patients have also been reported to demonstrate these cardiac abnormalities[3]. Cardiovascular dysfunction is observed in cirrhosis, but the underlying mechanisms are not still well understood. Despite the hyperdynamic systemic circulation and the absence of coronary artery or valvular disease and hypertension, cardiac hypertrophy and cardiomyocyte edema are observed in cirrhotic patients[3-7]. Furthermore, there is evidence for a concomitant decrease of inotropic effect along with impaired myocardial contractility[6]. Previous studies have shown that both portal hypertension and cirrhosis contribute to cardiomyopathy[1,8]. Cardiomyopathy is characterized by latent heart failure with impaired contractile responsiveness to pharmacological or physiological stress and/or altered diastolic relaxation with electrophysiological abnormalities, without any diagnosed cardiac disease and causes of cirrhosis[4,6].

A variety of mechanisms are responsible for the pathogenesis of cirrhotic cardiomyopathy. The major predisposing factors of cardiac contractility include alteration in ventricular receptor signal transduction (*i.e.*, β-adrenergic, muscarinic, and cannabinoid receptors)[9-12] and ionic channel function (*i.e.*, K^+ and L-type voltage-gated Ca^{2+})[13-15], cardiomyocyte plasma membrane fluidity changes[5,6], and complex alterations of carbon monoxide and nitric oxide (NO)[16,17]. Moreover, a rise in pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) is observed in this condition, resulting in stimulation of inducible nitric oxide synthase (iNOS) and NO overproduction[18].

Mammalian target of rapamycin (mTOR), a serine/threonine kinase component downstream of phosphotidylinositide 3-kinase (PI3K)/Akt signaling pathway[19,20], is a key regulator of mRNA translation and cell growth in cardiomyocytes[21,22]. Protein synthesis, a major factor for cardiac hypertrophic growth, is regulated by the PI3K/Akt/mTOR signaling pathway through inactivation of eukaryotic translation initiation factor 4E-binding proteins (4E-BPs)[23], leading to stimulation of polymerase Ⅰ and Ⅲ transcription[24], control of ribosome biogenesis and mitochondrial metabolism[25], and suppression of autophagy[26-28]. Zhang *et al*[29] found that mTOR knockout mice had improved baseline cardiomyocyte survival, decreased dilated cardiac hypertrophy, and less heart failure than control mice. Moreover, it was shown that activation of PI3K/Akt/mTOR signaling may lead to the development of cardiac

hypertrophy[30]. Indeed, the mTOR inhibitor rapamycin appeared to block the development of cardiomyocyte hypertrophy[29], and cohort studies have shown that rapamycin has cardioprotective effects in patients after liver transplantation[31,32].

Although our current knowledge of the predisposing factors of cirrhotic cardiomyopathy is somewhat understood, the role of other pathophysiological mechanisms underlying cardiac dysfunction induced by cirrhosis remains to be clarified. To this purpose, we examined the hypothesis that tetrachloride carbon (CCl_4)-induced cardiac inotropic dysfunction in response to adrenergic stimulation is associated with altered expression of cardiac p-mTOR in a rat model of cirrhotic cardiomyopathy. In this study, we demonstrate for the first time the positive inotropic effect of mTOR suppression by rapamycin and its ability to normalize cardiac levels of p-mTOR and the pro-inflammatory factor TNF-α in cirrhotic cardiomyopathy.

MATERIALS AND METHODS

Chemicals and reagents

The following compounds and reagents were applied in this investigation: rapamycin (Wyeth, Kildare, United Kingdom/Ireland), isoproterenol hydrochloride (Sigma, St. Louis, MO, United States), carbon tetrachloride (Merck, Darmstadt, Germany); TNF-α assay kit, polyclonal p-mTOR antibody (pSer2448), and horseradish peroxidase (HRP)-conjugated rabbit anti-rat Immunoglobulin G antibody (Biorbyt Co. Ltd., Cambridge, United Kingdom).

Animal model of cirrhosis

Male albino Wistar rats weighing 100-120 g were used with housing facilities (environment temperature at 21 ℃-23 ℃, 12-h regular light/dark cycle). Animals had unlimited access to food and water except for a brief time during injection and during the surgical procedure. The rats were divided into four main groups: control/drinking water, control/rapamycin, cirrhotic/drinking water, and cirrhotic/rapamycin. All experiments and manipulations were conducted in Prof. Dehpour's Hepatological Research Laboratory in accordance with the institutional animal care and use committee (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences) guidelines. This study was approved by the Ethics Committee of Tehran University of Medical Sciences.

To induce cirrhosis, CCl_4 (0.4 g/kg; a solution of 1:6 in mineral oil) was intraperitoneally injected to the animals three times a week for 8 wks until the appearance of ascites[33]. Rapamycin (2 mg/kg per day) was freshly dissolved in normal saline and daily administered in drinking water in a constant volume of 14 mL/100 g body weight during the 8-wk period[34,35]. Twenty-four hours after cessation of CCl_4, animals were sacrificed by guillotine decapitation. The liver was removed, sectioned, and stained with hematoxylin-eosin (HE). Light microscopy of stained liver sections confirmed the induction of cirrhosis in rats[4].

Twenty-four hours after the last administration of either CCl_4 or N/S, a lead Ⅱ electrocardiogram (ECG) was recorded for 15 min using three stainless steel subcutaneous electrodes attached to a bioamplifier (ADInstrument, Sydney, Australia) from the anesthetized rats. The signals were digitized at a sampling rate of 10 kHz by a Powerlab system and were displayed using Lab Chart 7 software (ADInstrument). The Q-T intervals, presented as corrected Q-T (QT_c), were calculated in a 5-min ECG. The QT_c was presented using Bazett's formula ($QT_c = QT/\sqrt{R\text{-}R}$)[36].

Preparation of isolated papillary muscle

Briefly, animals' hearts were excised following decapitation and left ventricular papillary muscles were dissected in cold oxygenated physiological salt solution (PSS) containing (in mmol/L) NaCl, 112; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; NaH_2PO_4, 0.5; KH_2PO_4, 0.5; $NaHCO_3$, 25; glucose, 10; and EDTA, 0.004[37,38]. The isolated papillary muscles were suspended in a 25-mL organ bath chamber containing PSS buffer solution bubbled with a gas mixture of 95% O_2: 5% CO_2 at 37 ℃ for 90 min to reach equilibrium. The contractility was induced by electrical field stimulation (Grass 88 Stimulator; Grass Instruments, West Warwick, RI, United States) at 1 Hz and 30 V, 20% higher than the threshold. After achievement of baseline contractile force, the muscle contraction was stimulated by addition of cumulative concentrations of isoproterenol (10^{-10} to 10^{-5} mol/L). The contractile force induced by the highest concentration of isoproterenol (10^{-5} mol/L) was considered as maximal contractility[16]. The resulted contractile forces were expressed as a percentage of the baseline papillary muscle contractility.

Immunohistochemistry

The ventricle samples were immediately fixed in freshly prepared 10% formalin and paraffin-embedded blocks. After deparaffinizing in xylene and rehydrating in decreasing concentrations of ethanol, 3% hydrogen peroxidase was added for 5 min to block dual endogenous peroxidase activity. Then, the immunohistochemical staining was performed based on the Avidin-Biotin peroxidase method. Polyclonal p-mTOR antibody (pSer2448) (1:50 dilution) was reacted for 1 h at room temperature followed by secondary HRP-conjugated rabbit anti-rat Immunoglobulin G antibody (1:50 dilution) for 30 min at room temperature. The sections were washed three times with Tris (pH 7.4), incubated with diaminobenzidine (DAB) solution for 10 min, and then incubated with 5% $CuSO_4$ for 5 min. Ultimately, the slides were washed and counterstained with H&E to obtain brown-colored precipitation for examination under light microscopy.

Ventricular TNF-α quantification

To measure tissue TNF-α, the left ventricles were excised, rinsed in PSS, snap-frozen in liquid nitrogen, and stored at -80 ℃ for further analysis. The samples were then homogenized in ice-cold phosphate-buffered saline (PBS) and centrifuged at 14200 *g* for 30 min. Fifty microliters of the samples and standards were pipetted into a 96-well plate precoated with rat TNF-α specific antibody. Following addition of 50 μL of biotinylated anti-TNF-α solution, the plate of the enzyme linked immunosorbent assay kit was incubated for 90 min at room temperature. The wells were washed, exposed to 100 μL of streptavidin-peroxidase, incubated for 45 min at room temperature, and washed four times with PBS. Finally, 100 μL of both stabilized chromogen and stop solution were respectively added in two stages and incubated for 20 min for spectrophotometrically analysis at λ = 450 nm[16].

Western blot analysis

The dissection and snap-freezing of left ventricles were performed as described in the above section. Briefly, left ventricles were homogenized in buffer (20 mmol/L Tris-HCl (pH 7.2), 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol), centrifuged at 40000 *g*, and resuspended in Tris buffer containing proteinase inhibitor. Thirty micrograms of protein samples were loaded and separated on sodium dodecyl sulfate-10% polyacrylamide gel (SDS-PAGE) by electrophoresis and were wet electroblotted onto nitrocellulose membrane at 4 ℃ for 12 h[39,40]. The blots were blocked for 1 h at room temperature with 2% bovine serum albumin in 0.1% Tween Tris-buffered saline (TBS-T) (pH 7.5). Then, the membranes were washed and incubated overnight at 4 ℃ with polyclonal p-mTOR primary antibody (pSer2448) (1:100 dilution). After washing, these blots were exposed to HRP-conjugated anti-rat secondary antibody (1:1000 dilution). Detection of blots was performed using enhanced chemiluminescence (ECL kit, Amersham, Chalfont St. Giles, United Kingdom) method. The levels of p-mTOR in cirrhotic, control, and rapamycin-treated animals were semi-quantified using ImageJ software (National Institutes of Health, Bethesda, MD, United States), which was defined as the p-mTOR/glyceraldehyde 3 phosphate dehydrogenase (GAPDH) densitometric ratio (%).

Statistical analysis

All data are expressed as mean ± SD and analyzed using GraphPad Prism software (version 5.0, GraphPad Software, Inc., La Jolla, CA, United States). To examine the differences between three or more experimental groups, one-way analysis of variance (ANOVA) followed by a Tukey's post test was used. For two-group comparisons, Student's *t*-test was applied. Evaluation of the effects of two variables (cirrhosis *vs* control and type of treatment) was performed using two-way ANOVA followed by a Bonferroni post test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Presence of CCl_4-induced cirrhosis was confirmed by visual observation of lethargy, weight loss, jaundice, brown urine, and ascites along with liver stiffness and a significant increase in spleen weight (1.52 ± 0.13 g *vs* 2.74 ± 0.41 g in control *vs* cirrhotic rats, $P < 0.001$), which contributed to the development of portal hypertension. H&E staining of liver tissues sampled from cirrhotic rats demonstrated focal hepatocellular necrosis and apoptotic cells as well as enhanced inflammatory cell infiltration into the portal tract. Fatty degeneration areas and central vein dilation were also seen histologically (Figure 1). Moreover, cirrhosis model animals had significantly prolonged QT_c intervals compared to controls ($P < 0.001$; Figure 2). The prolonged QT_c interval in cirrhotic rats was decreased by rapamycin (2 mg/kg) ($P < 0.01$; Figure 2).

Effect of rapamycin on papillary muscle contractility

As shown in Figure 3A, baseline papillary muscle inotropic responses to isoproterenol stimulation in cirrhotic rats were significantly decreased compared to controls ($P < 0.001$). The order was in agreement with the maximum response (R_{max}) to isoproterenol (76.46% ± 10.08% *vs* 117.36% ± 8.25%, $P < 0.001$; Figure 3A). Rapamycin did not significantly alter R_{max} in control rats. Likewise there was no significant difference in the EC_{50} of isoproterenol ($4.08 \pm 1.35 \times 10^{-8}$ and $6.59 \pm 1.29 \times 10^{-8}$ in N/S- and rapamycin-treated non-cirrhotic control groups, respectively; $P > 0.05$; Figure 3B). In cirrhotic rats, there was a significant rise in papillary muscle contractility and a significant enhancement of R_{max} following chronic treatment with rapamycin (2 mg/kg) compared to cirrhotic rats treated with N/S ($P < 0.001$; Figure 3C). There were no significant differences in the EC_{50} of isoproterenol among all four studied groups ($P > 0.05$; Figure 3D).

Effect of rapamycin treatment on ventricular TNF-α concentration

As shown in Figure 4, there was a significant increase in ventricular levels of TNF-α in cirrhotic rats compared to controls ($P < 0.001$). Treatment with rapamycin (2 mg/kg) for 8 wk caused no marked enhancement in tissue TNF-α concentration in the control group ($P > 0.05$). In addition, rapamycin significantly decreased the elevation in tissue TNF-α concentration in animals with cirrhosis ($P < 0.05$).

Ventricular p-mTOR expression

As shown in Figure 5, expression of p-mTOR in the left

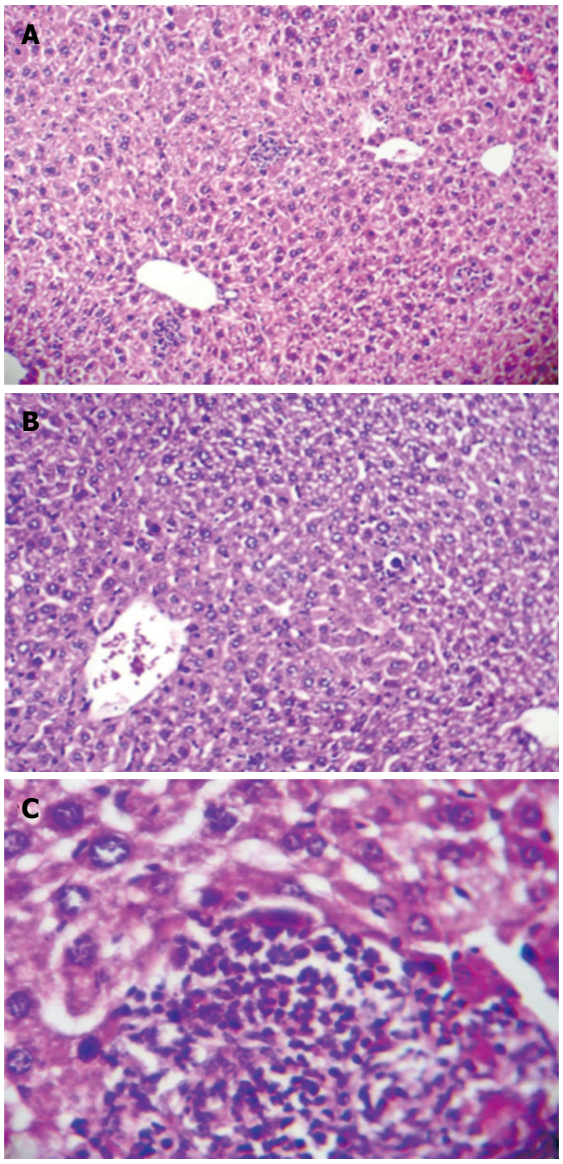

Figure 1 Histological change in liver tissue of CCl_4-induced cirrhotic rats (hematoxylin and eosin; magnification × 100 and × 400). A: Focal hepatocellular necrosis, apoptotic cells, and patchy inflammatory cell infiltration along with central vein dilation are observed; B: Fatty degeneration areas are clearly seen; C: Inflammatory cell infiltration into the portal tract.

ventricles of cirrhotic rats was increased compared to controls ($P < 0.001$). Rapamycin treatment reversed this increase in p-mTOR level in animals with cirrhosis ($P < 0.001$). Moreover, treatment of cirrhotic rats with rapamycin decreased p-mTOR protein expression to the level of control animals ($P > 0.05$).

To explore which cells express p-mTOR, immunohistochemical analysis was performed. Although almost no immunostaining was observed in the ventricular myocytes and endothelial cells in the control group (Figure 6A), p-mTOR immunostaining was markedly stronger in endothelial cells, but not in myocardial layer, of cirrhotic rats (Figure 6B). In the cirrhosis group, rapamycin could decrease p-mTOR immunostaining and induce mTOR phosphorylation in ventricular myocytes, as shown in Figure 6D.

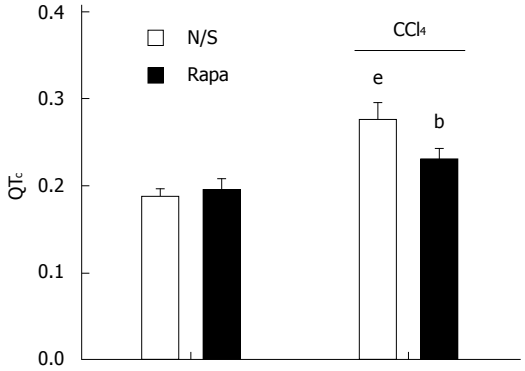

Figure 2 QT interval in control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). QT intervals were defined as corrected QT (QT_c) using Bazett's formula. The data are expressed as the mean ± SD. $^eP < 0.001$ *vs* control/normal saline group; $^bP < 0.01$ *vs* control/rapamycin and cirrhotic/saline group.

DISCUSSION

The main finding of the present study is the demonstration that cardiac mTOR expression and protein levels are increased in rats with cirrhotic cardiomyopathy. For the first time we showed that altered expression of p-mTOR in cirrhotic heart contributed to cardiac contractile suppression. This effect was confirmed by immunohistochemical assay, which showed a strong p-mTOR signal in cirrhotic left ventricles, especially in endothelial cells. Interestingly, the data from an *in vitro* papillary muscle study suggested that the enhanced expression of p-mTOR caused cardiac dysfunction. Consistent with that finding, we found a relationship between changes of mTOR activity and hypertrophic cardiomyopathy and heart failure[20,30,40-44]. Moreover, an increase in cardiac tissue TNF-α was observed in cirrhotic animals, which was accompanied by cardiomyocyte contractile dysfunction. Recently, several studies have investigated the role of TNF-α in the pathogenesis of heart failure and impaired cardiac contractility and have demonstrated that increased NO synthesis, an underlying mechanism for cirrhosis, in cardiac tissues of cirrhotic mice is attributed to elevated TNF-α level[4].

We also showed that repeated treatment with rapamycin normalized the cardiac contractile force defect in cirrhotic rats. To our knowledge, this is the first investigation to examine the hypothesis that rapamycin, *via* mTOR suppression, improves cardiac inotropic responsiveness to isoproterenol β-adrenergic stimulation and shortens the prolonged QT_c in rats with cirrhosis. Since mTOR phosphorylation was not obviously detectable in ventricular cardiomyocytes taken from CCl_4-induced cirrhotic rats, rapamycin caused significantly greater increases in p-mTOR protein level in cardiomyocytes than endothelial cells. Interestingly, despite the abundant expression of p-mTOR in cardiomyocytes, but not in endothelial cells, of rapamycin-treated rats with cirrhosis, total

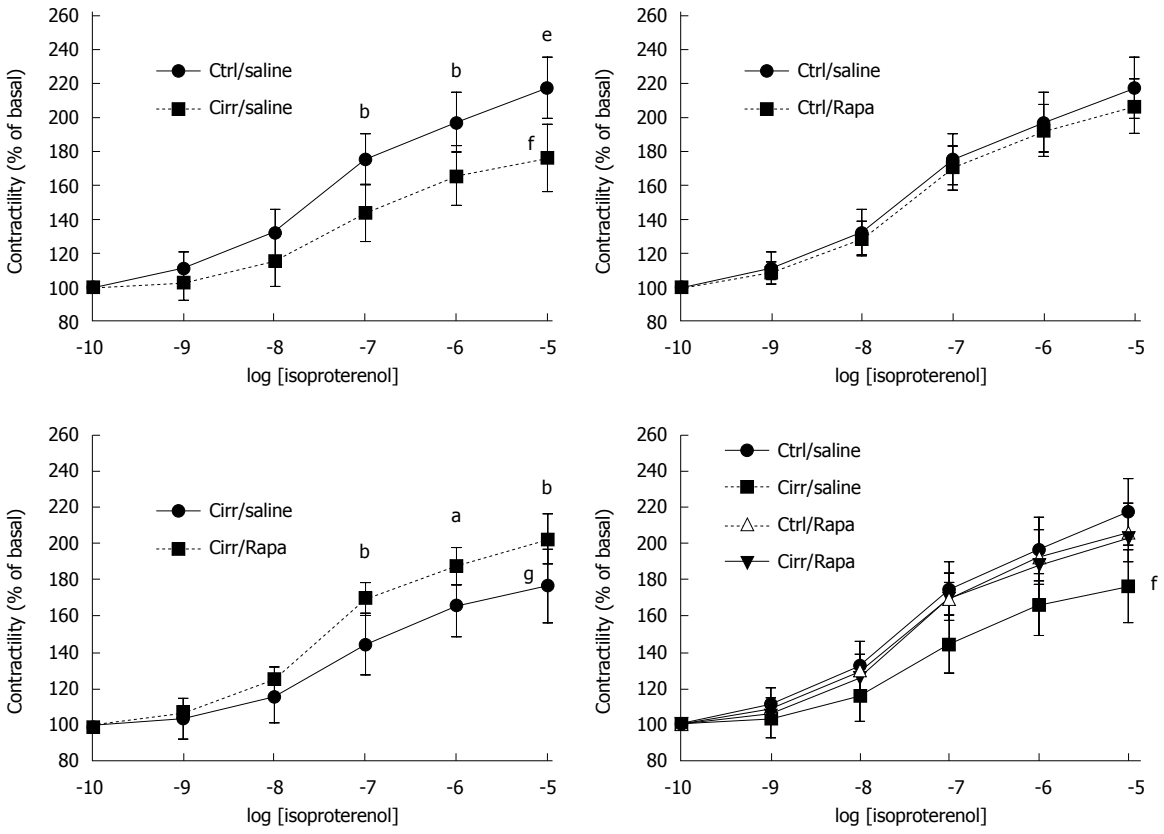

Figure 3 Contractile force in response to β-adrenergic stimulation in cirrhotic and control rats treated with normal saline or rapamycin (2 mg/kg). Inotropic responsiveness to β-adrenergic stimulation with isoproterenol in the isolated papillary muscle from cirrhotic and control rats treated with normal saline or rapamycin (2 mg/kg) was analyzed to determine the contractile force (% of basal). The data are expressed as the mean ± SD. Maximal response (R_{max}) in the CCl_4-induced cirrhotic rats was significantly lower than the other groups. There were no significant differences in EC_{50} values among the four studied groups. $^{f}P < 0.001$ *vs* the control group receiving normal saline; $^{g}P < 0.001$ *vs* the cirrhosis group receiving normal saline; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the cirrhotic group receiving normal saline in that concentration.

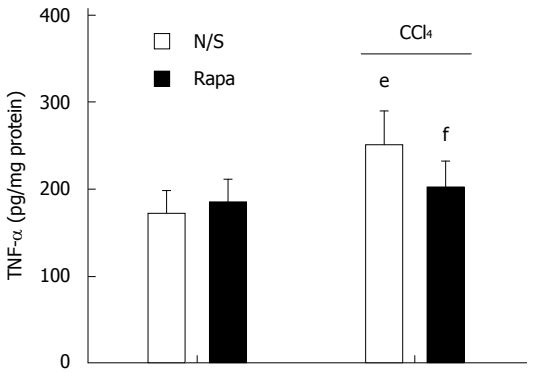

Figure 4 Left ventricular tumor necrosis factor-α levels in control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). The data are expressed as the mean ± SD. $^{e}P < 0.001$ *vs* control/normal saline group; $^{f}P < 0.001$ *vs* cirrhosis/normal saline group. TNF-α: Tumor necrosis factor-α.

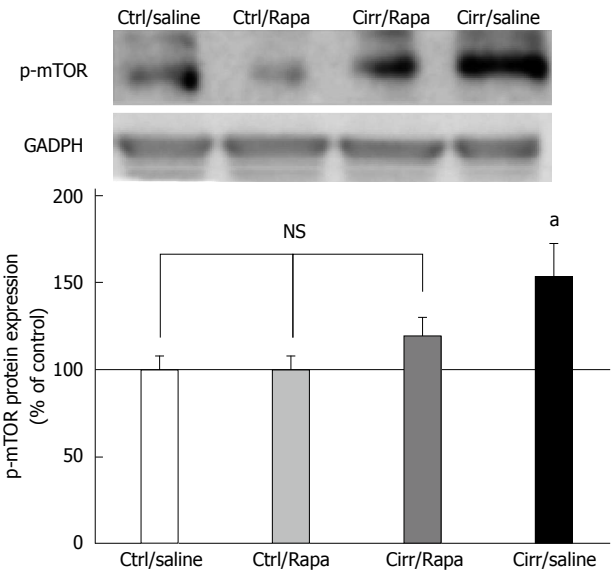

Figure 5 Western blot analysis of p-mammalian target of rapamycin protein in the left ventricles of control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). The upper panels demonstrate the representative immunoblots of p-mTOR and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) proteins in the control, control + rapamycin, cirrhotic and cirrhotic + rapamycin. The lower panel shows the densitometric analysis after normalization with GAPDH. Values are expressed as p-mTOR/GAPDH ratio (%) and normalized to the control group receiving normal saline (mean ± SD). $^{a}P < 0.05$ *vs* the other three groups; NS: Non-significant.

cardiac p-mTOR protein was reduced in comparison with cirrhotic rats receiving N/S. This finding was correlated with the positive inotropic effects of rapamycin in this paradigm. Decreased tissue levels of TNF-α after treatment with rapamycin confirmed the hypothesis that reduction in overproduced cytokines, such as TNF-α and interleukin-1β, from hepatic and systemic reticuloendothelial cells can reverse

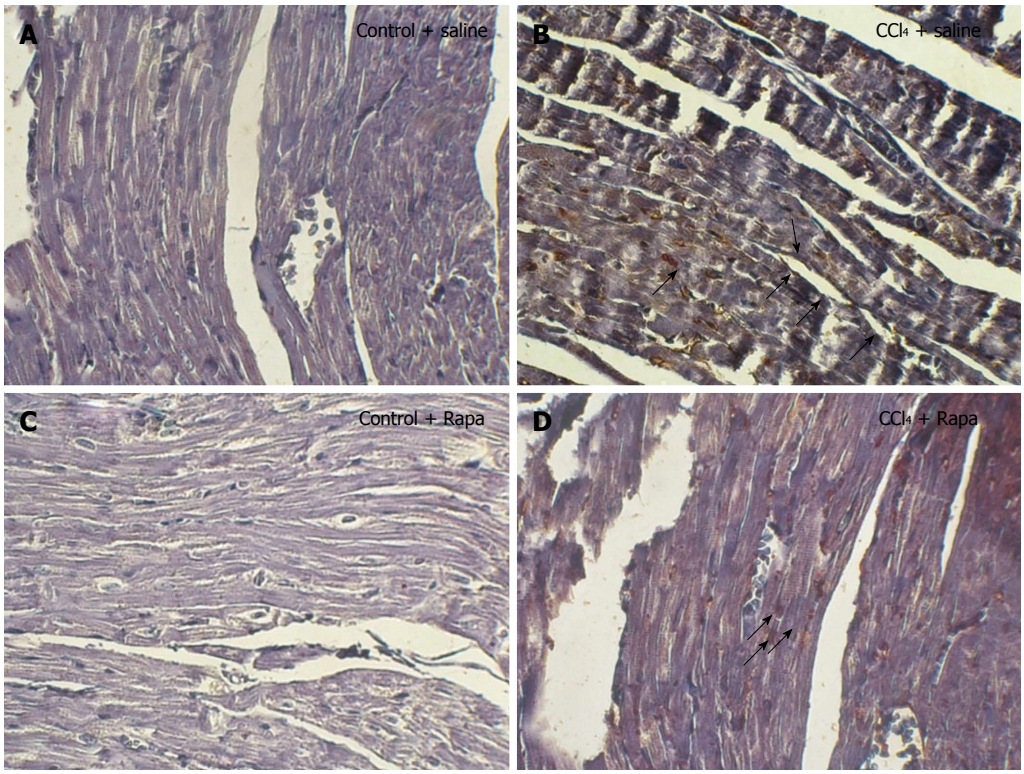

Figure 6 Immunohistochemical staining for p-mTOR in the ventricles of the rats in the following groups: control, cirrhotic, control + rapamycin and cirrhotic + rapamycin (× 400 magnification). Human gastric tissue was used as the positive control. Note the increased immunostaining of p-mTOR in the myocytes of the rats with cirrhosis. No significant immunostaining was localized to the cardiomyocytes of the untreated cirrhotic rats. In contrast, treatment with rapamycin caused significant immunostaining in the cardiomyocytes of the cirrhotic rats. The black arrows indicate to the p-mTOR immunoblots in rat ventricles.

their negative inotropic effects[16,45,46]. Evidence has shown that rapamycin acts as an effective agent, like isoproterenol, to raise intracellular cyclic adenosine monophosphate by reducing the expression and release of the pro-inflammatory cytokine TNF-α from human heart tissue[47]. Also, rapamycin may inhibit nuclear factor-kappa B (NFκB) activation and TNF-α, a potent inducer of in vascular smooth muscles[48].

During the last two decades, many investigations have been performed to explore the possible manifestations and potential mechanisms underlying cirrhotic cardiomyopathy. For instance, a decrease in isolated papillary muscle contractile force was observed in response to adrenergic stimulation in bile duct-ligated rats[12,36,49-51]. These results were similar to our observation that negative inotropic responsiveness to adrenergic stimulation is a result of CCl_4-induced cirrhosis. Although most of the studies are based on the hypothesis that defects of cardiac contractile force may result from downregulation of β-adrenergic receptors[10,37] as well as increased cardiac NO synthesis[16], we tried to investigate the role of mTOR inhibition in a rat model of cirrhosis to attenuate the impairment in cardiac contractile performance. Previous studies have reported the protective effects of rapamycin on the development of left ventricle hypertrophy and ischemia/reperfusion injury after myocardial infarction[21,22,42-44,52]. Blockade of NFκB and PI3K/Akt/mTOR signaling pathway may play an essential role in ameliorating myocardial hypertrophy induced by p70S6K, a main component downstream of mTOR, activation in the infarcted hearts[21,22,30,43]. In addition to the role of mTOR in cardiomyocyte hypertrophy, p-mTOR played a role in the impairment of cardiac survival and structure and also myocardial contractile dysfunction[53]. Inhibition of mTOR activated 4E-BP1, another downstream target of mTOR, resulting in inhibition of protein synthesis, pathogenesis of cardiomyopathy, and subsequent complications of cardiac hypertrophy[29,43].

Moreover, increment of autophagy and autophagosome formation upon mTOR inhibition with rapamycin is considered to be other protective mechanisms in heart failure[43,54]. Regarding the requirement of the ubiquitin proteasome system for activation of NFκB, rapamycin can restrict the myocardial infarction size and remodeling by inhibiting the ubiquitin proteasome and subsequent NFκB activity[43,55,56].

In addition to the observed positive effect of rapamycin on electrophysiological and mechanical cardiac function in cirrhosis, it is noteworthy that rapamycin has protective effects on human liver fibrosis and inhibits the progression of fibrosis, especially at early stages[35,57,58]. Rapamycin exerts this effect by inhibiting cell proliferation, deposition of extracellular matrix, and the profibrogenic pathway and factors[59-62]. Additionally, cohort studies have reported that patients receiving rapamycin after liver

transplantation had no cardiovascular problems. They showed that rapamycin not only did not increase the risk of congestive heart failure and myocardial infarction but plays a role as a cardioprotective agent[31,32]. In our study, the positive role of rapamycin on cirrhotic cardiomyopathy was attributed to a direct effect on cirrhotic heart, and it was assumed that a part of this phenomenon was associated with the anti-fibrogenic effect of this drug. This assumption is strongly amplified since cardiac and liver diseases share a common etiology[6]. Although experimental and clinical investigations on cirrhotic patients revealed latent heart failure with impaired response to provocations and subsequent mortality, no effective treatment has been found for improving cardiac contractility in patients with cirrhotic cardiomyopathy and evident ventricular failure[6]. As the prolongation in QT interval is considered an important life-threatening element in patients with cirrhotic cardiomyopathy, early identification and treatment of patients are necessary. Therefore, due to the anti-cytokine and beneficial role of rapamycin in correcting the abnormal cardiac contractile force and QT interval, rapamycin is expected to be the subject for further clinical investigations in patients with cirrhotic cardiomyopathy.

The present study has provided evidence that an increase in p-mTOR is responsible for the impaired cardiac contractility in animals with CCl_4-induced cirrhosis. Moreover, mTOR blockade corrected the cardiac contractile dysfunction in liver cirrhosis, highlighting the possible therapeutic potential for the mTOR antagonist rapamycin in this condition. This treatment may increase survival in cirrhosis-associated heart failure until a transplant becomes available. In addition, our utilization of an experimental model of cirrhotic cardiomyopathy and its translation to clinical benefits may guide future research studies.

COMMENTS

Background

"Cirrhotic cardiomyopathy" has been recognized as cardiac dysfunction in liver cirrhosis, which commonly occurs in patients suffering from cirrhosis. Unfortunately, the responsible mechanisms underlying the pathophysiology of cirrhotic cardiomyopathy are not well understood. Therefore, understanding these mechanisms may help to develop possible treatments for this disease.

Research frontiers

To date, a variety of mechanisms have been described that are responsible for the pathogenesis of cirrhotic cardiomyopathy. The major predisposing factors of cardiac contractility include alterations in ventricular receptor signal transduction and ionic function, cardiomyocyte plasma membrane fluidity changes, and complex alterations in carbon monoxide and nitric oxide.

Innovations and breakthroughs

Although the current knowledge of the mechanisms underlying cirrhotic cardiomyopathy is somewhat understood, the role of other pathophysiological mechanisms remains to be clarified. To this purpose, the authors examined the hypothesis that CCl_4-induced cardiac inotropic dysfunction in response to adrenergic stimulation is associated with altered expression of cardiac phosphorylated-mammalian target of rapamycin (mTOR) in a rat model of cirrhotic cardiomyopathy. Therefore, this study is the first to demonstrate the positive inotropic effect of mTOR suppression by rapamycin and its ability to normalize cardiac levels of phosphorylated-mTOR as well as the pro-inflammatory factor TNF-α in cirrhotic cardiomyopathy.

Applications

mTOR blockade corrected the cardiac contractile dysfunction in liver cirrhosis, highlighting the therapeutic potential of the mTOR antagonist rapamycin in this condition. Treatment with rapamycin may increase survival in those with cirrhosis-associated heart failure until a transplant becomes available. This study may guide researches to utilize the experimental model of cirrhotic cardiomyopathy translating to clinical benefits.

Peer-review

This is an interesting study about the role of mTOR in the pathogenesis of cirrhotic cardiomyopathy and the potential use of rapamycin for improving cardiac dysfunction.

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ORIGINAL ARTICLE

Basic Study

Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Shanghai University of Traditional Chinese Medicine (No. 2013031).

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Abstract

AIM: To investigate the mechanism by which Qinggan Huoxue Recipe (QGHXR) inhibits epithelial-to-mesenchymal transition (EMT) in rats with alcoholic liver fibrosis (ALF).

METHODS: A total of 75 male SD rats were used to induce ALF. Serum biochemical indicators, including

alanine aminotransferase, aspartate aminotransferase, laminin and hyaluronidase, were measured. Liver histopathological changes were evaluated using hematoxylin-eosin and Sirius red staining. EMT was examined by analyzing the expression of the epithelial marker E-cadherin and the mesenchymal markers vimentin and fibronectin using RT-PCR and Western blot. The inhibitory effect of QGHXR on EMT markers, as well as its effect on molecules associated with the transforming growth factor (TGF)-β1/Smad signaling pathway, including TGF-β1, Smad3, snail, occludin, ZO-1 and claudin, was also examined.

RESULTS: Compared with normal control rats, ALF rats exhibited a decrease in E-cadherin levels (mRNA: ALF 0.16 ± 0.05 *vs* control 1.00 ± 0.08; protein: ALF 0.09 ± 0.05 *vs* control 0.70 ± 0.17, $P < 0.01$) and an increase in vimentin and fibronectin levels (mRNA: 11.43 ± 0.39 *vs* 1.00 ± 0.19 and 9.91 ± 0.34 *vs* 1.00 ± 0.44, respectively, $P < 0.01$; protein: 1.13 ± 0.42 *vs* 0.09 ± 0.03 and 1.16 ± 0.43 *vs* 0.09 ± 0.00, respectively, $P < 0.01$). This indicates that EMT occurred in ALF rats. In addition, the TGF-β1/Smad signaling pathway was activated in ALF rats, as evidenced by the increase in TGF-β1 and snail levels (mRNA: 1.76 ± 0.12 *vs* 1.00 ± 0.05 and 6.98 ± 0.41 *vs* 1.00 ± 0.10, respectively, $P < 0.01$; protein: 1.43 ± 0.05 *vs* 0.12 ± 0.03 and 1.07 ± 0.29 *vs* 0.07 ± 0.02, respectively, $P < 0.01$) and the decrease in Smad3 levels (mRNA: 0.05 ± 0.01 *vs* 1.00 ± 0.12, $P < 0.01$; protein: 0.06 ± 0.05 *vs* 0.89 ± 0.12, $P < 0.01$). Furthermore, levels of the tight junction markers occludin, ZO-1 and claudin decreased in ALF rats compared with healthy control rats (mRNA: 0.60 ± 0.09 *vs* 1.00 ± 0.12, 0.11 ± 0.00 *vs* 1.00 ± 0.12 and 0.60 ± 0.01 *vs* 1.00 ± 0.08, respectively, $P < 0.01$; protein: 0.05 ± 0.01 *vs* 0.87 ± 0.40, 0.09 ± 0.05 *vs* 0.89 ± 0.18 and 0.04 ± 0.03 *vs* 0.95 ± 0.21, respectively, $P < 0.01$). In ALF rats treated with QGHXR, E-cadherin levels increased (mRNA: QGHXR 0.67 ± 0.04 *vs* ALF model 0.16 ± 0.05, $P < 0.01$; protein: QGHXR 0.66 ± 0.21 *vs* ALF model 0.09 ± 0.05, $P < 0.01$), and vimentin and fibronectin levels decreased (mRNA: 6.57 ± 1.05 *vs* 11.43 ± 0.39 and 1.45 ± 1.51 *vs* 9.91 ± 0.34, respectively, $P < 0.01$; protein: 0.09 ± 0.03 *vs* 1.13 ± 0.42 and 0.10 ± 0.01 *vs* 1.16 ± 0.43, respectively, $P < 0.01$). In addition, QGHXR inhibited the expression of TGF-β1 and increased the expression of Smad3 (mRNA: 1.03 ± 0.11 *vs* 1.76 ± 0.12, 0.70 ± 0.10 *vs* 0.05 ± 0.01, respectively, $P < 0.05$ and $P < 0.01$; protein: 0.12 ± 0.03 *vs* 1.43 ± 0.05 and 0.88 ± 0.20 *vs* 0.06 ± 0.05, respectively, $P < 0.01$). QGHXR treatment also reduced the levels of the EMT-inducing transcription factor snail (mRNA: 2.28 ± 0.33 *vs* 6.98 ± 0.41, $P < 0.01$; protein: 0.08 ± 0.02 *vs* 1.07 ± 0.29, $P < 0.01$) and increased the occludin, ZO-1 and claudin levels (mRNA: 0.73 ± 0.05 *vs* 0.60 ± 0.09, 0.57 ± 0.04 *vs* 0.11 ± 0.00 and 0.68 ± 0.03 *vs* 0.60 ± 0.01, respectively, $P < 0.01$, $P < 0.01$ and $P < 0.05$; protein: 0.92 ± 0.50 *vs* 0.05 ± 0.01, 0.94 ± 0.22 *vs* 0.09 ± 0.05 and 0.94 ± 0.29 *vs* 0.04 ± 0.03, respectively, $P < 0.01$). The effects of QGR and HXR on the TGF-β1/Smad signaling pathway were similar to that of QGHXR; however, the QGR- and HXR-induced changes in vimentin mRNA levels, the QGR-induced changes in fibronectin mRNA levels and the HXR-induced changes in snail and TGF-β1 mRNA levels were not significant.

CONCLUSION: Qinggan Huoxue Recipe inhibits EMT in ALF rats by modulating the TGF-β1/Smad signaling pathway, suggesting that the mechanism underlying the amelioration of ALF induced by QGHXR is associated with this pathway.

Key words: Alcoholic liver fibrosis; QGHXR; Epithelial-to-mesenchymal transition; Snail; Transforming growth factor-β1/Smad

Core tip: Epithelial-to-mesenchymal transition (EMT) is a dynamic process by which mature epithelial cells lose their distinct characteristics and acquire a mesenchymal phenotype. EMT is characterized by loss of the expression of the epithelial marker E-cadherin and up-regulation of the mesenchymal markers α-SMA, collagen Ⅰ, vimentin and fibronectin. Our study provided evidence that QGHXR inhibits EMT in alcoholic liver fibrosis by regulating the transforming growth factor-β1/Smad signaling pathway and that QGHXR-mediated inhibition of EMT might be a promising approach to ameliorating alcoholic liver injury.

Wu T, Chen JM, Xiao TG, Shu XB, Xu HC, Yang LL, Xing LJ, Zheng PY, Ji G. Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway. *World J Gastroenterol* 2016; 22(19): 4695-4706 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4695.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4695

INTRODUCTION

Alcoholic liver disease (ALD) has become a major cause of morbidity and mortality worldwide[1]. ALD progresses from a healthy liver to alcoholic steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[2]. Although great efforts to explore potential therapeutic targets for alcoholic liver fibrosis (ALF) have been made in recent decades, effective therapies for ALF remain an unmet need.

Epithelial-to-mesenchymal transition (EMT) is a remodeling process that occurs in adult tissues in response to pathological states, such as carcinogenesis and fibrosis[3]. EMT, a phenotypic conversion of the epithelium to a fibroblastic or myofibroblastic phenotype, plays a well-established role in the induction of fibrogenesis[4]. EMT generally begins with the dissociation of adhesions between epithelial cells, the decrease of

apical-basal polarity, the reorganization of the actin cytoskeleton and the increase of cell motility[5]. Although multiple studies have reported the significance of the EMT in fibrogenesis, the precise mechanisms underlying EMT in this context are only partially understood.

Numerous studies have demonstrated that cytokines, including transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), can induce EMT[6-8]. In addition, various signaling pathways associated with EMT have been found to be activated in EMT, including the TGF-β/Smad signaling pathway.

The traditional Chinese medicine formula Qinggan Huoxue Recipe (QGHXR) exerts many pharmacological effects that can ameliorate ALD, including reversing steatosis, mediating lipid peroxidation resistance and decreasing the levels of inflammatory cytokines[9,10]. A recent study demonstrated that QGHXR activated the lipopolysaccharide-Kupffer cell (LPS-KC) signaling pathway in rats with ALD by reducing serum alanine transaminase (ALT), aspartate transaminase (AST) levels and modulating CD14, NF-κB, TLR4, ERK and TNF-α expression and that QGHXR alleviated pathological changes associated with ALD[11].

However, the molecular mechanisms by which QGHXR inhibits ALD have yet to be fully elucidated. Here, we investigated the potential physiological and molecular mechanisms underlying QGHXR-mediated inhibition of EMT, especially regulation of the TGF-β/Smad signaling pathway, in an ALF rat model.

MATERIALS AND METHODS

Materials

QGHXR (bupleurum root 9 g, scutellaria root 9 g, red sage root 15 g, *Carapax trionycis* 9 g and *Radix puerariae* 15 g), Qinggan Recipe (QGR: bupleurum root 9 g and scutellaria root 9 g) and Huoxue Recipe (HXR: red sage root 15 g, *Carapax trionycis* 9 g and *Radix puerariae* 15 g), were used at concentrations of 4.75, 1.5 and 3.25 g/mL, respectively, and they were generated at the Department of Pharmacy of Longhua Hospital (Shanghai, China). Antibodies against E-cadherin, vimentin, fibronectin, TGF-β1, Smad3, snail, occludin, zona occludens-1 (ZO-1), claudin and GAPDH were purchased from Abcam (Cambridge, MA, United States). Parazole was purchased from Sigma-Aldrich Co. LLC (St Louis, MO, United States). All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Animals and experimental design

All experiments were approved by the Local Ethics Committee for Animal Research Studies at the Shanghai University of Traditional Chinese Medicine. Male specific pathogen-free SD rats were purchased from Slac Laboratory Animal Center, Inc. (Shanghai, China). A rat ALF model was established by treating the rats with an alcohol mixture (500 mL/L alcohol, 8 mL/kg per day; pyrazole, 24 mg/kg per day; corn oil, 2 mL/kg per day) once per day, accompanied with administering intraperitoneal injections with a 25% solution of CCl_4 in olive oil (0.25 mL/kg) twice a week for 12 wk as previously described[12]. After 8 wk, the ALF rats were divided into 4 groups: control, QGR, HXR and QGHXR groups (n = 15 rats per group). Rats in the QGR, HXR, and QGHXR groups were administered a daily treatment dose of 137.5, 62.5, 200 mg/kg, respectively, by gastric lavage. An equal volume of normal saline was administered to the control ALF and healthy control groups (n = 15) by gastric lavage. The treatment lasted 4 wk. All rats were then anesthetized with 2% pentobarbital sodium (2 mL/kg) and sacrificed, and blood and liver tissue specimens were subsequently collected. One section of the liver tissue was fixed for histopathology, and another section was used for real-time polymerase chain reaction (PCR) and Western blot assays. Serum biochemical assays including measurements of ALT, AST levels were conducted using an automatic biochemistry analyzer (Hitachi Ltd, Tokyo, Japan). Serum laminin and hyaluronidase levels were detected by Shanghai Adicon Clinical laboratories Inc.

Liver histological analysis

Hematoxylin and eosin (HE) and Sirius red staining were routinely performed following standard procedures. Histological analysis was performed by HE staining of paraffin-embedded liver sections. Fibrosis was assessed in tissue sections stained with Sirius red (0.3%). Briefly, the fresh liver tissue samples were fixed in 10% formalin and embedded in paraffin. The samples were sliced into 4 μm to 5 μm sections and stained with HE staining solution or with a 0.1% Sirius red-picric solution. The latter sections were washed rapidly with acetic acid. The stained sections were observed and photographed under a light microscope at a magnification of × 200.

RNA preparation and RT-PCR analysis

Total RNA was isolated from rat liver tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed into cDNA using a Reverse Transcription System (Promega, Madison, WI, United States). The thermal cycling conditions were as follows: 95 ℃ for 3 min followed by 34 cycles at 95 ℃ for 30 s, 55 ℃ for 40 s and 72 ℃ for 40 s. The mRNA expression levels of E-cadherin, vimentin, fibronectin, TGF-β1, Smad3, snail, occludin, ZO-1 and claudin were quantitatively analyzed and normalized to GAPDH levels. All assays were performed in triplicate. The forward and reverse primer sequences are provided in Table 1.

Western blot analysis

Protein extracts from rat liver tissues were quantified

Table 1 Primer sequences

E-cadherin	Forward 5′-CACACTGATGGTGAGGGTACAAGG-3′
	Reverse 5′-GGGCTTCAGGAACACATACATGG-3′
Vimentin	Forward 5′-ACCGCTTCGCCAACTACATC-3′
	Reverse 5′-GCAACTCCCTCATCTCCTCCT-3′
Fibronectin	Forward 5′-GACTCGCTTTGACTTCACCAC-3′
	Reverse 5′-ATCTCCTTCCTCGCTCAGTTC-3′
TGF-β1	Forward 5′-GAGGCGGTGCTCGCTTTGTA-3′
	Reverse 5′-GCACTGCTTCCCGAATGTCTG-3′
Smad3	Forward 5′-ATACGGATGTTCAAGTGTTCG-3′
	Reverse 5′-ACTGGGTCCTCTTTGGTTTT-3′
Snail	Forward 5′-GTCCTTGCTCCACAAACACCA-3′
	Reverse 5′-CTGCCTTCCATCAGCCATCT-3′
Occludin	Forward 5′-AGATGCTGGTTGCTGGAGAAGT-3′
	Reverse 5′-TGGAGACAGGAAACGGATGGT-3′
ZO-1	Forward 5′-CGGAGCAGAGAGGAAGAGC-3′
	Reverse 5′-GGCAGAACACCATCAGAAGG-3′
Claudin-1	Forward 5′-AGGTCTGGCGACATTAGTGG-3′
	Reverse 5′-TGGTGTTGGGTAAGAGGTTG-3′
GAPDH	Forward 5′-TGAGGACCAGGTTGTCTC-3′
	Reverse 5′-TCCACCACCCTGTTGCTGTA-3′

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

using the bicinchoninic acid (BCA) method, separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, NJ, United States). Nonspecific binding was blocked with 5% nonfat milk in TBST (Tris-buffered saline with Tween) buffer for 2 h at room temperature. The membranes were incubated with primary antibody against E-cadherin (1:1000), vimentin (1:1000), fibronectin (1:1000), TGF-β1 (1:1000), Smad3 (1:1000), snail (1:1000), occludin (1:1000), ZO-1 (1:1000) or claudin (1:1000) overnight at 4 ℃ and then incubated with the horseradish peroxidase-conjugated secondary antibodies. Finally, blots were visualized using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Amersham, United Kingdom), and GAPDH was used as a loading control. Each experiment was repeated three times independently.

Statistical analysis

All the data are presented as the mean ± SD and analyzed by one-way analysis of variance using SPSS17 software (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant. Histograms were generated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

QGHXR ameliorates liver injury in rats with ALF

To evaluate the effect of QGHXR on ALF, we generated an alcohol-induced rat model of ALF and measured serum levels of ALT, AST, laminin and hyaluronidase. Rats in the untreated ALF group exhibited signs of alcohol-induced liver injury, such as fibrosis, and the ALT, AST, laminin and hyaluronidase levels in these rats were significantly higher than those in the healthy control group of rats without ALF (Figure 1). Although the QGR, HXR and QGHXR interventions reduced the levels of ALT, AST, and hyaluronidase in rats with ALF, no significant differences in laminin levels were observed (Figure 1).

QGHXR ameliorates pathological changes associated with ALF in rats

Histological images of liver pathology were also obtained. Liver sections were stained with HE or Sirius staining solution.

In the control group of rats without ALF, no detectable fatty deposits or inflammatory cell infiltrates were observed in images obtained by microscopy. In contrast, relative to the normal control group, the liver sections of rats in the untreated ALF group displayed fat droplet accumulations in hepatocytes and scattered inflammatory cell infiltrates (Figure 2A and B). In addition, collagen fibers surrounding the central vein and portal area, and overt signs of perisinusoidal fibrosis were observed in the untreated ALF group (Figure 3A and B).

Compared with the untreated ALF group, the ALF groups treated with QGR, HXR or QGHXR exhibited improvements in ALF-associated pathological changes, with the greatest improvement observed in the QGHXR-treated group (Figures 2C-E and 3C-E).

QGHXR regulates the expression of EMT-associated transcription factors

Next, we investigated whether QGHXR ameliorates liver injury in ALF rats by inhibiting TGF-β1-induced EMT. To evaluate EMT, we analyzed the expression of the epithelial marker E-cadherin, and the mesenchymal markers vimentin and fibronectin using PCR and Western blot.

PCR analysis demonstrated that E-cadherin mRNA expression decreased, and vimentin and fibronectin mRNA expression increased in rats with ALF compared with the healthy control group. However, the expression of E-cadherin in ALF rats treated with QGR, HXR or QGHXR gradually returned to the levels observed in the healthy control group (Figure 4A). In addition, a stepwise decrease in the mRNA expression of vimentin and fibronectin was observed in ALF rats treated with QGR, HXR or QGHXR. However, significant changes in the mRNA expression levels of these genes were observed only in the QGHXR-treated group (Figure 4A).

Western blot analysis revealed that E-cadherin protein levels decreased and vimentin and fibronectin protein levels increased in untreated ALF rats than those in the healthy control group, and that treatment with QGHXR, QGR or HXR reversed this effect (Figure 4B).

Collectively, these findings indicate that QGR, HXR and QGHXR inhibited the EMT in ALF rats. However,

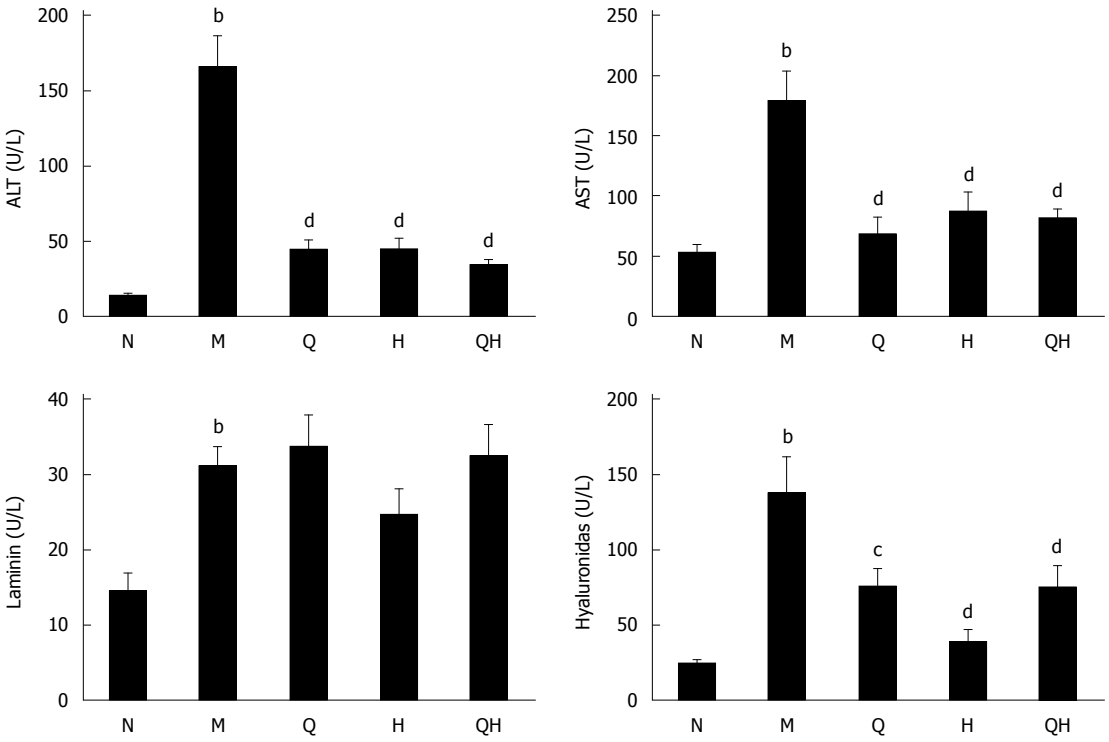
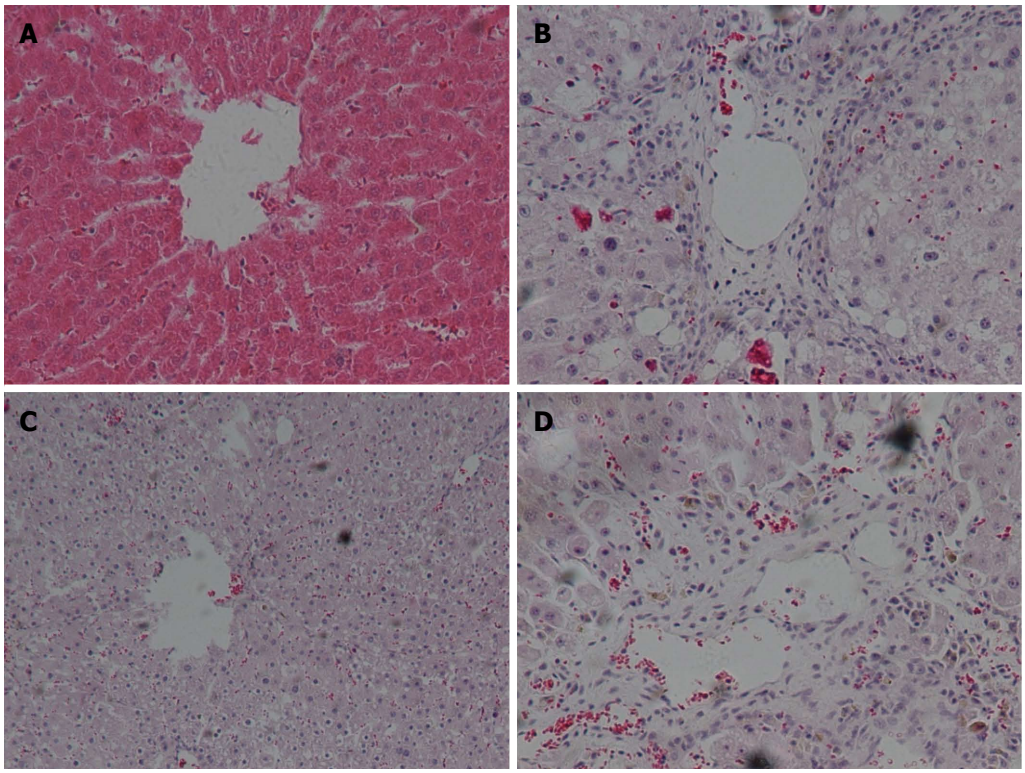


Figure 1 Qinggan Huoxue recipe ameliorates liver injury associated with alcoholic liver fibrosis with respect to alanine transaminase, aspartate transaminase, laminin and hyaluronidase levels. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.



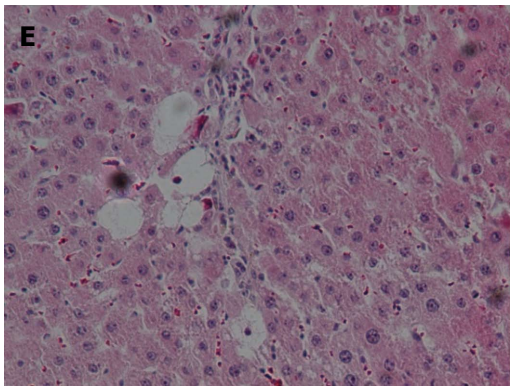


Figure 2 Changes in liver histopathology detected by hematoxylin and eosin staining and light microscopy (× 200 magnification). A: Normal healthy control group; B: Model ALF group; C: Qinggan Recipe group; D: Huoxue Recipe group; E: Qinggan Huoxue Recipe group. ALF: Alcoholic liver fibrosis.

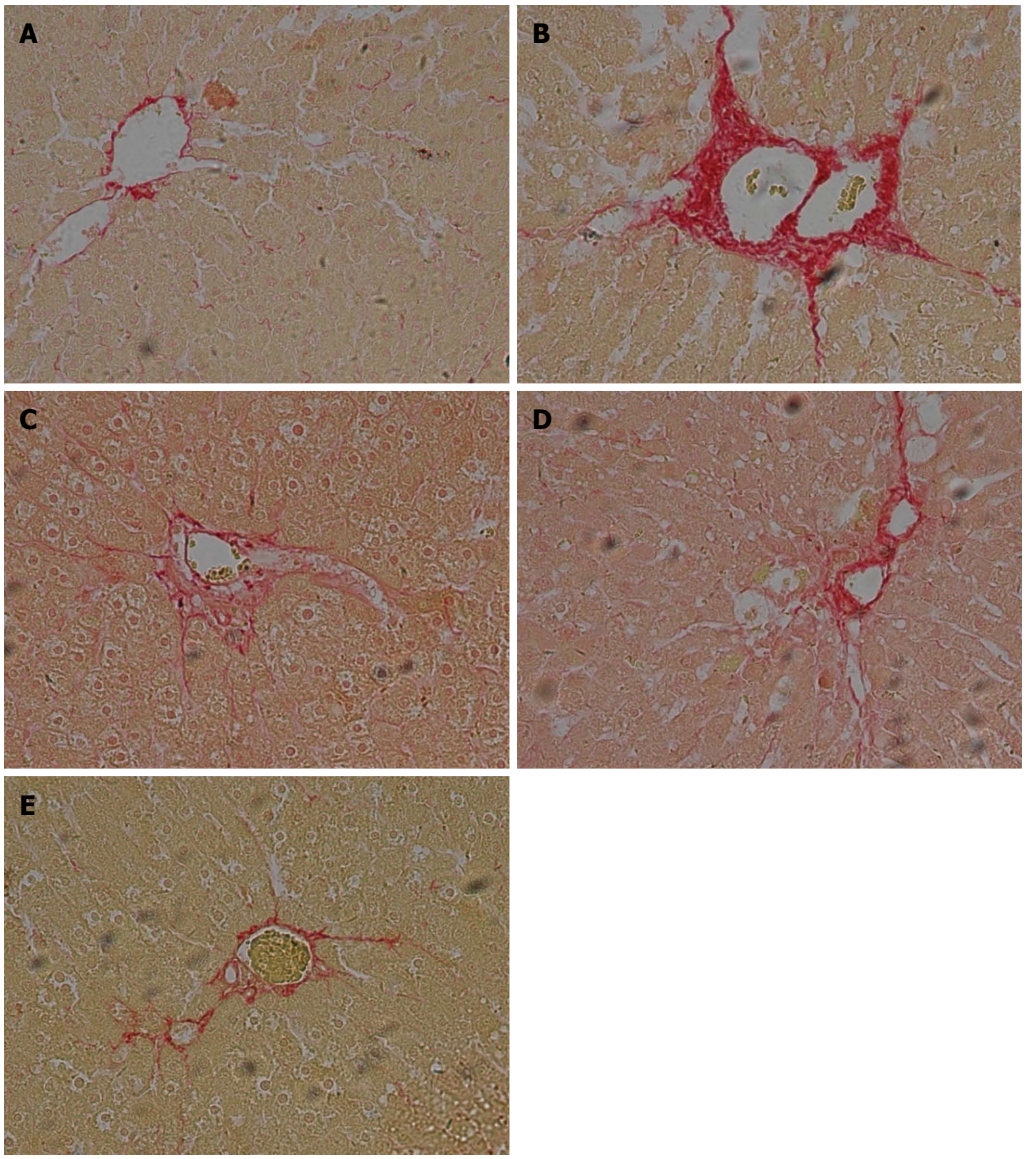


Figure 3 Changes in liver histopathology observed by Sirius Red staining and light microscopy (× 200 magnification). A: Normal healthy control group; B: Model ALF group; C: Qinggan Recipe group; D: Huoxue Recipe group; E: Qinggan Huoxue Recipe group.ALF: Alcoholic liver fibrosis.

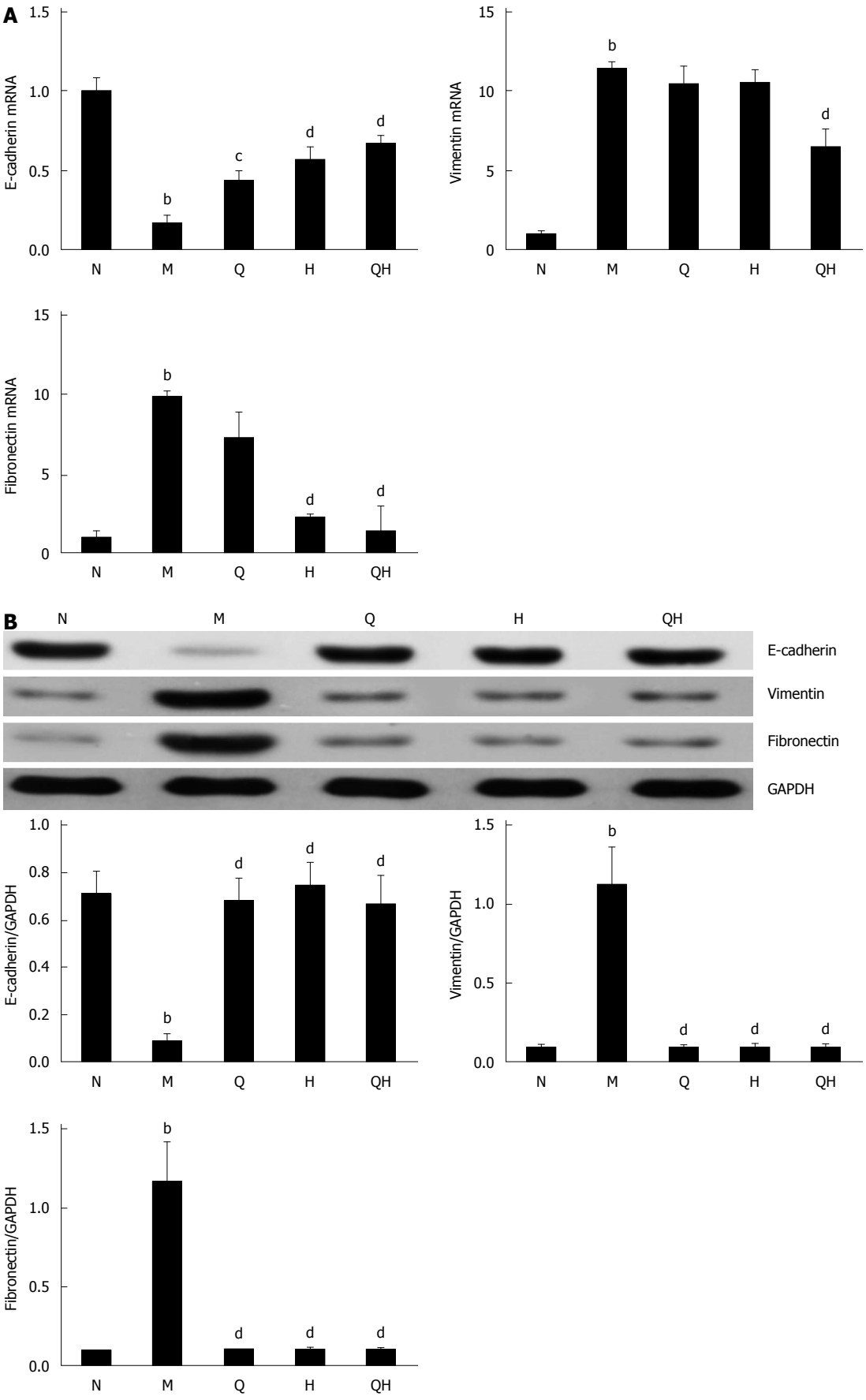


Figure 4 Qinggan Huoxue Recipe-induced expression of epithelial-to-mesenchymal transition-associated factors. A: E-cadherin, vimentin and fibronectin mRNA expression evaluated by RT-PCR; B: E-cadherin, vimentin and fibronectin protein expression evaluated by Western blot. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.

significant differences in the mRNA and protein levels of the three EMT markers were observed only in the group treated with QGHXR.

QGHXR suppresses EMT by inhibiting the TGF-β1/Smad signaling pathway

To examine the inhibitory effect of QGHXR on the expression of EMT-associated transcription factors, the expression of Snail, TGF-β1 and Smad3 was evaluated using PCR and Western blot. Compared with the healthy control group, the mRNA and protein expression of Snail was significantly up-regulated in the untreated ALF group (Figure 5); however, treatment with QGR or QGHXR inhibited the Snail mRNA and protein levels, and HXR inhibited the ALF-induced change in Snail protein levels (Figure 5). As the TGF-β/Smad signaling pathway is a critical pathway triggered by the phosphorylation of Smads, we measured the activation status of the Smad signaling pathway. As expected, TGF-β1 levels increased and Smad3 levels decreased in the liver of ALF rats. When QGR, HXR or QGHXR was given to rats with ALF, TGF-β1 and Smad3 protein levels returned to levels similar to those observed in the healthy control group (Figure 5B). The PCR results showed a similar regulation of related molecules; however, in HXR-treated rats, no significant differences in TGF-β1 mRNA levels were observed compared with the untreated control ALF group (Figure 5A). Similar trends in the mRNA and protein expression levels were observed between the three treatment groups.

In addition, the mRNA and protein expression levels of the tight junction markers occludin, ZO-1 and claudin were significantly reduced in the untreated ALF group compared with the healthy group, and treatment with QGR, HXR, or QGHXR reversed these changes (Figure 5).

DISCUSSION

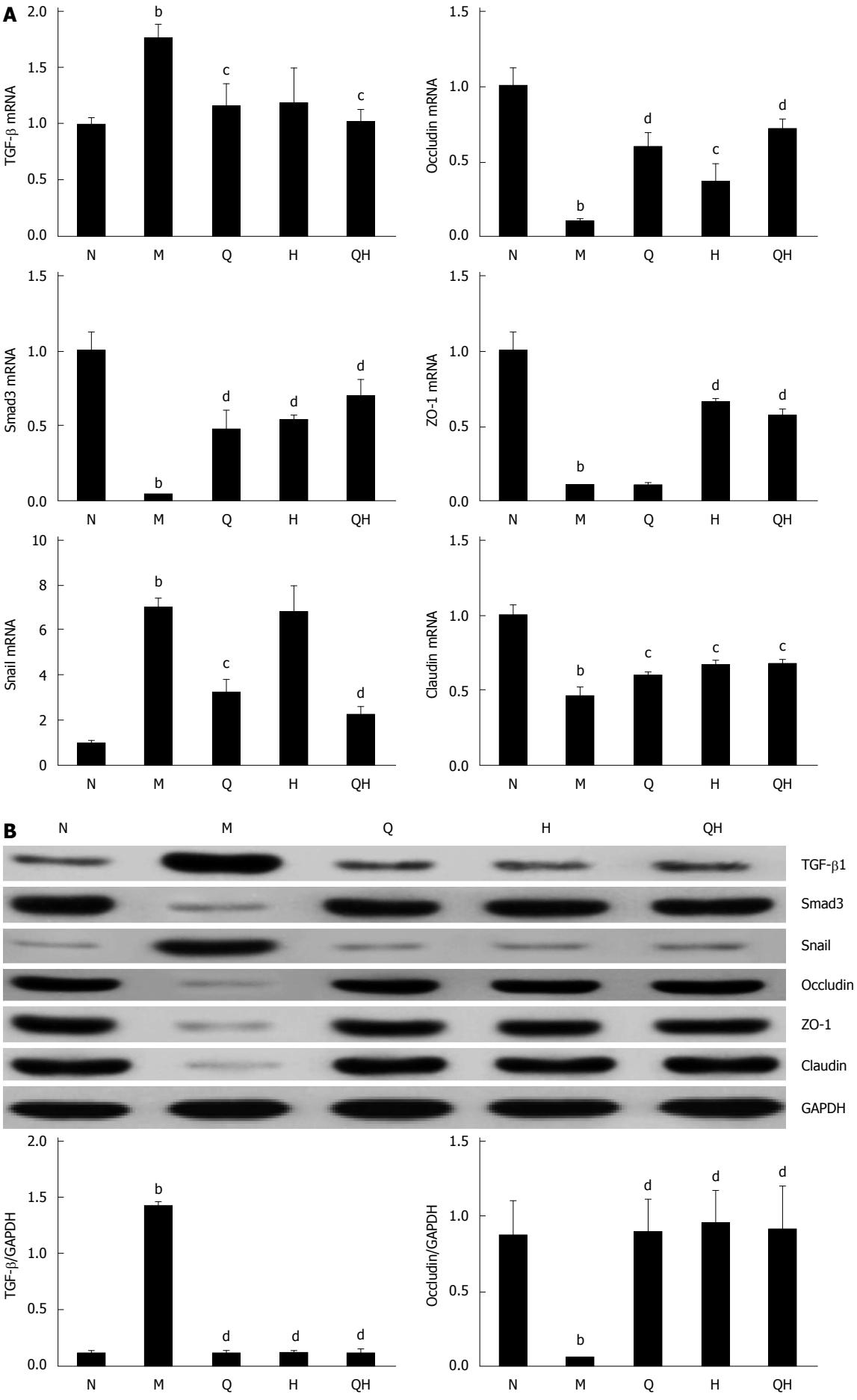
In the present study, we demonstrated that EMT was observed in rats with ALF and that QGHXR treatment can rescue the mesenchymal phenotype.

EMT is a dynamic process by which mature epithelial cells lose their defining characteristics and acquire a mesenchymal phenotype[13]. During the development of EMT, epithelial cells usually lose their adhesive capability and undergo cytoskeletal rearrangements. Previous studies have reported that EMT is characterized by loss of the expression of the epithelial marker E-cadherin and the up-regulation of the mesenchymal markers α-SMA, collagen I, fibronectin and vimentin[14,15]. In this study, E-cadherin expression decreased and vimentin and fibronectin expression increased in a rat model of ALF, indicating that an EMT model was successfully constructed.

An increasing body of evidence indicates that Chinese medicine formulas have potential value as therapeutic agents or adjuvants in ALD treatment[16]. Our previous studies revealed that QGHXR potentially exerts its therapeutic effect against liver injury *via* the LPS-KC signal transduction pathway in rats with ALD[11]. In the present study, we demonstrated that ALF rats undergo classic EMT characterized by the up-regulation of the mesenchymal markers vimentin and fibronectin, and the down-regulation of the epithelial marker E-cadherin. We observed that QGR, HXR, and QGHXR ameliorate alcoholic liver injury by reversing EMT, as demonstrated by the increase in E-cadherin expression and the decrease in vimentin and fibronectin expression (except that QGR and HXR showed no significant changes in vimentin mRNA expression, and QGR elicited no changes in fibronectin mRNA expression).

Increasing evidence indicates that TGF-β1 is the primary mediator of EMT and that the TGF-β1/Smad3 signaling pathway is important in the EMT process[17,18]. TGF-β1 binds and phosphorylates cell-surface receptors (TGF-βRI/TGF-βRII), activates TGF-βRI, and phosphorylates Smad2 or Smad3, which subsequently form a complex with Smad4[19,20]. After activation by phosphorylation and partnering with a co-Smad4, the Smad complex translocates to the nucleus and, in conjunction with other transcription factors, directs the activation and repression of genes regulated by TGF-β1[21,22]. Snail, a gene whose expression is regulated by the TGF-β/Smad signaling pathway[23], inhibited E-cadherin expression and promoted EMT. A strong inverse correlation between snail and E-cadherin expression has been reported in a panel of epithelial and dedifferentiated cells derived from carcinomas of different etiologies[24]. In the present study, the protein and mRNA expression levels of TGF-β1 increased in rats with ALF compared with normal rats. These findings are consistent with previous studies reporting that ALF is characterized by excess accumulation of collagen and other extracellular matrix proteins, steatosis, and fibrosis, and the release of the key pro-fibrogenic cytokine TGF-β1, which is mostly produced by bone marrow-derived macrophages and resident Kupffer cells[2,25,26]. The increase in snail and TGF-β1 and the decrease in Smad3 levels observed in the model ALF group further verified that the TGF-β/Smad signaling pathway is activated in ALF rats. In addition, our results demonstrated that the expression of the EMT-inducing transcription factor snail and TGF-β1 was inhibited, and the expression of Smad3 was enhanced in ALF rats treated with QGR, HXR, or QGHXR. However, the differences in snail and TGF-β1 mRNA expression induced by HXR alone were not significant (Figure 6).

EMT is characterized by the downregulation of E-cadherin expression, which results in the disruption of cell-cell junctions and the dissemination of cells from the primary tumor[27]. In hepatocytes, tight junction proteins are critical for maintaining cell polarity. It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TGF-β mRNA
Occludin mRNA
Smad3 mRNA
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Snail mRNA
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TGF-β/GAPDH
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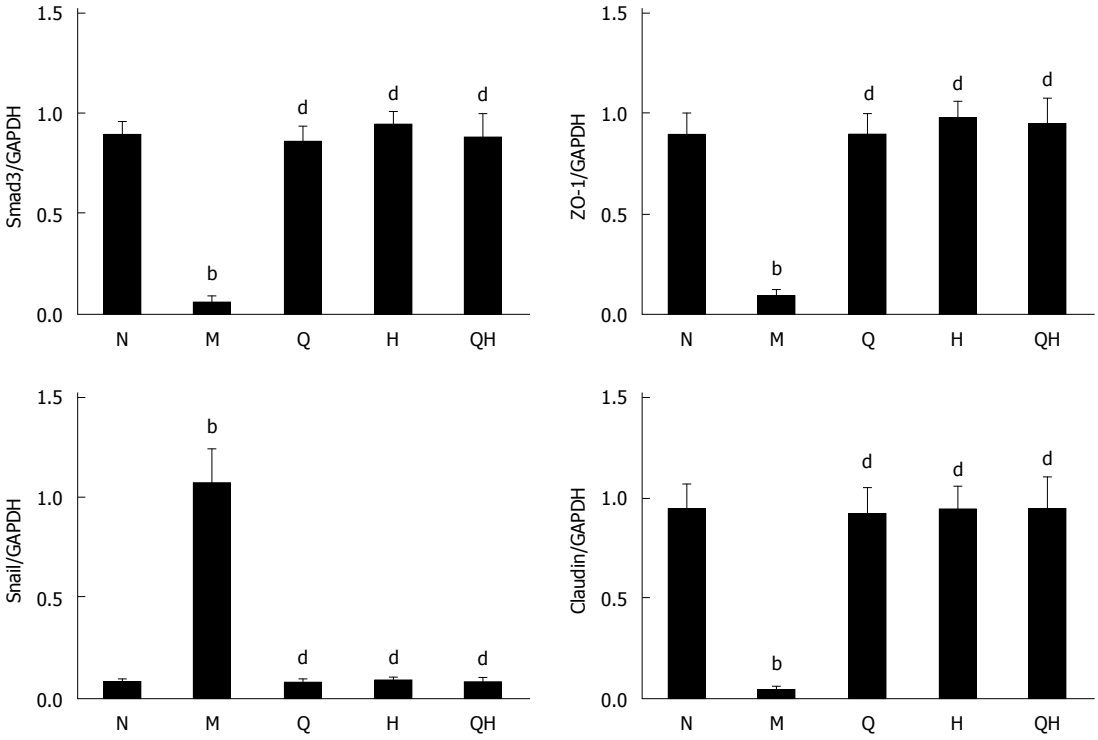

Figure 5 Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition by inhibiting the Smad signaling pathway. A: TGF-β1, Smad3 and Snail mRNA expression evaluated by RT-PCR; B: TGF-β1, Smad3 and Snail protein expression evaluated by Western blot. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.

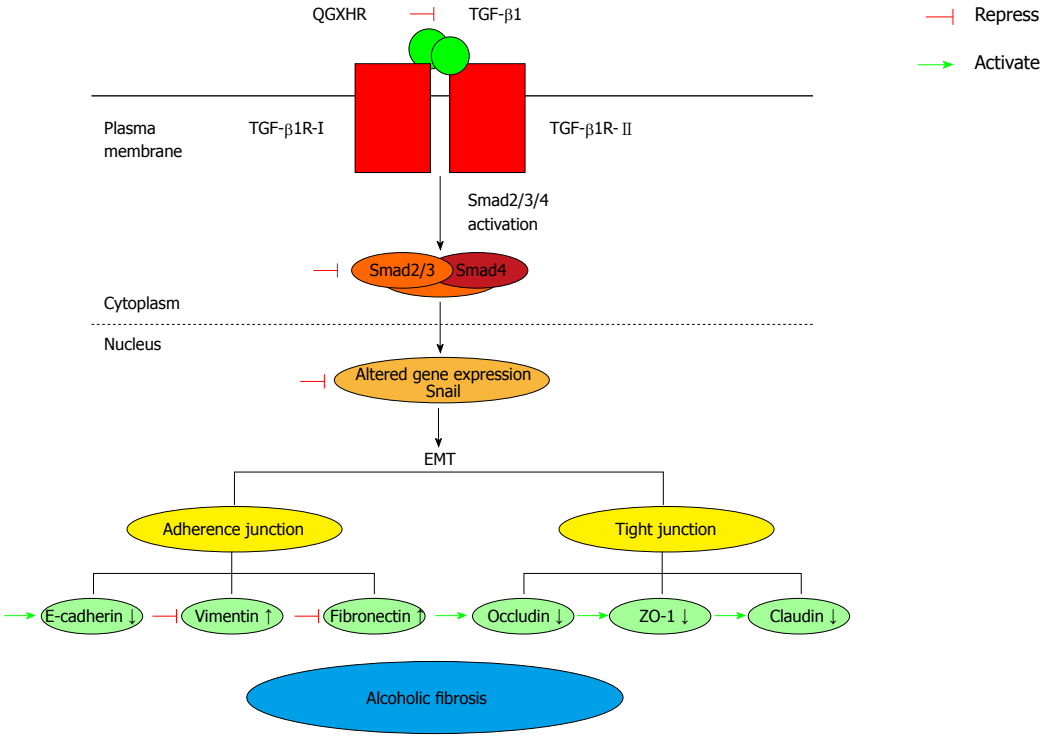

Figure 6 Potential mechanism underlying Qinggan Huoxue Recipe-mediated inhibition of epithelial-to-mesenchymal transition in alcoholic liver fibrosis rats. TGF-β1 stimulates responsive cells through binding and activating the transmembrane receptors TGF-β type I (TGF-βR-I) and type II (TGF-βR-II). Receptor ternary complexes phosphorylate and activate Smad2/3. Once activated, Smad2/3 forms heterocomplexes with Smad4, and these translocate to the nucleus and activate TGF-β1 signaling. Snail, a gene associated with the TGF-β/Smad signaling pathway, inhibits E-cadherin expression, increases vimentin and fibronectin levels, promotes EMT and decreases the levels of occludin, ZO-1 and claudin. QGR, HXR and QGHXR suppressed the effects of ALF-induced modulation of the TGF-β/Smad signaling pathway and ameliorated EMT-induced alcoholic fibrosis. QGR, HXR and QGHXR affected molecules associated with the TGF-β/Smad signaling pathway in the same manner. However, QGR and HXR exerted no significant changes in vimentin mRNA expression, QGR exerted no significant changes in fibronectin mRNA expression, and HXR exerted no significant changes in Snail and TGF-β1 mRNA expression.

is well known that claudin proteins are important components of tight junctions in paracellular transport for maintaining the structure and function[28]. Occludin and junction adhesion molecule A are transmembrane proteins that are directly involved in paracellular transport, and ZO-1 is a protein that contains a domain that forms a binding site for other tight junction proteins[29]. In the model ALF group, the mRNA and protein expression of E-cadherin, occludin and ZO-1 decreased compared with the normal healthy control group, which further verified the establishment of EMT in the ALF rat model. QGR, HXR, and QGHXR increased the mRNA and protein levels of tight junction molecules.

There are several deficiencies in our study. First, we did not evaluate the EMT markers E-cadherin, vimentin and fibronectin using immunofluorescence or immunohistochemistry. Second, we did not use gene knockout rats to verify the specific functions of the molecules analyzed in the current study. Third, although liver disease can disrupt endothelial function[30], we did not evaluate the effect of QGHXR on this parameter. Therefore, further studies are needed to verify the findings of this study.

In conclusion, our study provides evidence that QGHXR inhibits EMT in ALF by modulating the TGF-β1/Smad signaling pathway, suggesting that QGHXR ameliorates alcoholic liver injury *via* this mechanism.

COMMENTS

Background

Alcoholic liver disease has become a major cause of morbidity and mortality worldwide. EMT, a well-characterized dynamic process by which epithelial cells transform into a tissue with a fibroblastic or myofibroblastic phenotype, might have a pivotal role in inducing fibrogenesis. Therefore, targeting EMT might be a promising strategy for inhibiting the generation of extracellular matrix, which can provide a new therapeutic target for liver fibrosis.

Research frontiers

The TGF-β/Smad signaling pathway plays a pivotal role in EMT, cell proliferation and metastasis. This pathway is currently a hot topic in carcinogenesis and fibrosis studies.

Innovations and breakthroughs

EMT is a dynamic process by which mature epithelial cells lose their defining characteristics and acquire a mesenchymal phenotype. The present study demonstrated that the EMT is characterized by the down-regulation of the epithelial marker E-cadherin and the up-regulation of the mesenchymal markers vimentin and fibronectin in ALF rats. QGHXR reversed the EMT-induced changes in E-cadherin, vimentin and fibronectin levels and inhibited ALF by regulating the TGF-β1/Smad signaling pathway.

Applications

The present study provides evidence that QGHXR, a traditional Chinese medicine recipe, inhibits ALF-induced EMT by modulating the TGF-β1/Smad signaling pathway, suggesting that the potential mechanism by which QGHXR ameliorates alcoholic-induced liver injury is associated with this pathway. Thus, EMT represents a novel therapeutic target for ALF and the findings of this study provide new insight into the pathogenesis of ALF.

Terminology

EMT is a phenotypic conversion of the epithelium to a fibroblastic or myofibroblastic phenotype. EMT begins with the dissociation of adhesions between epithelial cells, the decrease of apical-basal polarity, the reorganization of the actin cytoskeleton and the increase of cell motility.

Peer-review

This study "Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway" is very interesting.

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ORIGINAL ARTICLE

Basic Study

Laparoscopic colonic anastomosis using a degradable stent in a porcine model

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Author contributions: Ma L performed the majority of experiments and analyzed the data; Ma L, Wang HH, Huang DY, Ge GJ, Hu HY and Yu SC contributed equally to treatment of animals; Cai XJ and Ma L designed and coordinated the research; Ma L and Yu YL wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Sir Run Run Shaw Hospital, Hangzhou, China.

Institutional animal care and use committee statement: All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Center for Drug Safety Evaluation and Research of Zhejiang University with the following reference number: IACUC-13001.

Conflict-of-interest statement: None of the authors disclosed any conflict of interest.

Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the feasibility and safety of laparoscopic colonic anastomosis using a degradable stent in a porcine model.

METHODS: Twenty Bama mini-pigs were randomly assigned to a stent group (n = 10) and control group (hand-sewn anastomosis, n = 10). The anastomotic completion and operation times were recorded, along with histological examination, postoperative general condition, complications, mortality, bursting pressure, and the average anastomotic circumference (AC).

RESULTS: All pigs survived postoperatively except for one in the stent group that died from ileus at 11 wk postoperatively. The operation and anastomotic completion times of the stent group were significantly shorter than those of the control group (P = 0.004 and P = 0.001, respectively). There were no significant differences in bursting pressure between the groups (P = 0.751). No obvious difference was found between the AC and normal circumference in the stent group, but AC was significantly less than normal circumference

in the control group (P = 0.047, P < 0.05). No intestinal leakage and luminal stenosis occurred in the stent group. Histological examination revealed that the stent group presented with lower general inflammation and better healing.

CONCLUSION: Laparoscopic colonic anastomosis with a degradable stent is a simple, rapid, and safe procedure in this porcine model.

Key words: Laparoscope; Colon; Anastomosis; Stent; Porcine model

Core tip: We explored the feasibility and safety of laparoscopic colonic anastomosis using a degradable stent in a porcine model. Twenty Bama mini-pigs were randomly assigned to a stent group and hand-sewn anastomosis group. The operation and anastomotic completion times of the stent group were significantly shorter than those of the control group. There was no significant difference between the anastomotic and normal circumference in the stent group. No intestinal leakage and luminal stenosis occurred in the stent group. Histological examination of anastomoses revealed that the stent group presented with less general inflammation and better healing than the control group.

Ma L, Cai XJ, Wang HH, Yu YL, Huang DY, Ge GJ, Hu HY, Yu SC. Laparoscopic colonic anastomosis using a degradable stent in a porcine model. *World J Gastroenterol* 2016; 22(19): 4707-4715 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4707.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4707

INTRODUCTION

There is substantial evidence that laparoscopic surgery plays a pivotal role in abdominal surgical disease[1,2]. Laparoscopic intestinal anastomosis is a basic technique in gastrointestinal procedures. The hand-sewn method has been the most widely accepted procedure for intestinal anastomosis for the past 160 years. Although surgical skills have been significantly improved and developed, hand-sewn anastomoses are still complicated and time-consuming[3]. An effective alternative technique is stapled anastomosis. Although this is relatively convenient and time-saving, stapler devices are more expensive than the hand-sewn method and have a higher risk of anastomotic stenosis[4,5]. Besides, the foreign materials in anastomosis may induce chronic inflammation[6,7], which could slow down the process of healing or lead to secondary leakage and stenosis[8,9]. In addition, intestinal staplers cannot be used for hemostasis, and suturing after stapling may be required[10]. Another feasible technique is sutureless anastomosis, such as compression rings, tissue glue, and laser anastomosis, which have been in use since Murphy's button in 1892[10-13]. However, sutureless anastomosis is less applied clinically due to some safety issues, such as weak anastomotic strength, necrosis, and stricture. Therefore, an ideal laparoscopic surgical procedure that can achieve the desired results is urgently needed.

In our previous experiments, we have exhibited the feasibility and safety of colonic anastomosis and primary repair of colonic perforation using a degradable stent in a porcine model undergoing open surgery[14-16]. In this study, we further explored the application of this stent in laparoscopic colonic anastomosis using a degradable stent.

Figure 1 Appearance of degradable stent.

MATERIALS AND METHODS

Animals

Twenty healthy experimental Bama mini-pigs of either sex, weighing approximately 30 kg, were purchased from Shanghai Multi-Bio-Sci-Tech Co. Ltd., China. Animal were raised separately in clean cages at the Experimental Animal Center of Zhejiang University and provided with a liquid diet for 5 d before surgery. They were then were starved for 12 h and fed with 5% magnesium sulfate to clean the colonic lumen. Cefazolin sodium was administered intramuscularly for preoperative antibiotic prophylaxis. All the protocols were approved by the Experimental Animal Ethics Committee of Zhejiang University.

Features of the degradable stent

The stent was developed by the Institute of Polymer Science of Zhejiang University (Figure 1). The material properties were demonstrated in our previous studies[14-16]. The stent is synthesized from 1,3-propanediol, 1,2-propanediol, and sebacic acid and decomposes finally to CO_2 and water. *In vivo*, we found that the stents were damage free on day 10 and were degraded and broken on day 28. The stent was approved by the State Food and Drug Administration of

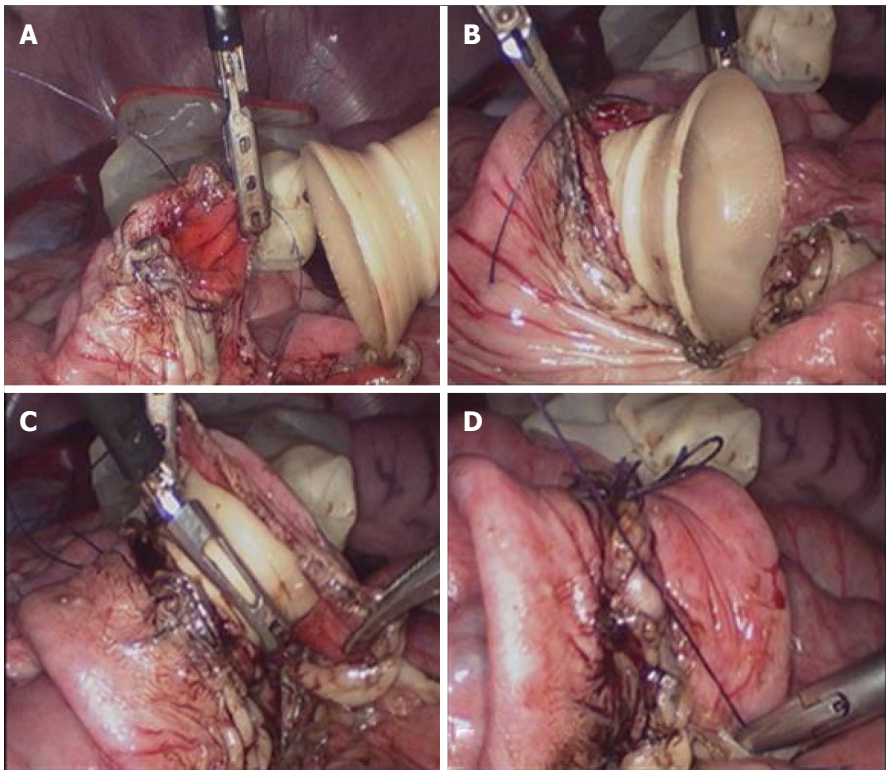

Figure 2 Operative procedure in the stent group. A: A purse-string suture was performed circularly around the intestine; B: One end of the stent was embedded in one side of the intestinal cavity, and the purse string was tightened, knotted, and fixed; C: The other side of the stent was placed in the contralateral intestinal cavity, and the purse string was tightened, knotted, and fixed; D: Both sides of the purse-string were knotted and fixed.

China for its biocompatible qualities (No. G20090993)[16].

Experimental design and procedure

Twenty pigs were randomized and assigned to a degradable stent group (n = 10) and control group (hand-sewn anastomosis, n = 10). The pigs in each group were divided evenly into two subgroups according to the time of sacrifice (2 wk and 3 mo postoperatively), and each subgroup included five pigs. A laparoscopic colonic anastomosis with a degradable stent was performed in the stent group, and laparoscopic hand-sewn anastomosis was performed in the control group.

The animals were anesthetized by intramuscular injection of midazolam (total 5 mg, Jiangsu Enhua Pharmaceutical Group, China) and inhalation anesthesia with isoflurane, with subsequent intubation and mechanical ventilation. The animals were in the supine position on a disposable disinfectant towel. Laparotomy was performed *via* left lower quadrant incision (about 2.5 cm), and pneumoperitoneum was established using CO_2 to create a sufficient operating space in the abdominal cavity. The appropriate intestinal segment was selected, and then the colon and partial mesentery was dissociated. The tissue with bad blood supply was cut, and the colonic contents were removed. The colonic lumen was cleaned, and the incisal edge was disinfected. At 0.5 cm from both ends of the bowel transection, a purse-string suture using 3-0 absorbable thread sutures was performed circularly around the intestine at the seromuscular layer of the colon (Figure 2A). One end of the stent was embedded into the intestinal cavity, and the purse string was tightened, knotted, and fixed (Figure 2B). Afterwards, the other end of the stent was placed into the contralateral intestinal cavity, and the purse string was tightened, knotted, and fixed (Figure 2C). Finally, both sides of the purse-string were knotted and fixed, and another one or two stitches were added intermittently, if necessary, to avoid intestinal volvulus (Figure 2D). No abdominal drain was placed, and the incisions were closed. Pigs in the control group received a hand-sewn, end-to-end colonic anastomosis. The procedure was performed as previously described[15].

After the operation, pigs were given free access to water for 24 h and returned to a fluid diet 24 h later, with normal diet being resumed on postoperative day 7. The anastomotic completion and operation times of each group were recorded as well as postoperative general condition, complications, and mortality. Five pigs in each group were sacrificed 2 wk postoperatively to evaluate the bursting pressure, and the rest was sacrificed 3 mo postoperatively to assess the average anastomotic circumference (AC) and healing of the anastomosis. All the procedures were conducted by the same operators. The circumference was approximately 10 cm above and below the anastomotic stoma, and the narrowest circumference was measured. After

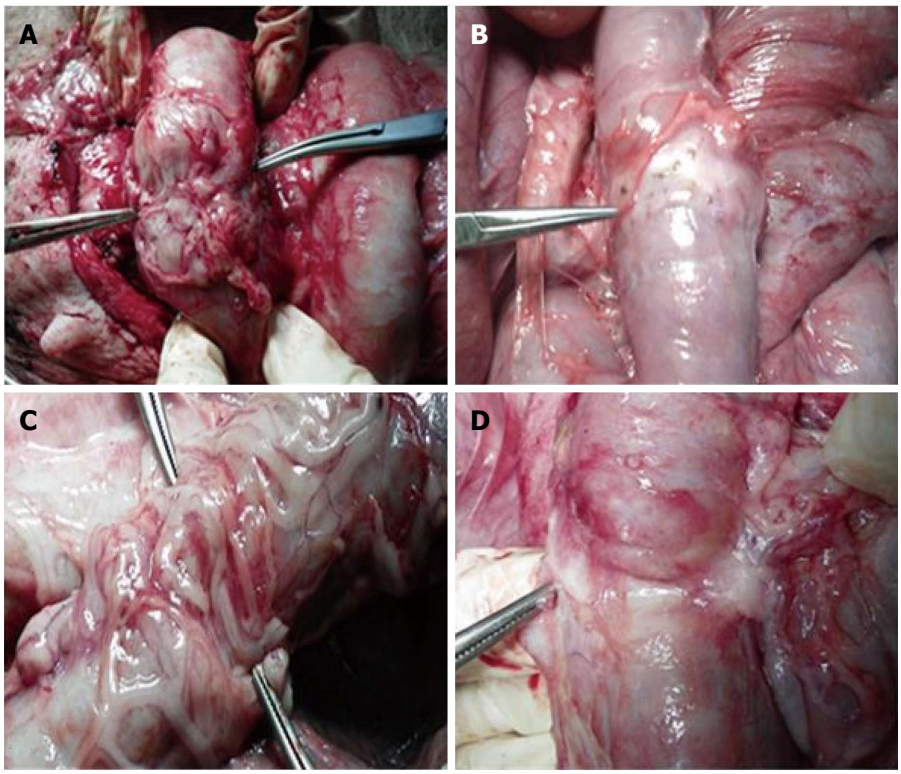

Figure 3 General observation of the anastomosis. A: Stent group at postoperative week 2; B: Stent group at postoperative month 3; C: Control group at postoperative week 2; D: Control group at postoperative month 3.

sacrifice, an approximately 5-cm anastomotic segment was resected for histological examination, including hematoxylin-eosin (HE), Masson's trichrome stain, and immunohistochemical staining, performed by the Pathology Department of Sir Run Run Shaw Hospital, Hangzhou, China. The samples were fixed, embedded, and sliced into 5-μm thick sections. Immunohistochemical staining was performed with mouse anti-porcine anti-α-smooth muscle actin (SMA) antibody (1:300; Abcam, Cambridge, United Kingdom), anti-basic fibroblast growth factor (b-FGF) antibody (1:300, Santa Cruz Biotechnology, Dallas, TX, United States), or anti-transforming growth factor (TGF)-β1 antibody (1:300, Santa Cruz Biotechnology). The second antibody was performed using rabbit anti-mouse (1:1000, Zhongshan Goldenbridge Biotechnology Co. Ltd., Beijing, China).

Statistical analysis

Continuous variables, presented as mean ± SD, were compared using Student's *t* test. Statistical analysis was performed by using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States).

Animal care and use statement

All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Center for Drug Safety Evaluation and Research of Zhejiang University with the reference number: IACUC-13001.

RESULTS

General condition

General observation of anastomosis is shown in Figure 3. One pig in the stent group died from ileus at postoperative week 11. Autopsy found that the ileus was located below the anastomosis, and the cause of death was congenital intestinal valvular disease. The other animals all survived.

Anastomotic completion and operation times

As shown in Table 1, the mean operation time of the stent group was significantly shorter than that of the control group ($P = 0.004$, $P < 0.01$). Within the stent group, there were significant differences between the two subgroups ($P = 0.010$). The operation time in the 2 wk and 3 mo subgroups was 86.6 ± 10.9 min and 56.6 ± 16.6, respectively. However, no significant differences were found in the two subgroups of the control group ($P = 0.426$). The mean anastomotic completion time of the stent and control groups was 33.1 ± 18.5 min and 65.5 ± 19.9 min, respectively. There was a significant difference between the two groups ($P = 0.001$). In the stent group, the anastomotic completion time of the 2 wk subgroup was less than that of the 3 mo subgroup ($P = 0.016$). In addition, there were no significant differences in the

Table 1 Parameters compared between different groups

Groups	Subgroups	Operation time(min)	Completion time (min)	Bursting pressure (cmH_2O)	Anastomotic circumference (cm)	Normal circumference (cm)
Stent group		71.6 ± 20.6	33.1 ± 18.5	-	-	-
	Week 2	56.6 ± 16.6	23.6 ± 14.8	108.0 ± 34.9		-
	Month 3	86.6 ± 10.9	42.6 ± 18.0	-	7.7 ± 0.4	7.7 ± 0.2
Control group		108.3 ± 27.8	65.5 ± 19.9	-	-	-
	Week 2	100.8 ± 34.1	57.2 ± 24.3	97.5 ± 60.2	-	-
	Month 3	115.8 ± 20.8	73.8 ± 11.5	-	7.1 ± 1.1	8.3 ± 1.1

two subgroups of the control group (P = 0.204).

Bursting pressure and AC

The bursting pressure of the 2 wk subgroup in the stent and control groups was 108.0 ± 34.9 and 97.5 ± 60.2 cm H_2O, respectively, and there were no significant differences in the two subgroups (P = 0.751). The normal intestinal circumference and AC of the 3 mo subgroups in the stent and the control groups were 7.7 ± 0.2, 7.7 ± 0.4, 8.3 ± 1.1, and 7.1 ± 1.1 cm, respectively. There were no significant differences between the normal intestinal circumference and the AC in the stent group (P = 0.344). However, the AC was lower than the normal intestinal circumference in the control group (P = 0.047).

Colonic anastomotic healing and stent degradation

No intestinal leakage or luminal stenosis occurred in the stent group; while leakage appeared in three pigs in the 2 wk subgroup of the control group, and two pigs achieved healing. Bowel tapering was found in two pigs in the 3 mo subgroup of the control group. No stents were dislocated in the 2 wk subgroups of both groups, but the stents presented with partial breakages because the material properties were fragile. All the stents were absorbed in anastomotic stoma at 3 mo postoperatively.

Microscopic findings at 2 wk and 3 mo postoperatively

HE staining at postoperative week 2 in both groups showed that the anastomotic mucosa was absent, and granulation tissue formed with infiltration of a large number of lymphocytes, plasma cells, and neutrophils. Fibrous tissue hyperplasia, collagen deposition, and muscularis propria interruption were present. However, the degree of inflammatory infiltration in the stent group was lower than in the control group (Figure 4A-D). Collagen deposition was similar in the two groups (Figure 5A-D). Immunohistochemical staining demonstrated that α-SMA had higher immunostaining intensity and wider range than b-FGF and TGF-β1 and no significant differences were found between the two groups (Figure 6A and B).

HE staining at postoperative month 3 showed that colonic mucosa was present at the site of anastomosis, along with fibrous tissue hyperplasia and collagen deposition that extended through the adventitial layer. Smooth muscle bundles were also interleaved between the mucosa and adventitial layer, fusing with the muscularis mucosa. However, submucosa was absent at the site of the anastomosis. The amount of smooth muscle tissue in the stent group was higher than in the control group (Figure 4E-H). There was a large amount of collagen deposition and scar tissue formation and no obvious differences were found between the two groups (Figure 5E-H). Immunohistochemical staining showed that the intensity of α-SMA staining decreased compared with that at postoperative week 2, but the range was still extensive. Positive staining smooth muscle cells presented with brown color and were arranged in a fascicular pattern. The immunostaining intensity and range of b-FGF and TGF-β1 were lower than those at week 2 (Figure 6).

DISCUSSION

In the present study, the safety and feasibility of laparoscopic colonic anastomosis using a degradable stent was investigated by comparing with hand-sewn anastomosis. The operation and anastomotic completion times using a degradable stent were significantly shorter than those for the hand-sewn method, while no significant differences were found in bursting pressure. Besides, no obvious significance was found between the AC and normal circumference in the stent group, whereas the AC was significantly less than the normal circumference in the control group. Severe complications, such as intestinal leakage and luminal stenosis, were also less frequent than those with the hand-sewn method. Therefore, we conclude that the simple surgical procedure with a degradable stent was a simple, rapid, feasible, and safe procedure in this porcine model.

The methods for intestinal anastomosis are numerous, and each has its advantages and disadvantages. Extracorporeal anastomosis is simple but is highly invasive. Therefore, totally laparoscopic procedures have been gradually paid more attention. Laparoscopic stapled anastomosis may elicit local inflammation caused by a foreign body reaction and result in leakage and stenosis[17]. The biodegradable compression ring (BAR Valtrac) is hard to perform and is not suitable for laparoscopic operation. Another novel compression anastomosis clip (Hand CAC 30), which is made of a shape-memory alloy of nickel-titanium, is suitable for laparoscopic operation[18-20].

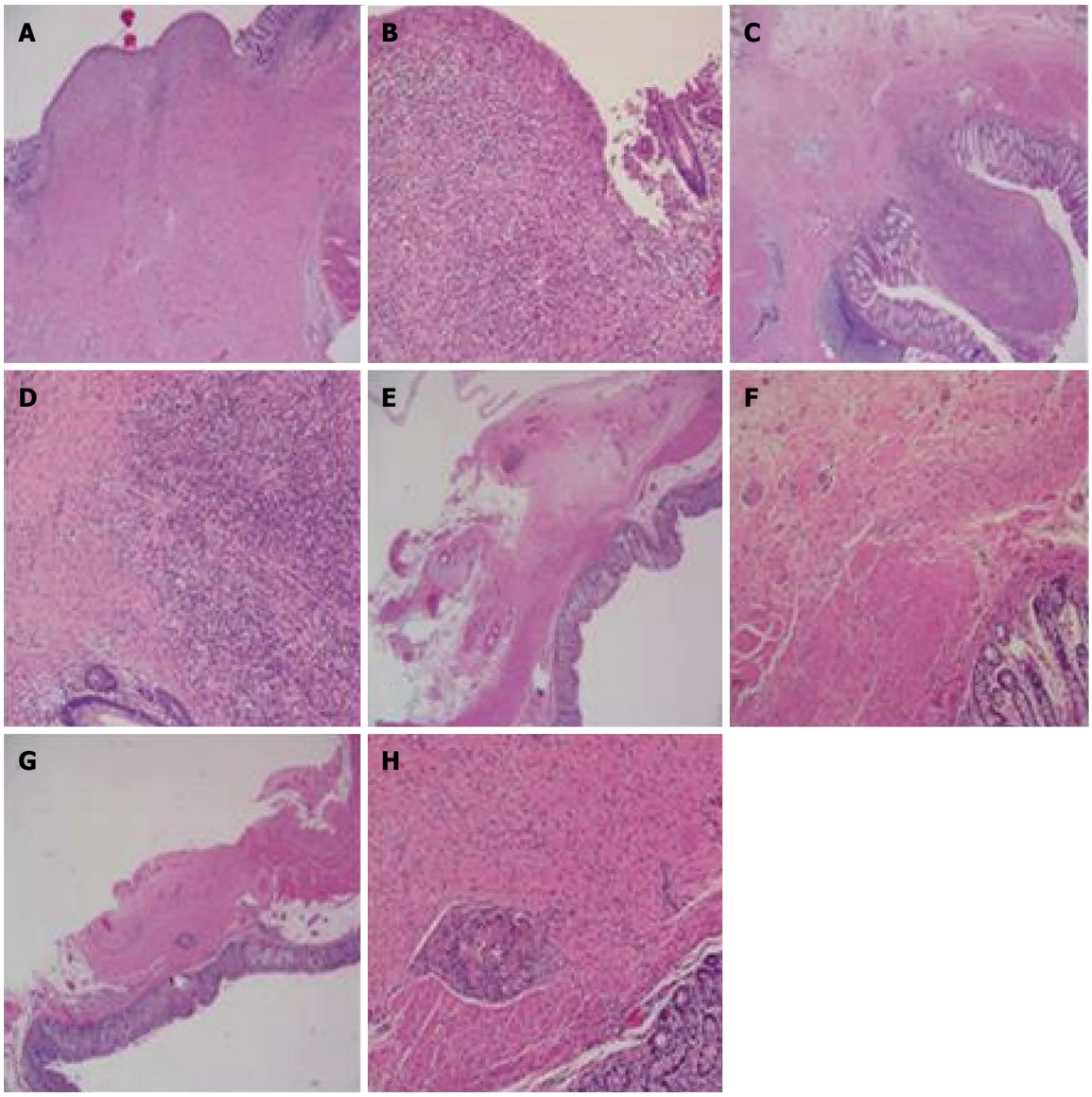

Figure 4 Hematoxylin and eosin staining of anastomosis. A, B: Hematoxylin and eosin (HE) staining of the stent group at 2 wk postoperatively (× 100, × 200); C, D: HE staining of the control group at 2 wk postoperatively (× 100, × 200); E, F: HE staining of the stent group at 3 mo postoperatively (× 100, × 200); G, H: HE staining of the control group at 3 mo postoperatively (× 100, × 200).

However, CAC is metallic, which cannot be degraded and removal and discharge are difficult[21]. Therefore, it is essential to develop an ideal surgical procedure that is easy to perform. In the present study, we introduced a novel degradable stent that was synthesized from 1,3-propanediol, 1,2-propanediol and sebacic acid, which can decompose finally to CO_2 and water. Like the biodegradable anastomosis, the degradable stent leaves no residual foreign body in the anastomosis and can isolate the intestinal contents from the anastomotic stoma, subsequently reducing the chance of infection. In addition, the appropriate stent diameter can be selected, which may avoid anastomotic stenosis and achieve the ideal effect.

Twenty healthy experimental Bama mini-pigs were used in our study. Only one pig died from ileus caused by congenital intestinal valvular disease. Hence, the death was not directly related to the experimental method. The operation and anastomotic completion times in the stent group were significantly shorter than in the control group, indicating that the stent procedure was easier and simpler to perform compared with the hand-sewn method. In the stent group, the operation time in the 3 mo subgroup declined 34.65% more than in the 2 wk subgroup, while the anastomotic completion time declined by 44.6% in both subgroups. The anastomotic completion time for the last four pigs in the stent group was 18, 15, 18, and 17 min, respectively. This was superior to the hand-sewn method (24.5 ± 11.3 min) and close to the stapled intestinal anastomosis (mean 15.5 ± 7.8 min)[22]. The results indicated the potential of the laparoscopic colonic anastomosis stent method. No significant differences were found in bursting pressure between the groups. Bursting pressure is one of the most reliable parameters for measuring the quality of the healing process. The

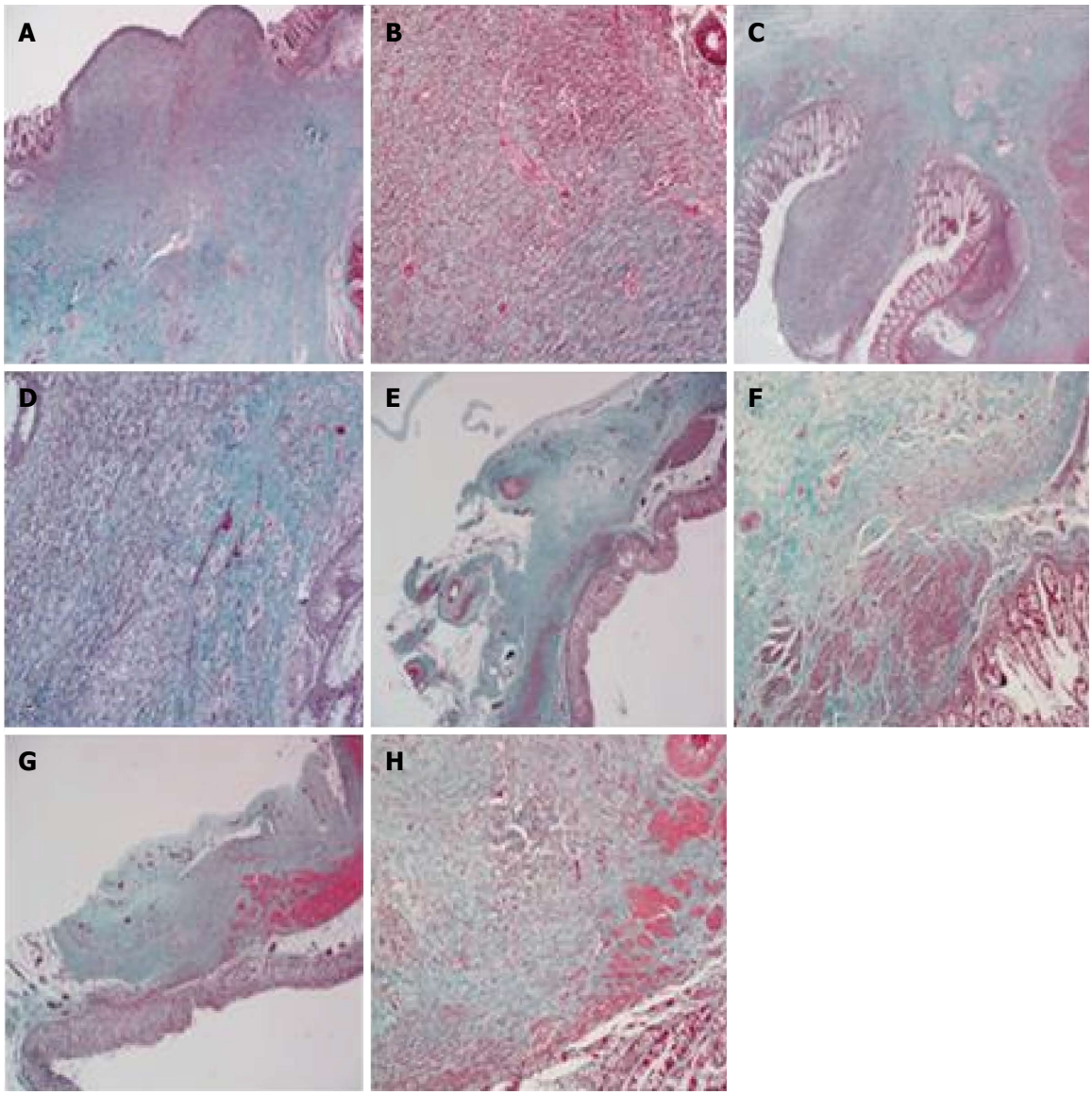

Figure 5 Masson's trichrome staining of anastomosis. A, B: Masson's trichrome staining of the stent group at 2 wk postoperatively (× 100, × 200); C, D: Masson's trichrome staining of the control group at 2 wk postoperatively (× 100, × 200); E, F: Masson's trichrome staining of the stent group at 3 mo postoperatively (× 100, × 200); G, H: Masson's trichrome staining of the control group at 3 mo postoperatively (× 100, × 200). The hyperplastic fibrous tissue and collagenous fibers are shown with green or blue, while hyperplastic smooth muscle cells with red.

healing process in the stent and control groups was uneventful. There were no significant differences between the average AC and normal circumference in the stent group at 3 mo postoperatively, which showed that no luminal stenosis occurred in the presence of the stents. However, the average AC of the control group at 3 mo postoperatively was less than the normal average circumference, which may be the reason for bowel tapering in two pigs.

Histological examination is the gold standard for confirming anastomotic healing. We found that the mucosa of the anastomosis was absent at postoperative week 2, and granulation tissue formed with a large amount of inflammatory cell infiltration. In addition, fibrous tissue hyperplasia and collagen deposition were noted, but the smooth muscle layer was not formed. The degree of inflammatory infiltration in the stent group was lower than in the control group. The mucosa had grown completely, and smooth muscle was already formed at 3 mo postoperatively. The amount of smooth muscle in the stent group was more than in the control group, and foreign body granuloma was noted in the control group. The results indicated that the healing was similar in the two groups, but the inflammatory reaction was lighter and the growth of intestinal smooth muscle was better in the stent group than in the control group. Therefore, the stent method had some advantages. No foreign body, lower tension, and isolation from intestinal contents might have contributed to the satisfactory results in the stent group.

However, there were some limitations in our study. The sample of animals was small, and the experimental animals were Bama mini-pigs. Although

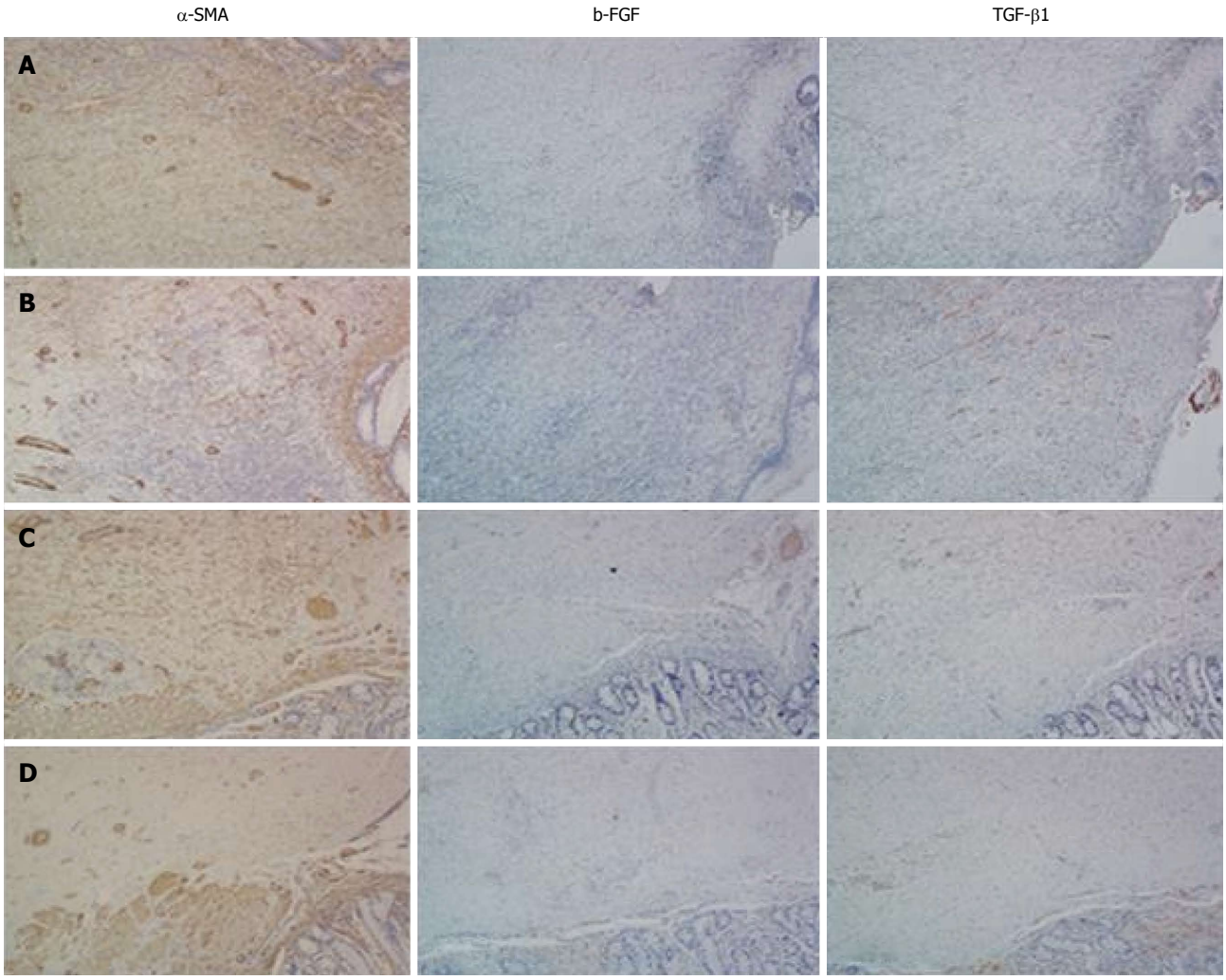

Figure 6 Immunohistochemical staining of anastomosis. A: Immunohistochemical staining for α-SMA, b-FGF, and TGF-β1 of the stent group at 2 wk postoperatively; B: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 of the control group at 2 wk postoperatively; C: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 of the stent group at 3 mo postoperatively; D: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 in the control group at 3 mo postoperatively (all × 200). α-SMA: α-smooth muscle actin; b-FGF: Basic fibroblast growth factor; TGF: Transforming growth factor.

the animals have similar physiological features to humans and comparable operating procedures, the healing ability and anti-infective activity in the animals might not be consistent with those of humans. Hence, further research should be done before the procedure is applied clinically.

In conclusion, laparoscopic colonic anastomosis with degradable stents could be a potential alternative procedure for intestinal anastomosis.

COMMENTS

Background

Laparoscopic intestinal anastomosis is a basic technique in gastrointestinal procedures. Although surgical skills have been significantly improved and developed, hand-sewn anastomoses are still complicated and time-consuming. Therefore, an ideal laparoscopic surgical procedure that can achieve the desired results is urgently needed.

Research frontiers

Many procedures have been developed, including stapling devices, compression rings, tissue glue, and laser anastomosis. However, stapler devices are expensive and require introduction of a permanent foreign body. Sutureless anastomosis has weak anastomotic strength, necrosis, and stricture. Tissue glue and laser anastomosis are not used clinically because of weak anastomotic strength.

Innovations and breakthroughs

The authors developed a degradable stent and have exhibited its feasibility and safety in colonic anastomosis and primary repair of colonic perforation in a porcine model undergoing open surgery. With this simple technique, suturing time was greatly decreased, the anastomosis was free of compressive pressure, and the damage to the submucosal vascular plexus and mesenteric vessels were minimized. In this study, the application of this stent in laparoscopic colonic anastomosis was explored.

Applications

Laparoscopic colonic anastomosis with degradable stents could be a potential alternative procedure for intestinal anastomosis. Further research should be done before the procedure is applied clinically.

Terminology

Anastomotic circumference: The circumference of the anastomosis. Bursting pressure: the maximum pressure the segment resisted or the pressure at the

moment the first leakage.

Peer-review

This is a nice and novel study with good practical value. The novel technique described has potential applications in the future.

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ORIGINAL ARTICLE

Basic Study

Plasma long noncoding RNA expression profile identified by microarray in patients with Crohn's disease

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Author contributions: Chen D and Liu J performed the majority of experiments; Zhao HY, Chen YP and Xiang Z participated in clinical sample collection, microarray, qRT-PCR and data analysis; Jin X designed the study and wrote the manuscript; Liu J and Jin X contribute equally as the first author.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the expression pattern of plasma long noncoding RNAs (lncRNAs) in Chrohn's disease (CD) patients.

METHODS: Microarray screening and qRT-PCR verification of lncRNAs and mRNAs were performed in CD and control subjects, followed by hierarchy clustering, GO and KEGG pathway analyses. Significantly dysregulated lncRNAs were categorized into subgroups of antisense lncRNAs, enhancer lncRNAs and lincRNAs. To predict the regulatory effect of lncRNAs on mRNAs, a CNC network analysis was performed and cross linked with significantly changed lncRNAs. The overlapping lncRNAs were randomly selected and verified by qRT-PCR in a larger cohort.

RESULTS: Initially, there were 1211 up-regulated and 777 down-regulated lncRNAs as well as 1020 up-regulated and 953 down-regulated mRNAs after microarray analysis; a heat map based on these results showed good categorization into the CD and control groups. GUSBP2 and AF113016 had the highest fold change of the up- and down-regulated lncRNAs, whereas TBC1D17 and CCL3L3 had the highest fold

change of the up- and down-regulated mRNAs. Six (SNX1, CYFIP2, CD6, CMTM8, STAT4 and IGFBP7) of 10 mRNAs and 8 (NR_033913, NR_038218, NR_036512, NR_049759, NR_033951, NR_045408, NR_038377 and NR_039976) of 14 lncRNAs showed the same change trends on the microarray and qRT-PCR results with statistical significance. Based on the qRT-PCR verified mRNAs, 1358 potential lncRNAs with 2697 positive correlations and 2287 negative correlations were predicted by the CNC network.

CONCLUSION: The plasma lncRNAs profiles provide preliminary data for the non-invasive diagnosis of CD and a resource for further specific lncRNA-mRNA pathway exploration.

Key words: Crohn's disease; Long noncoding RNA; Inflammatory bowel disease; Plasma; Microarray

Core tip: The pathogenesis of Chrohn's disease (CD) is unclear while increasing evidence supports the involvement of epigenomic regulation. In this study, we jointly used microarray screening and qRT-PCR verification to achieve the plasma specific long noncoding RNAs expression profile of patients with CD and their potential regulation of downstream mRNAs. Our results would provide preliminary data for non-invasive diagnosis of CD and a reservoir for specific lncRNA-mRNA pathway exploration in the future.

Chen D, Liu J, Zhao HY, Chen YP, Xiang Z, Jin X. Plasma long noncoding RNA expression profile identified by microarray in patients with Crohn's disease. *World J Gastroenterol* 2016; 22(19): 4716-4731 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4716.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4716

INTRODUCTION

Crohn's Disease (CD) is a chronic and relapsing inflammatory disease that could affect any part of the intestine. The prevalence of CD is increasing in developing and developed countries, making it a global health care problem and an interesting research area[1,2]. However, the mechanism of CD remains vague; the involvement of genetic predisposition, immune response and environmental factors has been advocated[3]. Although the development of 5-amino-salicyte acid, prednisone and anti-inflammatory reagents has improved CD therapy, their effects are much more alleviative and sometimes are ineffective for refractory CD[4]. Therefore, the development of novel CD therapeutics is urgently needed, and the exploration of CD mechanisms is of clinical importance.

Recent genome-wide association studies (GWAS) identified novel susceptibility genes for CD[5], highlighting the important role of genomic factors. However, the majority of these studies focused on protein-coding genes and neglected the noncoding RNAs (ncRNAs) that were previously regarded as junk RNA or transcript noises[6]. With the development of high-throughput technologies, huge numbers of ncRNAs were identified, and many ncRNAs have been shown to be involved in physiological processes that maintain cellular and tissue homeostasis[7-9]. Generally, ncRNAs could be categorized into small ncRNAs (< 200 nt), such as microRNAs (miRNAs), and long noncoding RNAs (lncRNAs, > 200 nt). Research on lncRNAs has increased in recent years, showing their potency in regulating protein coding genes at the level of chromatin remodeling, transcriptional control and post-transcriptional processes[10].

A recent study revealed that lncRNAs play a pivotal role in immune function regulation and the progression of autoimmune related diseases, including CD[11]. An in-depth study by Mirza *et al*[12] reported the transcriptomic landscape of lncRNAs in inflammatory bowel disease (IBD). Furthermore, Qiao *et al*[13] identified the increased lncRNA DQ786243 level in CD patients and its effect on the function of regulatory T lymphocytes through changing CREB and Foxp3 levels. However, although evidence for plasma lncRNAs as noninvasive diagnostic biomarkers has accumulated[14,15], none has been reported in CD. Therefore, we conducted microarray screening, qRT-PCR verification and bioinformatics analysis of plasma lncRNAs and mRNAs from CD patients, aiming to provide preliminary data for noninvasive CD diagnosis and investigations into the underlying mechanism of CD.

MATERIALS AND METHODS

Ethics statement

The protocol on human beings was approved by the institutional review board of the First Affiliated Hospital of Zhejiang University and conducted in accordance with the Declaration of Helsinki. The study design and manuscript preparation fully followed the guidelines from the STROBE statement. Written consent was obtained before beginning the study.

Patient sample preparation

CD patients (n = 12) were selected when first diagnosed in the Department of Gastroenterology, The First Affiliated Hospital of Zhejiang University between January 2013 and December 2014. The diagnosis of CD was based on endoscopy manifestations and biopsy, as adopted by the Asia-pacific consensus, with preclusion of intestinal tuberculosis, ulcerative colitis, Bechet's disease and ischemic colitis[16]. To reduce the bias caused by different severities and extents of disease, we narrowed our selection to severe CD with

Table 1 Clinical characteristics and laboratory tests of enrolled subjects

	CD (n = 12)	Control (n = 12)	P value
Gender (M/F)	6/6	7/5	> 0.050[1]
Age (yr)	37.8 ± 14.6	42.8 ± 14.8	0.420
BMI (kg/m^2)	20.8 ± 1.8	23.3 ± 1.4	0.001
CRP (mg/L)	47.3 ± 6.9	4.4 ± 2.1	< 0.001
WBC (10E9/L)	11.6 ± 1.6	5.7 ± 0.9	< 0.001

[1]$\chi^2 = 0.17$.

small intestine involvement and related comorbidities (3 aphthous, 3 perianal abscess, 2 anal fistula and 2 arthralgia). The CD severity degree was assessed based on Harvey-Bradshaw index (HBI) and HBI > 9 was regarded as severe[17]. In CD patients, the average HBI was 11.3. Control subjects (n = 12) were enrolled from healthy volunteers without any health problems during their health checkups at our hospital during the same period. The authors had access to identifying information during or after data collection (Table 1). In this study, 3 of 12 subjects were randomly chosen from each group for microarray analysis, and ensuing qRT-PCR verification of significantly dysregulated lncRNAs and mRNAs was performed for the whole group. All blood samples were collected in a separate vacuum tube and sequentially centrifuged at 3000 rpm for 10 min and at 12000 rpm for 10 min. The cell-free plasma from supernatant was then stored at -80 ℃ for further analysis.

RNA isolation and quality control

Total RNA was isolated from each plasma sample by separately mixing the sample with Polyacryl Carrier (MRC, OH, United States), TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and chloroform, according to the manufacturer's protocol. RNA purification was routinely performed with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was tested with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray analysis and computational analysis

A human 8 × 60 KLncRNA/mRNAV3.0 microarray (Arraystar, Rockville, Maryland, United States) containing 30586 human lncRNAs and 26109 protein-coding transcripts was used in our study. Each transcript was represented using 1-5 probes to improve the statistical confidence. Generally, sample labeling and array hybridization were performed (Supplementary method) according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Santa Clara, CA, United States), and Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. The quantile normalization and subsequent data processing were performed with the GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed lncRNAs and mRNAs with statistical significance between the two groups were identified through a paired t-test (P < 0.05), multiple hypothesis testing (FDR < 0.05) and fold change filtering (⩾ 2.0 or ⩽ 0.5). Further hierarchical clustering was performed to visualize numerical changes of lncRNAs and samples. The lncRNAs expression data have been deposited into Gene Expression Omnibus (GEO) under accession number GSE75459.

Based on the microarray data, the significantly differentially expressed lncRNAs were further categorized into antisense_LncRNAs, enhancer_LncRNAs and LincRNAs according to their potential effects and associations with downstream mRNAs. For the mRNA analysis, Gene Ontology (GO) that describes genes and gene products in any organism was used, covering the domains of Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). Pathway analysis was also carried out for a functional analysis of mapping genes to KEGG pathways. Fisher's exact/χ^2 test and FDR were jointly used for significance detection. The P-value denotes the significance of GO term and Pathway correlated to the conditions. The lower the P-value, the more significant the Pathway and the GO Term. The FDR indicates the false discovery rate; a smaller FDR indicates smaller error in judging the P-value.

Quantitative real-time polymerase chain reaction validation

The total RNA isolated from the CD and control groups was reverse transcribed using a PrimeScript RTreagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) in accordance with the manufacturer's instructions. U6 snRNA was amplified as a normalization control, and the relative amount of each lncRNA/mRNA to U6 RNA was calculated using the equation $2^{-\Delta CT}$, where $\Delta CT = C_{TmiRNA} - C_{Tu6}$. Based on combinational consideration of the fold change, raw data, FDR, P-value and clinical manifestation reported by previous studies, 10 mRNAs were selected for quantitative real-time polymerase chain reaction (qRT-PCR) verification (Table S1) with a SYBR Green PCR kit (TaKaRa), with 3 replicated each. The same procedure was performed on 14 lncRNAs for verification (Table S1); these lncRNAs were selected from the overlap between the CNC network predications and the lncRNAs microarray data, with further preclusion of fold change < 2 and raw data density < 200.

Co-expression network construction and statistics

The co-expression network of lncRNA-mRNA was constructed based on the correlation between significantly differentially expressed lncRNAs and mRNAs, as previously reported[18]. In the network, a pink node represents a significantly expressed mRNA, and a blue node represents the related

Table 2 Top 10 dysregulated long noncoding RNAs and mRNAs in Chrohn's disease patients

Gene name	Transcript	Fold change
Up-regulated lncRNAs		
GUSBP2	ENST00000466668	626.49
RP5-968D22.1	ENST00000422548	444.43
RP11-68L1.2	ENST00000502712	324.23
RP11-428F8.2	ENST00000425364	245.98
GAS5-AS1	NR_037605	236.81
RP11-923I11.5	ENST00000562996	196.25
DDX11-AS1	NR_038927	192.07
XLOC_005955	TCONS_00014043	175.81
XLOC_005807	TCONS_00012771	87.76
AC009133.20	ENST00000569039	82.92
Down-regulated lncRNAs		
AF113016	uc001ody.3	481.03
ALOX12P2	ENST00000575787	298.81
AGSK1	uc010bmo.1	208.70
CTC-338M12.3	ENST00000509252	172.74
AC064871.3	ENST00000413954	96.13
RP11-510H23.3	ENST00000431104	77.63
LOC729678	uc011dhd.2	69.95
XLOC_010037	TCONS_00020749	66.82
LOC283761	NR_027074	58.70
XLOC_013142	TCONS_00027621	45.60
Up-regulated mRNAs		
TBC1D17	NM_024682	488.98
GALNT8	NM_017417	303.23
DENND1A	NM_024820	301.75
VANGL1	NM_001172411	247.83
VPS29	NM_057180	189.17
EHD1	NM_006795	132.08
FAM84A	NM_145175	121.69
SAA4	NM_006512	105.50
ZNF33A	NM_006974	101.84
GKN1	NM_019617	91.51
Down-regulated mRNAs		
CCL3L3	NM_001001437	994.63
BGLAP	NM_199173	501.53
SLC51B	NM_178859	499.44
BAG4	NM_004874	134.54
MAU2	NM_015329	115.54
TSNARE1	NM_145003	106.10
DIXDC1	NM_033425	93.11
ZBTB25	NM_006977	82.42
CMTM8	NM_178868	75.71
ANXA1	NM_000700	69.07

The top 10 dysregulated lncRNAs and mRNAs are listed based on significantly differentially expressed lncRNAs and mRNAs ($P < 0.05$) and the fold change.

lncRNA. Moreover, the red solid line represents a direct connection of a positive correlation between specific lncRNAs and mRNAs, and the green line represents a direct connection of a negative correlation. SPSS (version 16.0, Chicago, IL, United States) was used for statistical analyses. The data are expressed as the mean ± SD. Variables of the microarray and qRT-PCR data between the two groups were compared by Student's *t*-test. In the microarray results, a fold change of lncRNAs/mRNAs $\geqslant$ 2.0 was chosen for further analysis, and $P < 0.05$ was considered statistically significant.

RESULTS

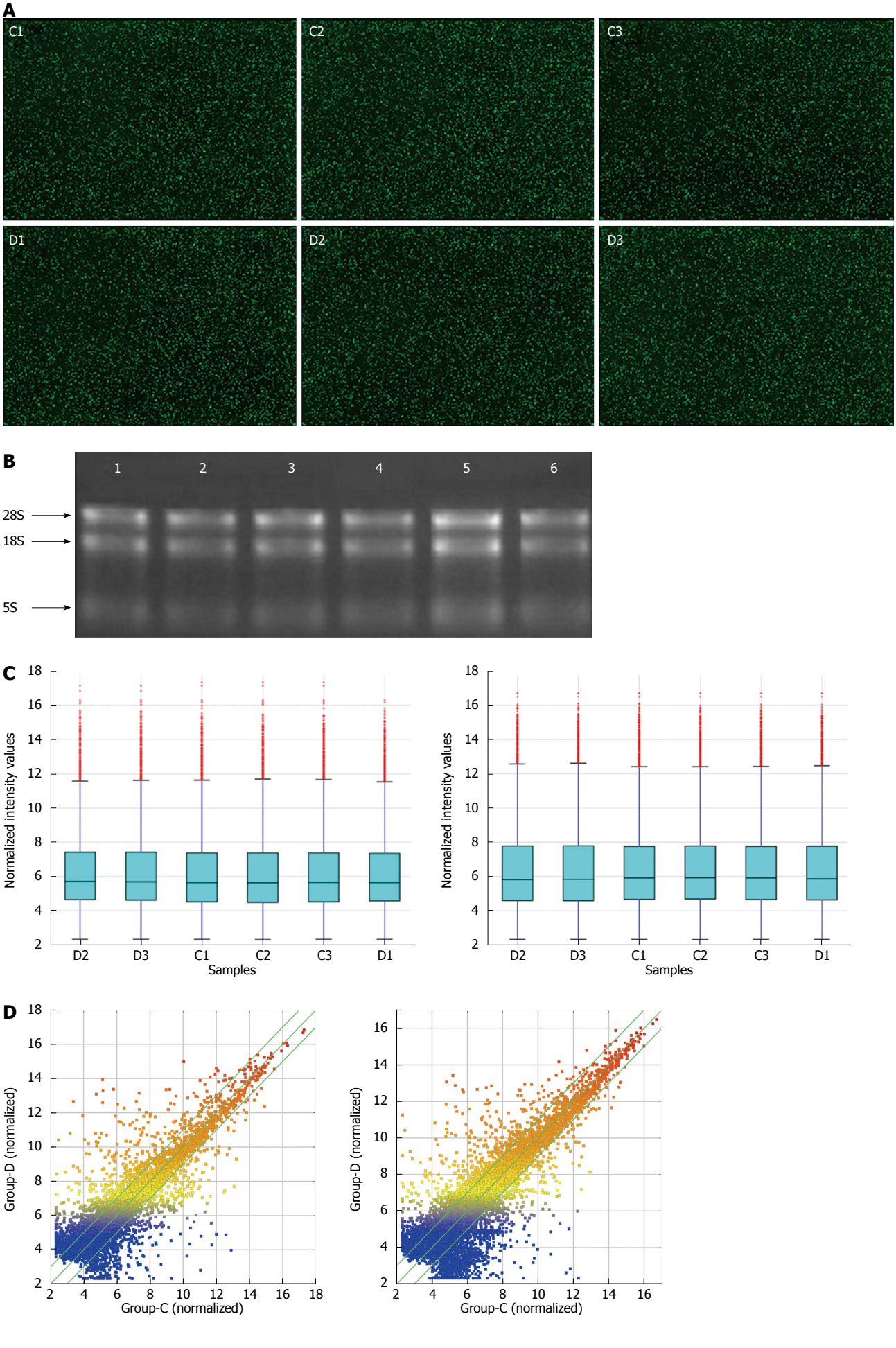
Expression pattern of lncRNAs and mRNAs in CD

Compared with the control group, the BMI was significantly lower and the WBC and CRP were significantly higher in CD patients (Table 2). A microarray analysis of lncRNAs and mRNAs was carried out in randomly selected subjects. As jointly evaluated by heat map, box plot, scatter plot and volcano plot, the differential expression of lncRNAs and mRNAs was well categorized in the CD and control groups with good RNA quality and microarray image control (Figure 1). Compared with the control group, there were 1211 up-regulated and 777 down-regulated lncRNAs in the CD group (fold change $\geqslant$ 2.0, $P < 0.05$; Table S2). Moreover, there were 1020 up-regulated and 953 down-regulated mRNAs (fold change $\geqslant$ 2.0, $P < 0.05$; Table S3). The top 10 dysregulated lncRNAs and mRNAs are summarized in Table 2.

Computational analysis of significantly dysregulated lncRNAs and mRNAs

To narrow the large number of lncRNAs retrieved from the microarray data, we further carried out lncRNAs subgroup analyses. Based on the association between an lncRNA and its nearby mRNA, lncRNAs were categorized into antisense lncRNAs, enhancer lncRNAs and lincRNAs, providing a more accurate source for further functional study (Table S4). Briefly, among the 15 antisense lncRNAs, up-regulated ENST00000569039 had the highest fold change of 82.92, targeting the nearby gene NM_001042539 (myc-associated zinc finger protein isoform 2); the down-regulated ENST00000555407 had the highest fold change of 4.97, targeting the nearby gene NM_001085471 (forkhead box protein N3 isoform 1). Of the 81 enhancer lncRNAs, up-regulated ENST00000422548 had the highest fold change of 444.43, targeting the nearby gene ENST00000367818 [chemokine (C motif) ligand 1], and the down-regulated ENST00000427085 had the highest fold change of 24.29, targeting the nearby gene ENST00000534062 (retrotransposon-like 1). Finally, in the 161 lincRNAs, the up-regulated TCONS_00027580 had the highest fold change of 54.09, targeting the nearby gene NM_001102599 (carcinoembryonic antigen-related cell adhesion molecule 20 isoform 4L precursor), and the down-regulated TCONS_00027621 had the highest fold change of 45.60, targeting the nearby gene NM_001164309 (zinc finger protein 415 isoform 2).

The top 10 dysregulated GO processes of each subgroup (BP, CC and MF) are presented in Figures 2 and 3. Because the GO manifestations in up- and down-regulated mRNAs varied, we analyzed them separately. In the up-regulated mRNAs as shown in Figure 2, the largest GO processes included single-organism process, neurogenesis and negative


A
C1
C2
C3
D1
D2
D3
B
1
2
3
4
5
6
28S
18S
5S
C
Normalized intensity values
Samples
D2
D3
C1
C2
C3
D1
D
Group-D (normalized)
Group-C (normalized)

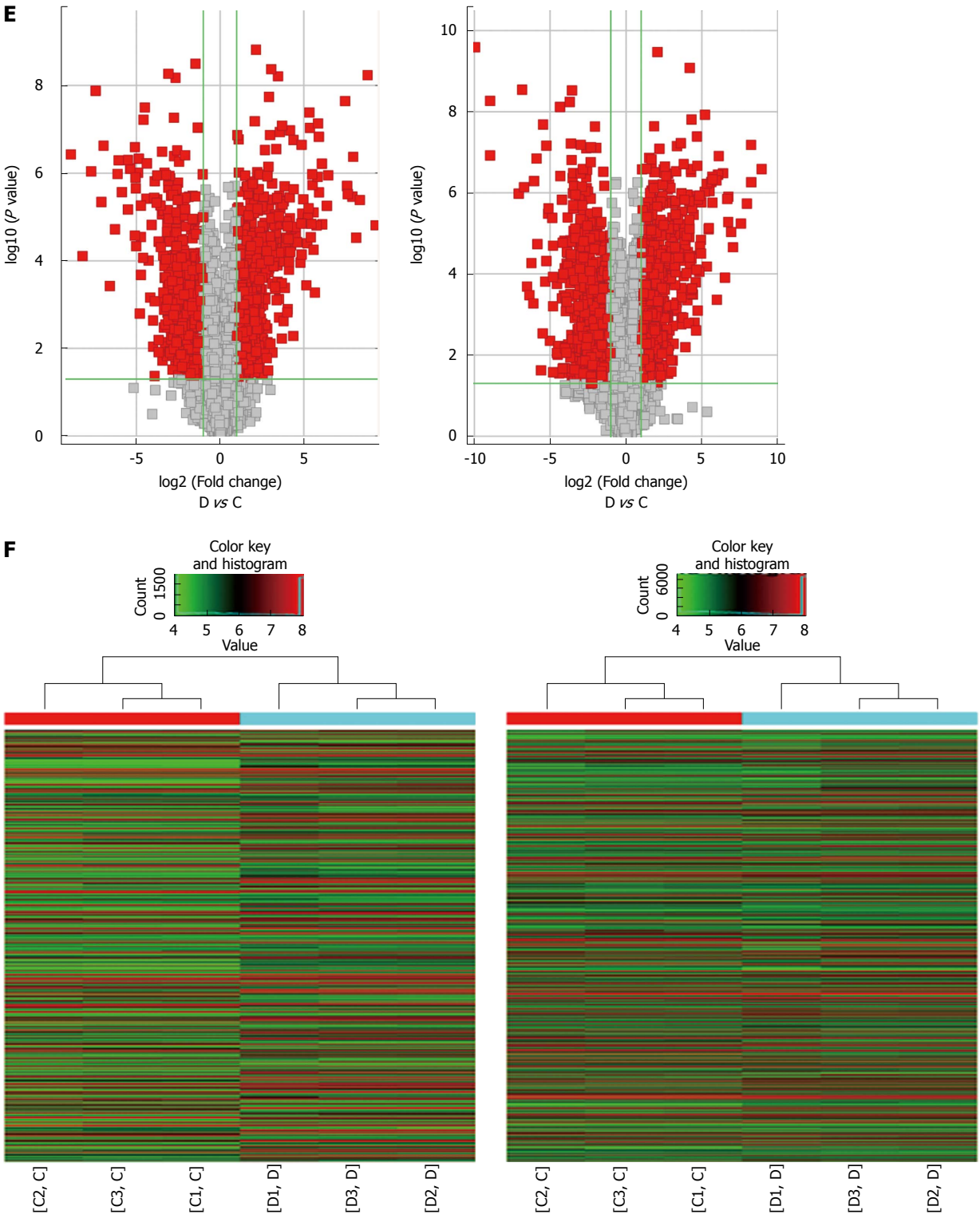
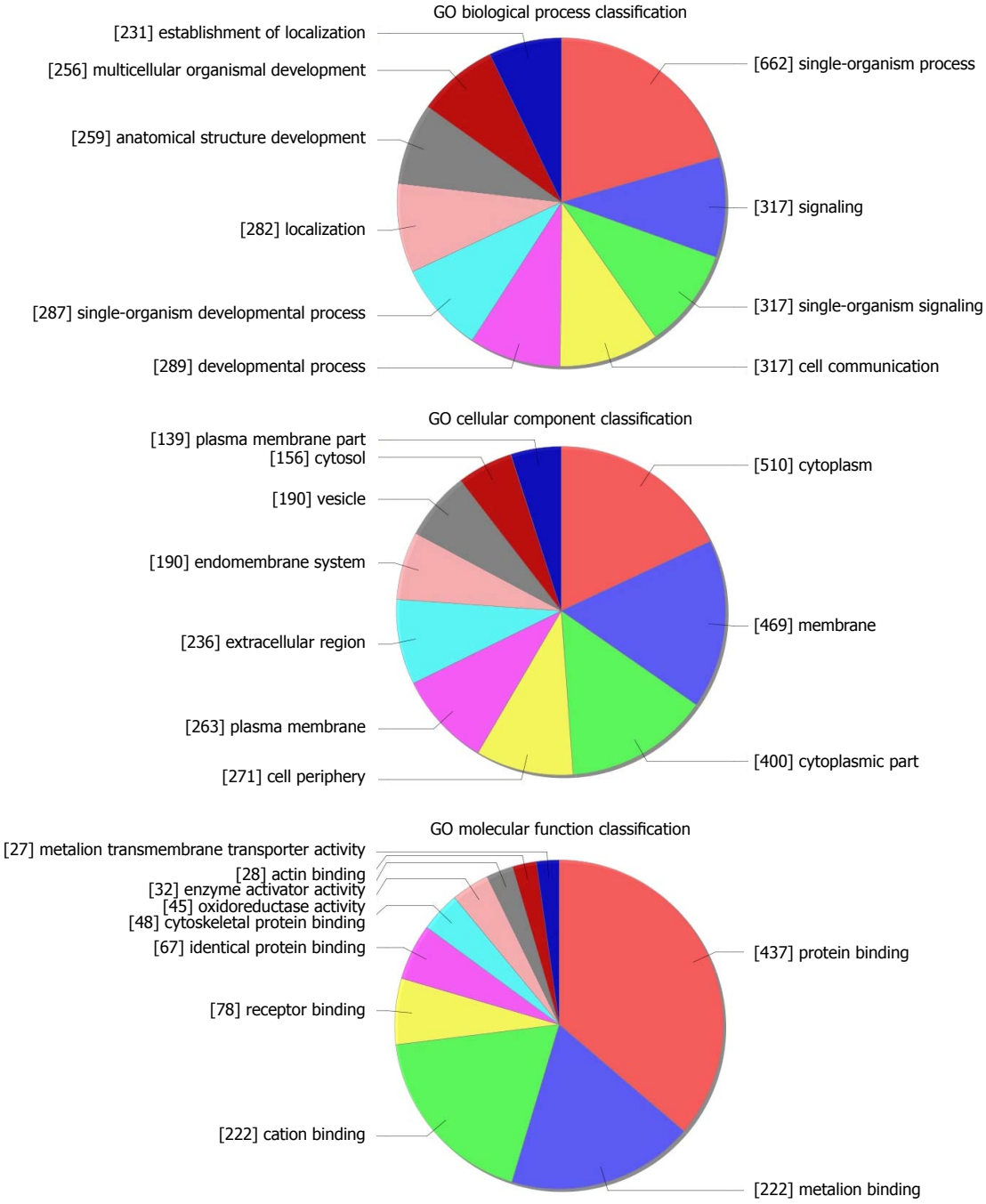
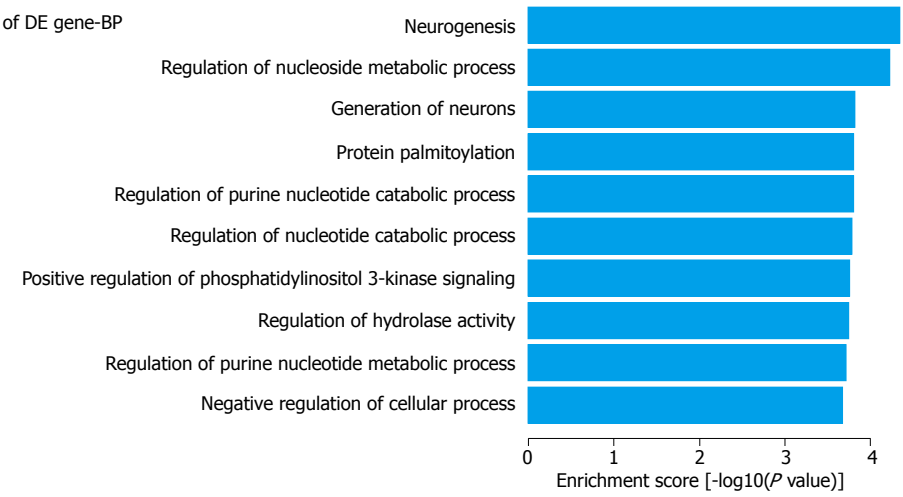


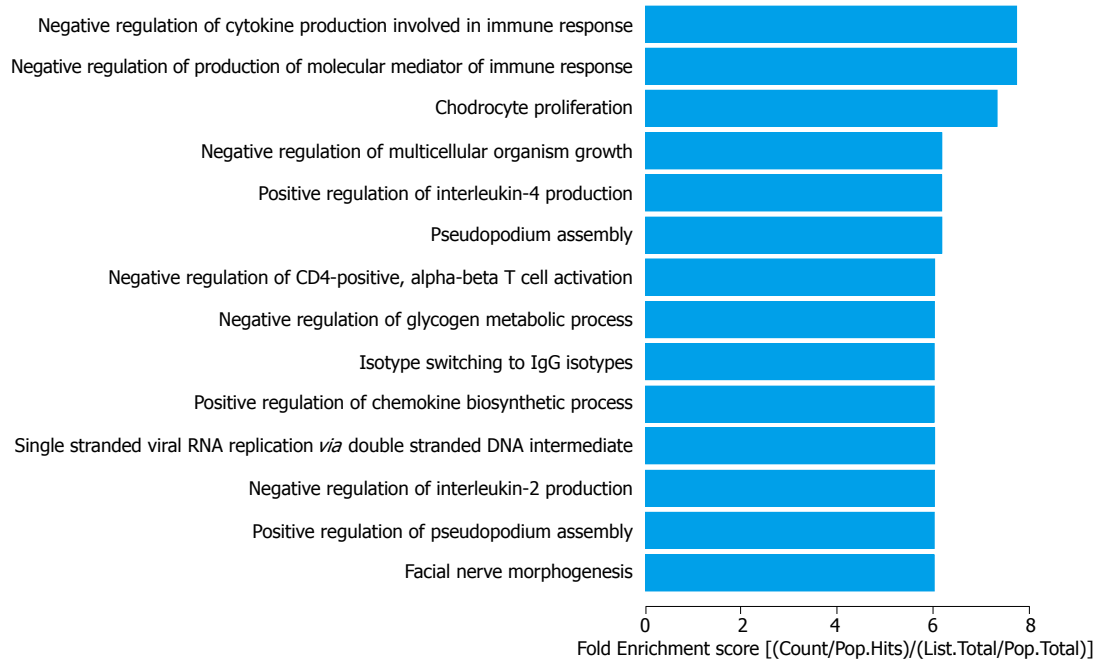
Figure 1 Raw image, RNA quality and bioinformatics analysis of differentially expressed long noncoding RNAs and mRNAs in Chrohn's disease patients. A: Raw image of the microarray analysis. The green dot on the black background represents a single lncRNA or mRNA. C1-C3 were control samples, and D1-D3 were CD samples; B: RNA elecotrophoretogram showing good RNA quality (1-3 were control, and 4-6 were CD); C: Box plot visualizing the lncRNA (left panel) and mRNA (right panel) expression variations; D, E: Scatter plot and volcano plot showing the distributions of lncRNAs (left panel) and mRNAs (right panel) in a more direct way. After normalization, the distributions of the log2 ratios among samples were nearly the same. The values of the X- and Y-axes in the scatter plot were the averaged normalized signal values of the group (log2 scaled). The green lines in the scatter plot and volcano plot represent the default significant fold change (2.0); F: Hierarchical cluster analysis of microarray data assessing the significant expression of lncRNAs (left panel) and mRNAs (right panel) between the CD and control groups. Red and green denote high and low expression, respectively. Each RNA is represented by a single row of colored boxes, and each sample is represented by a single column.

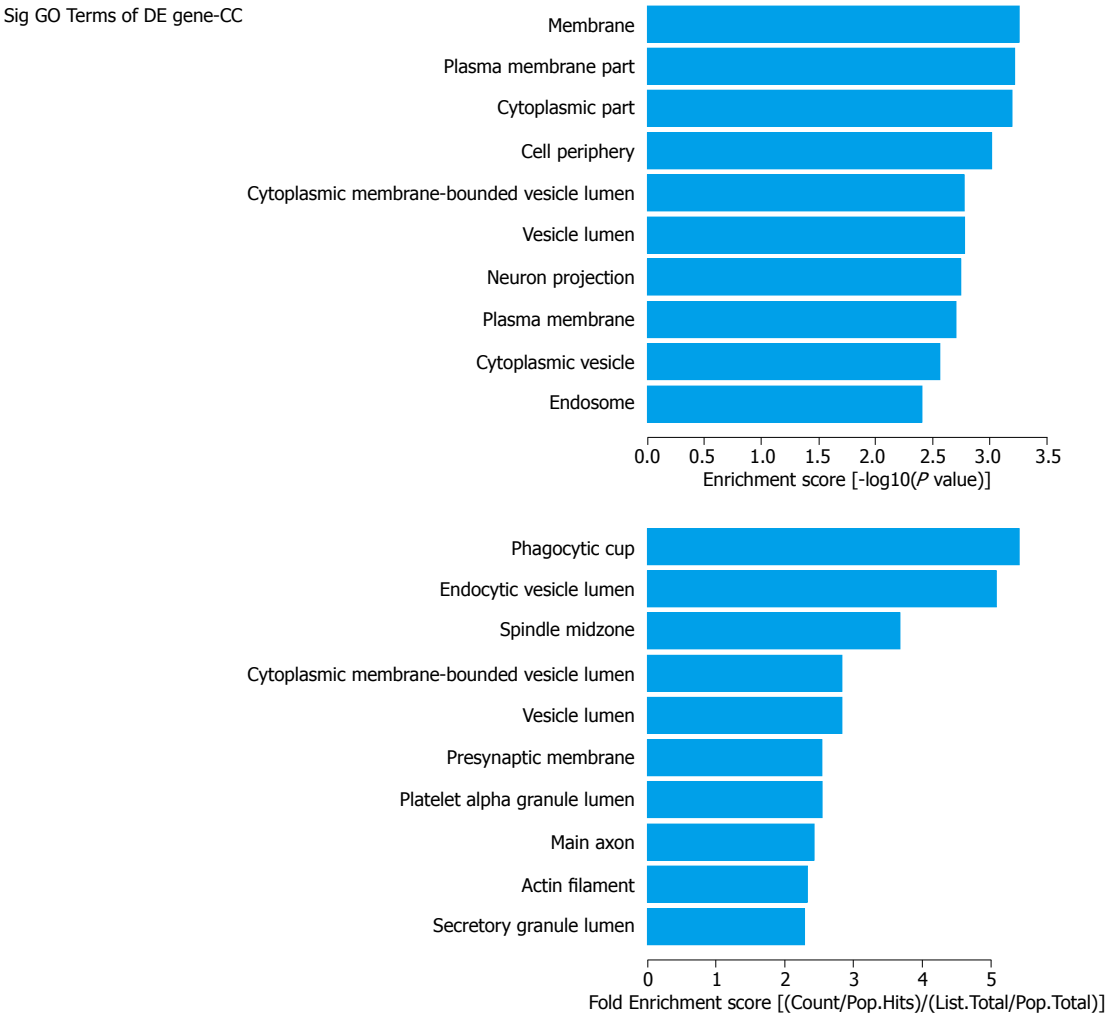
A


GO biological process classification
[231] establishment of localization
[256] multicellular organismal development
[259] anatomical structure development
[282] localization
[287] single-organism developmental process
[289] developmental process
[662] single-organism process
[317] signaling
[317] single-organism signaling
[317] cell communication
GO cellular component classification
[139] plasma membrane part
[156] cytosol
[190] vesicle
[190] endomembrane system
[236] extracellular region
[263] plasma membrane
[271] cell periphery
[510] cytoplasm
[469] membrane
[400] cytoplasmic part
GO molecular function classification
[27] metalion transmembrane transporter activity
[28] actin binding
[32] enzyme activator activity
[45] oxidoreductase activity
[48] cytoskeletal protein binding
[67] identical protein binding
[78] receptor binding
[222] cation binding
[437] protein binding
[222] metalion binding

B


Sig GO Terms of DE gene-BP
Neurogenesis
Regulation of nucleoside metabolic process
Generation of neurons
Protein palmitoylation
Regulation of purine nucleotide catabolic process
Regulation of nucleotide catabolic process
Positive regulation of phosphatidylinositol 3-kinase signaling
Regulation of hydrolase activity
Regulation of purine nucleotide metabolic process
Negative regulation of cellular process
0
1
2
3
4
Enrichment score [-log10(P value)]


Negative regulation of cytokine production involved in immune response
Negative regulation of production of molecular mediator of immune response
Chodrocyte proliferation
Negative regulation of multicellular organism growth
Positive regulation of interleukin-4 production
Pseudopodium assembly
Negative regulation of CD4-positive, alpha-beta T cell activation
Negative regulation of glycogen metabolic process
Isotype switching to IgG isotypes
Positive regulation of chemokine biosynthetic process
Single stranded viral RNA replication *via* double stranded DNA intermediate
Negative regulation of interleukin-2 production
Positive regulation of pseudopodium assembly
Facial nerve morphogenesis
0
2
4
6
8
Fold Enrichment score [(Count/Pop.Hits)/(List.Total/Pop.Total)]


Sig GO Terms of DE gene-CC
Membrane
Plasma membrane part
Cytoplasmic part
Cell periphery
Cytoplasmic membrane-bounded vesicle lumen
Vesicle lumen
Neuron projection
Plasma membrane
Cytoplasmic vesicle
Endosome
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Enrichment score [-log10(*P* value)]
Phagocytic cup
Endocytic vesicle lumen
Spindle midzone
Cytoplasmic membrane-bounded vesicle lumen
Vesicle lumen
Presynaptic membrane
Platelet alpha granule lumen
Main axon
Actin filament
Secretory granule lumen
0
1
2
3
4
5
Fold Enrichment score [(Count/Pop.Hits)/(List.Total/Pop.Total)]

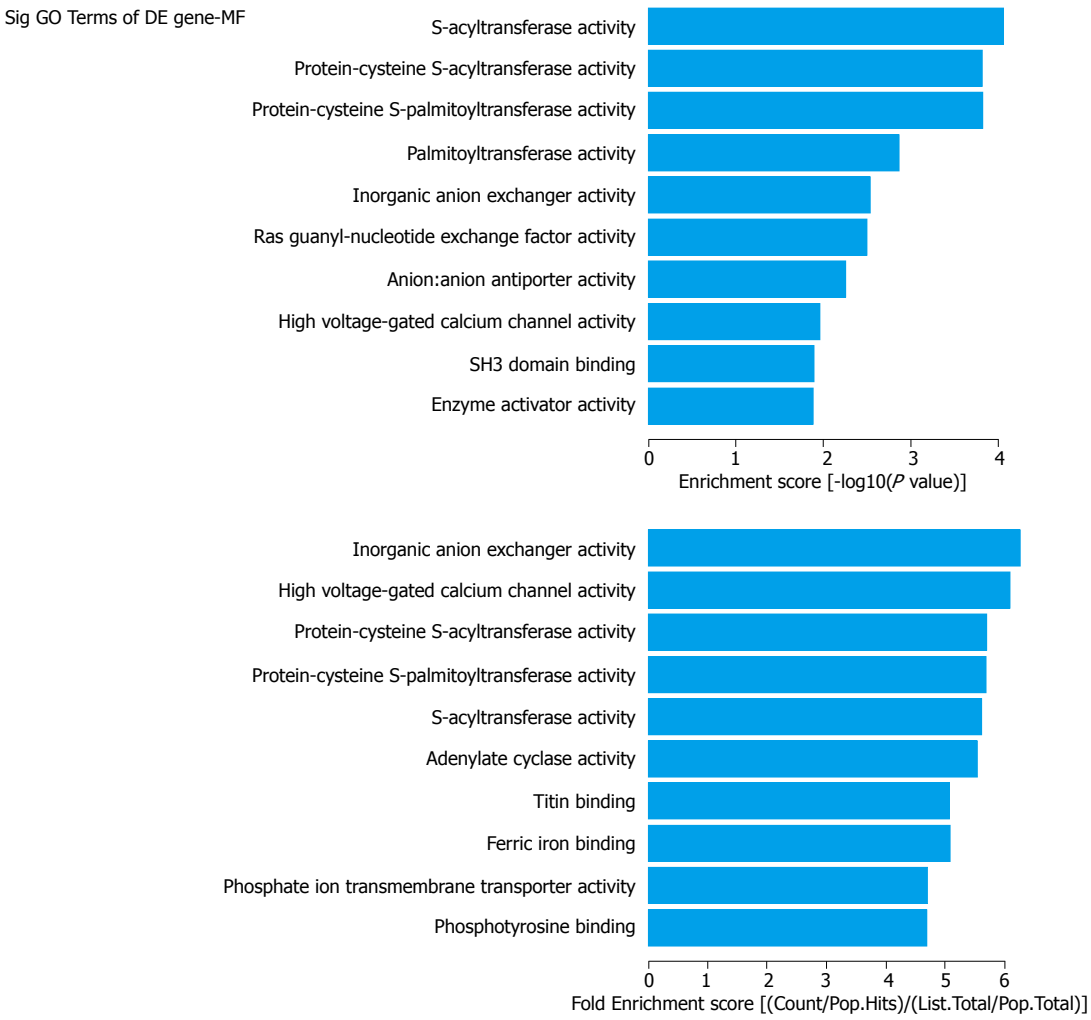


Figure 2 Gene ontology analysis of up-regulated mRNAs. A: The GO analysis includes classification of biological process (BP), cellular component (CC) and molecular function (MF); B: The summaries of significant GO terms (BP, CC and MF) of differentially expressed genes are shown in the up and down panels according to the values in the enrichment score and the fold enrichment.

regulation of cytokine production involved in immune response in BP; cytoplasm, membrane and phagocytic cup in CC; protein binding, S-acyltransferase activity and inorganic anion exchanger activity in MF, according to the different algorithms of routine classification, enrichment score and fold enrichment. Similarly, the largest GO processes of the down-regulated mRNAs were cellular process, presentation of exogenous peptide antigen *via* MHC class I TAP dependent, antigen processing and presentation of endogenous peptide antigen in BP; cell part, extracellular vesicular exosome and MHC class I protein complex in CC and binding, protein binding and RAGE receptor binding in MF (Figure 3).

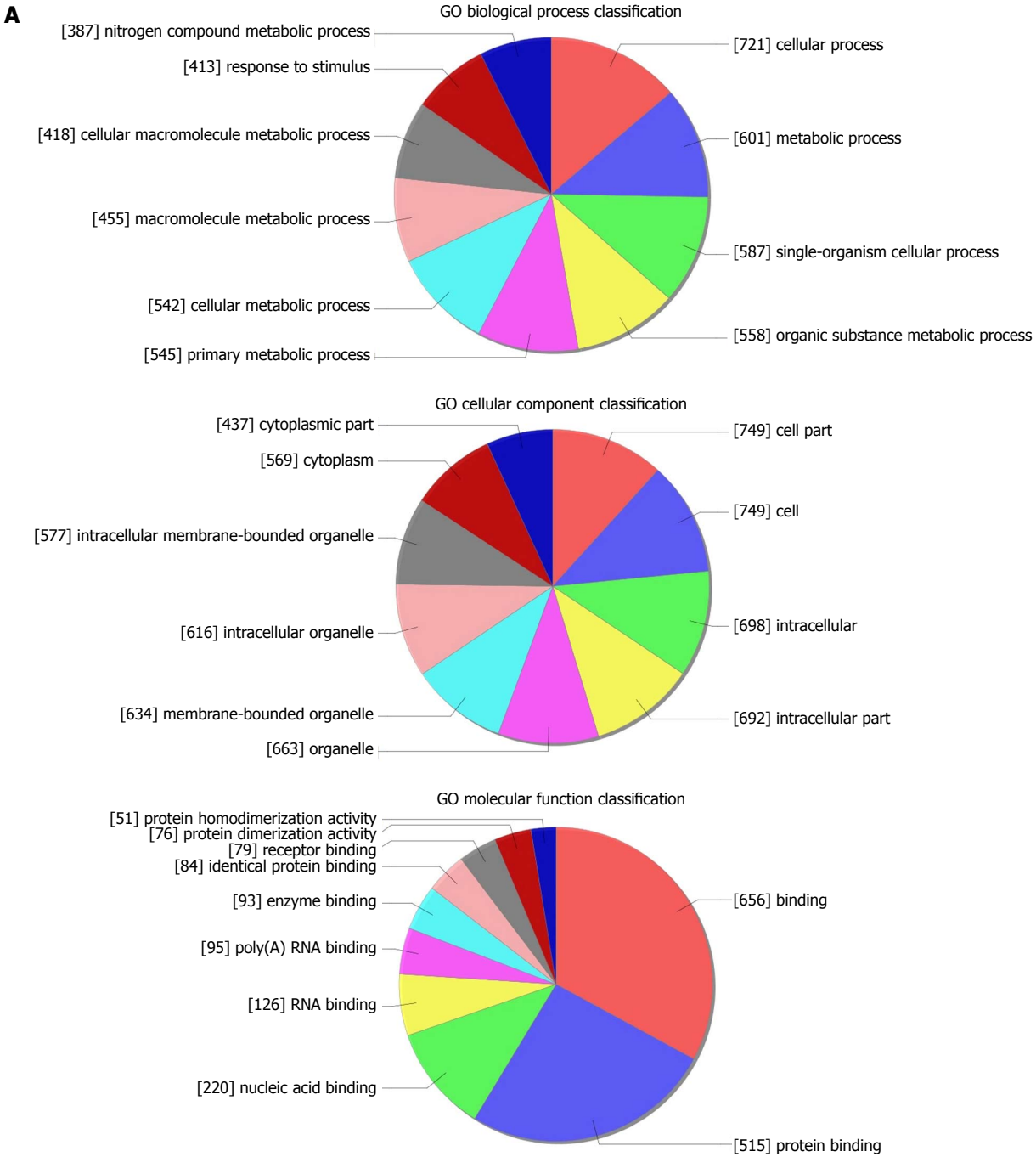
As further shown in Table S5, through the KEGG pathway analysis, 37 gene pathways were found to be targeted in up-regulated mRNAs; the top 3 processes were dilated cardiomyopathy, endocytosis and the estrogen signaling pathway (Figure 4A). More importantly, the IBD process itself was at the sixth of the top 10 pathways according to the enrichment score. Similarly, 32 gene pathways were found in down-regulated mRNAs; the top 3 processes were Huntington's disease, proteasome and oxidative phosphorylation (Figure 4B).

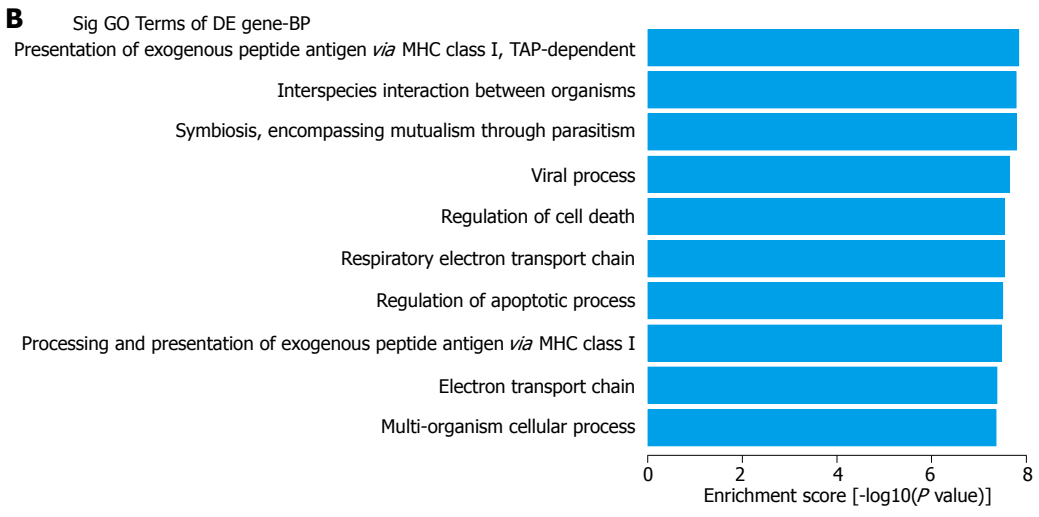
Downstream mRNAs selection and qRT-PCR verification

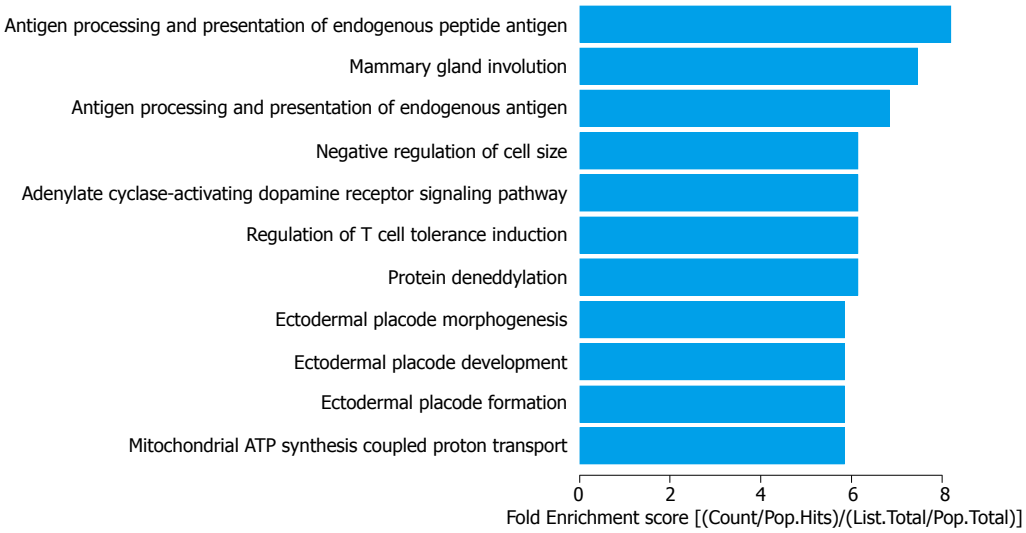
Based on the high normalized intensity in the raw data, high fold change, significant *P*-value and clinical meanings, we selected 10 mRNAs (SNX1, CYFIP2, CD6, CMTM8, AURKB, BGLAP, STAT4, WNT4, IGFBP7 and TGFβ-2) for qRT-PCR verification. As shown in Figure 5A, six mRNAs (SNX1, CYFIP2, CD6, CMTM8, STAT4 and IGFBP7) showed the same change tendency between the microarray and qRT-PCR results with statistical significance. AURKB and WNT4 showed the opposite change between the microarray and qRT-PCR results without statistical significance.

CNC network construction and predicted lncRNAs verification by qRT-PCR

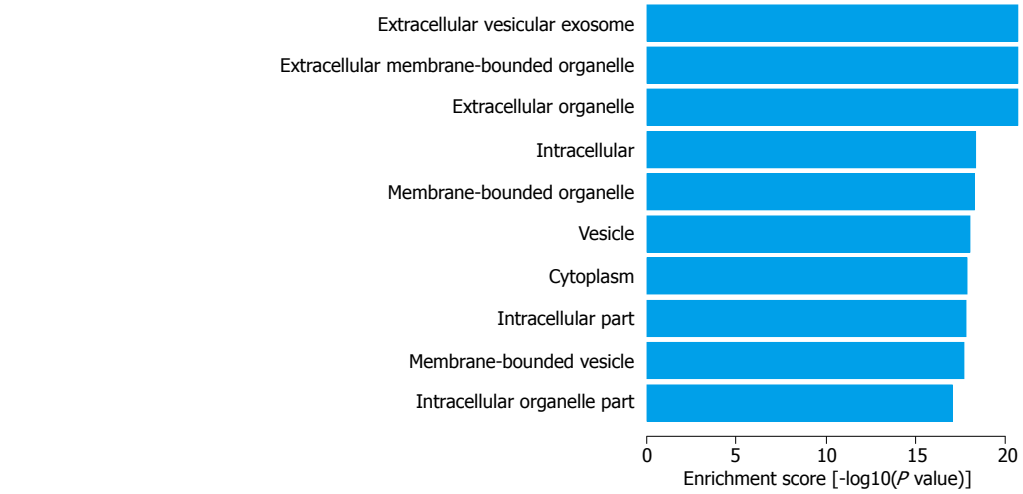
Because lncRNAs participate in the regulation of gene expression in transcriptional, epigenetic and posttranscriptional stages, it is plausible that certain

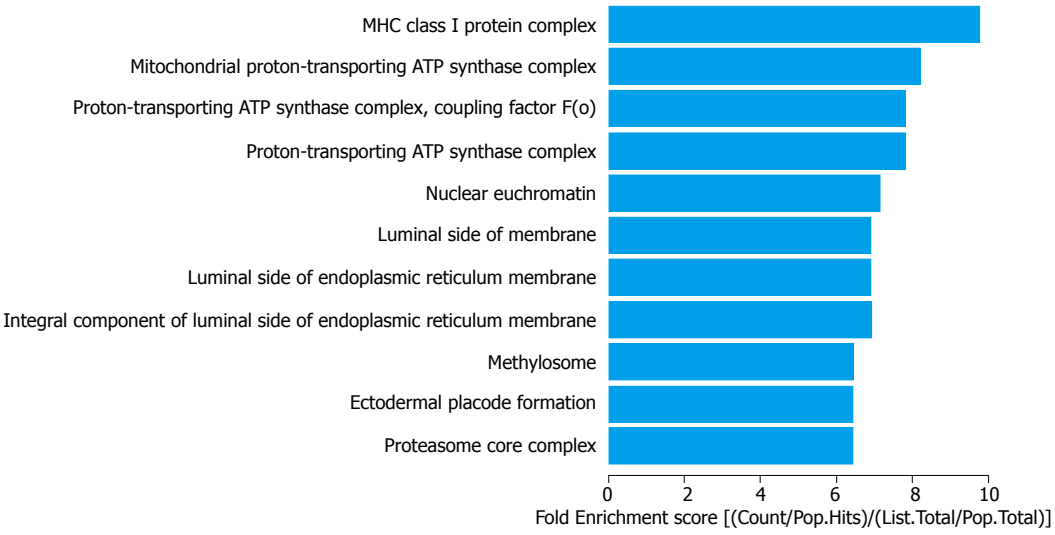

A
GO biological process classification
[387] nitrogen compound metabolic process
[413] response to stimulus
[418] cellular macromolecule metabolic process
[455] macromolecule metabolic process
[542] cellular metabolic process
[545] primary metabolic process
[721] cellular process
[601] metabolic process
[587] single-organism cellular process
[558] organic substance metabolic process
GO cellular component classification
[437] cytoplasmic part
[569] cytoplasm
[577] intracellular membrane-bounded organelle
[616] intracellular organelle
[634] membrane-bounded organelle
[663] organelle
[749] cell part
[749] cell
[698] intracellular
[692] intracellular part
GO molecular function classification
[51] protein homodimerization activity
[76] protein dimerization activity
[79] receptor binding
[84] identical protein binding
[93] enzyme binding
[95] poly(A) RNA binding
[126] RNA binding
[220] nucleic acid binding
[656] binding
[515] protein binding


B
Sig GO Terms of DE gene-BP
Presentation of exogenous peptide antigen *via* MHC class I, TAP-dependent
Interspecies interaction between organisms
Symbiosis, encompassing mutualism through parasitism
Viral process
Regulation of cell death
Respiratory electron transport chain
Regulation of apoptotic process
Processing and presentation of exogenous peptide antigen *via* MHC class I
Electron transport chain
Multi-organism cellular process
0
2
4
6
8
Enrichment score [-log10(*P* value)]



Sig GO Terms of DE gene-CC





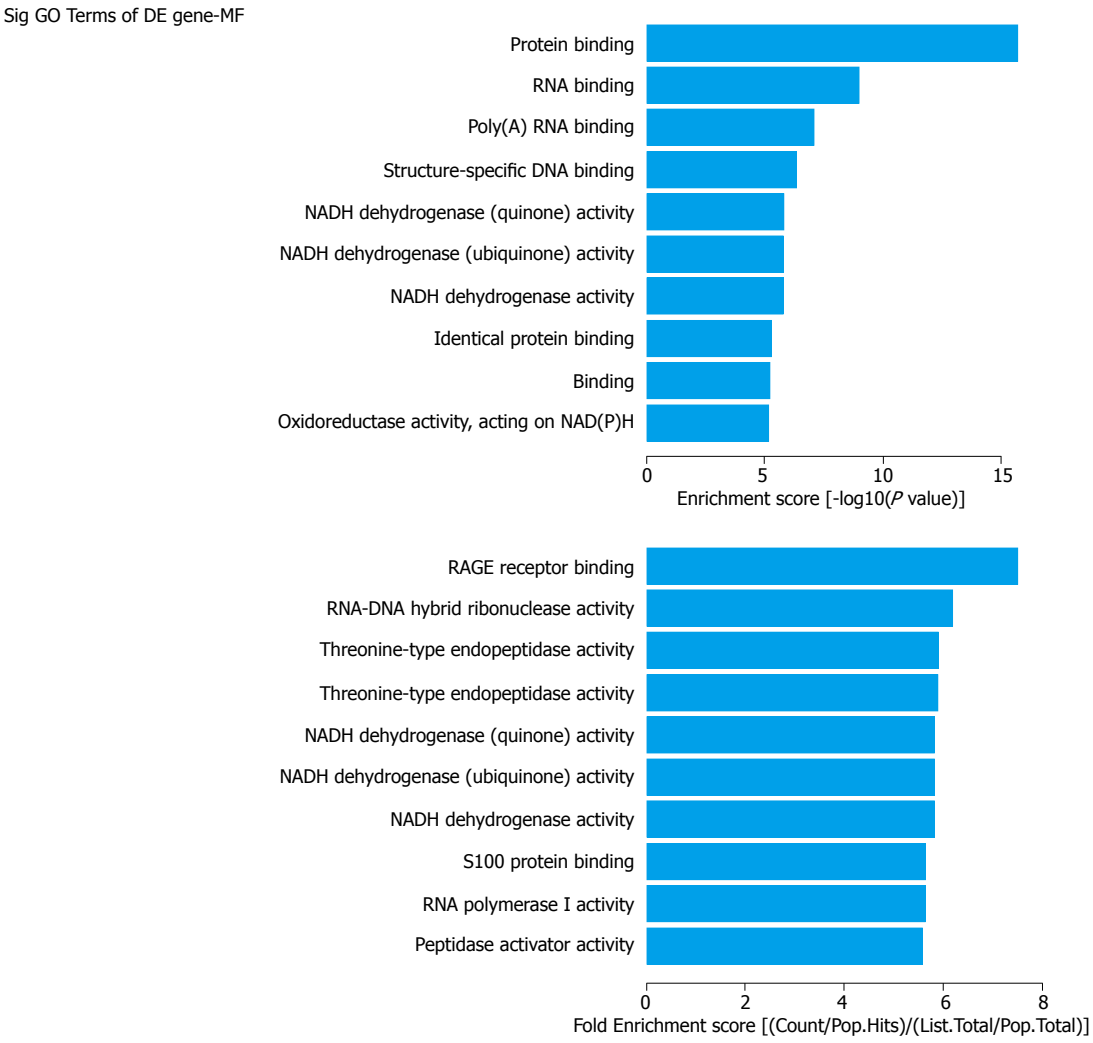


Figure 3 Gene ontology analysis of down-regulated mRNAs. A: The GO analysis includes classification of biological process (BP), cellular component (CC) and molecular function (MF); B: The summaries of significant GO terms (BP, CC and MF) of differentially expressed genes are shown in the up and down panels according to the values in the enrichment score and the fold enrichment.

lncRNAs are involved in CD pathogenesis. Based on the six qRT-PCR verified mRNAs, we predicted 1358 potential lncRNAs with 2697 positive correlations and2287 negative correlations between mRNA and lncRNAs by CNC network construction (Figure S1 and Table S6). After cross-linking between CNC predicted lncRNAs and lncRNAs microarray results, we selected 14 lncRNAs (NR_033913, NR_073047, NR_038927, NR_038218, NR_036512, NR_072994, NR_049759, NR_046052, NR_033951, NR_045408, NR_038377, NR_015413, NR_039976 and NR_038345) for qRT-PCR verification based on the selection criteria used for mRNAs. As shown in Figure 5B, 8 lncRNAs (NR_033913, NR_038218, NR_036512, NR_049759, NR_033951, NR_045408, NR_038377 and NR_039976) showed the same change tendency between the microarray and qRT-PCR results with statistical significance. Two lncRNAs (NR_073047 and NR_015413) showed the opposite change tendency without statistical significance. Four lncRNAs (NR_038927, NR_072994, NR_046052 and NR_038345) showed the same change tendency as in microarray results, but the results did not reach statistical significance (See detailed qRT-PCR and microarray results in Table S7).

DISCUSSION

CD is an important subtype of IBD with characteristics of intestinal full-thickness lesions and severe complications including perforation, fistula formation, malnutrition and carcinogenesis. Currently, next-generation sequencing and high-density microarrays have provided novel methods for CD study. For example, the NOD2/CARD15 gene mutation was identified to be associated with a CD phenotype[19], whereas a novel CD locus mapping to a gene desert on 5p 13.1 was also reported[20]. Nevertheless, according to the recent meta-analysis of GWAS studies, the number of confirmed genetic loci associated with CD was 150, although an overwhelming majority of these loci are located in noncoding regions[5], suggesting the importance of ncRNAs in CD research. A recent study showed that the loss of endogenous intestinal

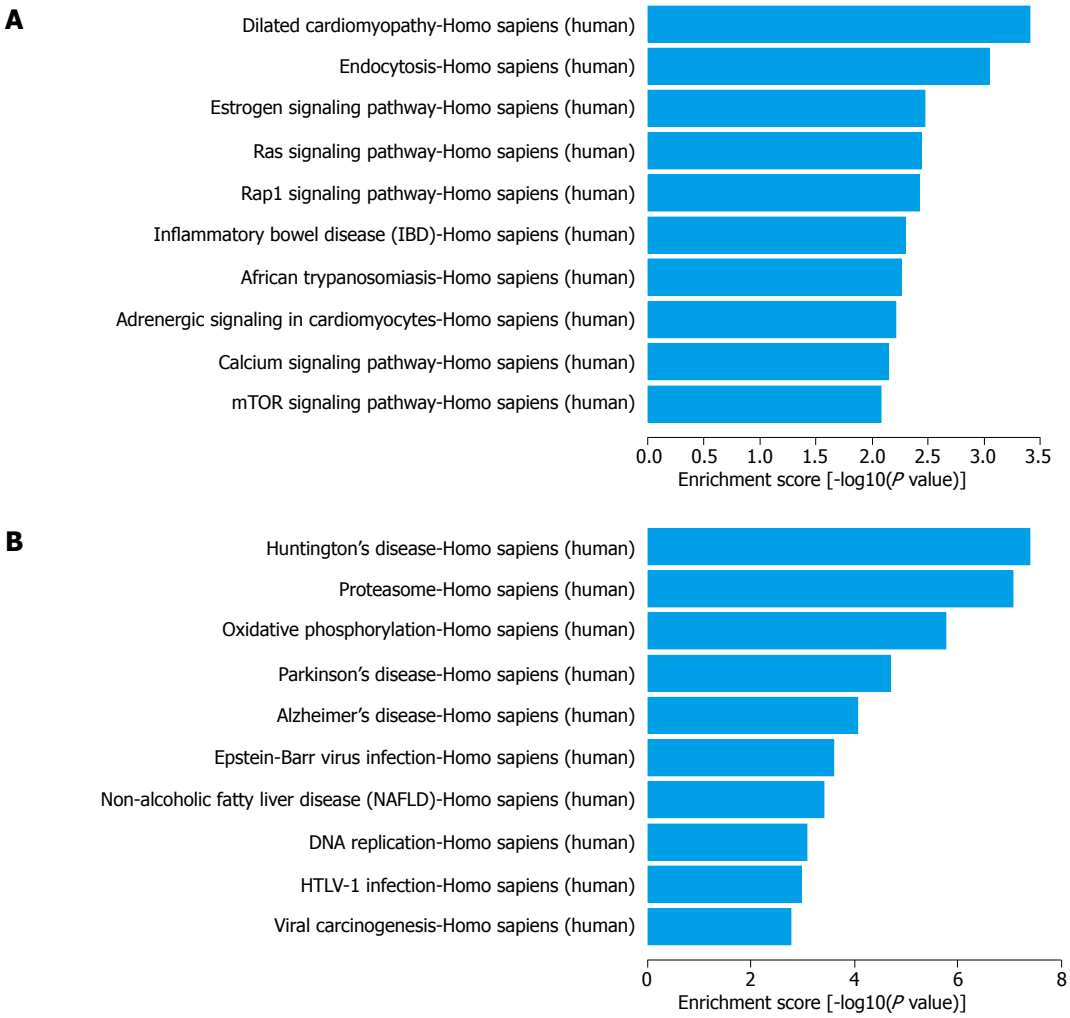


Figure 4 Top 10 KEGG pathways in up- (A) and down-regulated (B) mRNAs.

miRNAs caused impairment of epithelial barrier function, resulting in acute inflammation[21]. Further accumulating data supported an active role of miRNAs in the pathogenesis of IBD[22]. However, research into lncRNAs, another subgroup of ncRNAs with ability in binding protein, RNA and DNA, in CD has been rarely reported.

In this study, we used microarray screening and qRT-PCR verification to obtain the profile of plasma lncRNAs in carefully selected CD patients. We identified a total of 1988 and 2993 dysregulated lncRNAs and mRNAs between the CD and control groups; the numbers in our study were much higher than 450 lncRNAs and 1100 mRNAs from Mirza's report[12]. This difference might be due to the relatively lower sample size of our study, which makes the data more dispersed. Moreover, there were no overlap of dysregulated lncRNAs between our results and the top 10 dysregulated lncRNAs we extracted from Mirza's paper, except one lncRNA - DIO3OS that was up-regulated in our but down-regulated in their study. One possible explanation might be the secretion of DIO3OS from intestinal tissue to circulation, which needs further investigation. The intrinsic difference between our and Mirza's results is the source of the lncRNAs. We reported, for the first time, the plasma lncRNAs in CD patients. If we could enlarge sample size and narrow down the scale of plasma lncRNAs, this method would become a good candidate for the non-invasive diagnosis of CD. Circulating lncRNAs (serum and plasma) have already been used as non-invasive biomarkers for liver cancer[23], breast cancer[24] and lung cancer[25], implying their wide application potential. It is theoretically plausible to use plasma lncRNAs because they are quite stable when included in lipid or lipoprotein vesicles in the circulation. A recent study showed that lncRNAs might be protected by exosomes in blood[26]. Nevertheless, the detailed secretion mechanisms of lncRNAs remain vague.

The application of bioinformatics is pivotal for in-depth analyses of huge data from microarray results. For lncRNAs, we used subgroup analyses based on the category of antisense lncRNAs, enhancer lncRNAs and lincRNAs, according to the effect and gene locus of the lncRNAs. For mRNAs, we combined the GO and KEGG pathway for enrichment analysis. In the GO analysis, we found that the largest portion of mRNAs was located in the cytoplasm and was involved in

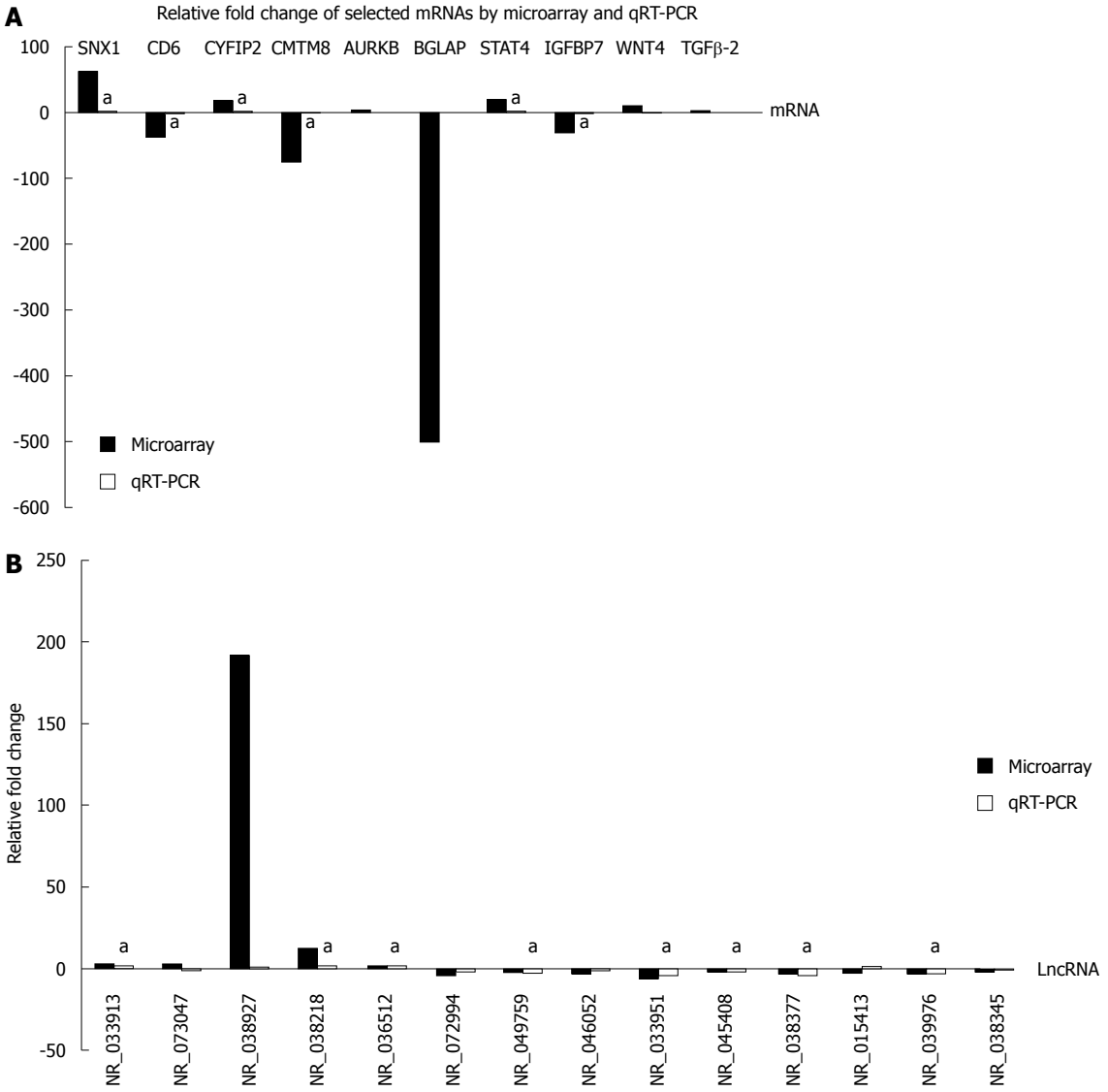


Figure 5 Relative fold changes of lncRNAs and mRNAs by microarray and qRT-PCR. The column upwards indicates up-regulation, and downwards indicates down-regulation. [a]*P* < 0.05, microarray *vs* qRT-PCR. A: mRNA data; B: LncRNA data.

single-organism process and protein binding activity. In the KEGG pathways, we found IBD and oxidative phosphorylationin the list of the top 10 dysregulated pathways. The former finding supported the effectiveness of our study whereas the latter finding revealed the possibility of oxidative stress and energy metabolism in CD pathogenesis.

Of the top 10 up- and down-regulated lncRNAs (Table 2), only GAS5-AS1 was reported to be associated with various cancers[27], leaving a large blank area for further study. Of the top 10 up-regulated mRNAs, VANGL1 is a planar cell polarity component with a developing role in cancer[28], and FAM84A is associated with the enhanced migration of human colon cancer cells[29]. These mRNAs might contribute to the more frequent occurrence of colon cancer in the background of CD. Plasma SAA4 is present mostly in high density lipoproteins[30]. Therefore, the increased expression of SAA4 in CD might imply its potential role in assisting the stable existence of lncRNAs in the circulation. Of the top 10 down-regulated mRNAs, CMTM8 was revealed to induce caspase related apoptosis through a mitochondrial–mediated pathway[31], whereas BAG4 is involved in mitochondrial apoptosis[32], indicating the importance of apoptosis in the aetiology of CD. Moreover, ZBTB25 was found as a novel NF-AT repressor that participates in T-cell development, differentiation and lineage-specific transcription[33], emphasizing the importance of T cell mediated immune imbalance in CD pathogenesis.

Because we listed the top 10 dysregulated lncRNAs and mRNAs according to the relative fold change, there might be some genes that actually have raw data of low density (< 200). Therefore, we first selected 10 mRNAs for qRT-PCR verification based on the joint consideration of the *P*-value (< 0.05), fold change (> 2) and raw data (> 200). Then, we used CNC network analysis with combination of the mRNAs selection criteria and selected 14 lncRNAs for further qRT-PCR verification. Of the finally verified 6 mRNAs, a significantly increased STAT4 level was also reported in previous report[34],

implying its importance in CD. Moreover, CD6 acts as a cell surface receptor and a target for regulating immune responses[35], whereas CYFIP2 is involved in T cell adhesion[36], further emphasizing the importance of T cell mediated immunity in CD. In 8 of 12 verified lncRNAs, NR_049759 is the transcript variant 2 of the coding gene IFITM3 that was up-regulated in colon mucosa of DSS induced mice[37], an animal model of IBD. NR_045408 is the antisense RNA of the coding gene RCNA3 that acts as a tumor suppressor[38], indicating the possibility of the NR_045408-RCNA3 pathway involvement in the occurrence of cancer in CD.

We identified the expression pattern of plasma lncRNAs based on microarray data. Further bioinformatics analyses successfully categorized the subjects into CD and control groups according to the lncRNAs profile, supporting the possibility of using this method as a non-invasive method for CD diagnosis. However, due to the small sample size, the retrieved profile contains many lncRNAs, which might become an obstacle for further application in clinics. Therefore, enlarging the experimental size to narrow the enrolled lncRNAs is urgently needed. Furthermore, because we only focused on severe CD subjects in this study, investigating lncRNAs expression in mild and moderate CD is suggested. Finally, it is better if we can compare the lncRNAs in plasma and intestinal tissue, which may be helpful for the mechanism exploration of CD. For the mRNA data, the GO and KEGG pathways were used to obtain more information. Approximately 60% of the microarray retrieved lncRNAs and mRNAs were verified by qRT-PCR, supporting the effectiveness of microarray screening. Several qRT-PCR verified lncRNAs and mRNAs were related with cancer or T-cell mediated immunity. Therefore, our data also provide a resource for further study of the lncRNA-mRNA pathway in CD pathogenesis.

COMMENTS

Background

Crohn's disease (CD) has been regarded as a chronic and relapsing inflammatory disease that could affect any part of the intestine. The prevalence of CD is increasing in developing and developed countries, making it a global health care problem and an interesting research area.

Research frontiers

The mechanism of CD remains vague; the involvement of genetic predisposition, immune response and environmental factors has been advocated. Currently, with the development of high-throughput technologies, the effect of noncoding RNAs, mainly divided into microRNAs and long noncoding RNAs, has been intensively investigated in CD pathogenesis.

Innovations and breakthroughs

Although evidence for plasma long noncoding RNAs (lncRNAs) as noninvasive diagnostic biomarkers has accumulated, none have been reported in CD. Therefore, we conducted, for the first time, microarray screening, qRT-PCR verification and bioinformatics analysis of plasma lncRNAs and mRNAs from CD patients, aiming to provide preliminary data for noninvasive CD diagnosis and investigations into the underling mechanism of CD.

Applications

Although further verification is needed in a larger independent cohort study, the profile of plasma lncRNAs would provide data for noninvasive diagnosis of CD and the potential lncRNA-mRNA pairs may shed light on the pathogenesis of CD.

Terminology

LncRNAs are a group of RNAs that have the length > 200 nt and do not encodes proteins but exert function of post-transcriptional regulation.

Peer-review

In this work, authors investigated the expression pattern of plasma lncRNAs in CD patients by microarray screening and qRT-PCR verification of lncRNAs and mRNAs, followed by hierarchy clustering, GO and KEGG pathway analysis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Veterans health administration hepatitis B testing and treatment with anti-CD20 antibody administration

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Abstract

AIM: To evaluate pretreatment hepatitis B virus (HBV) testing, vaccination, and antiviral treatment rates in Veterans Affairs patients receiving anti-CD20 Ab for quality improvement.

METHODS: We performed a retrospective cohort study using a national repository of Veterans Health Administration (VHA) electronic health record data. We identified all patients receiving anti-CD20 Ab treatment (2002-2014). We ascertained patient demographics, laboratory results, HBV vaccination status (from vaccination records), pharmacy data, and vital status. The high risk period for HBV reactivation is during anti-CD20 Ab treatment and 12 mo follow up. Therefore, we analyzed those who were followed to death or for at least 12 mo after completing anti-CD20 Ab. Pretreatment serologic tests were used to categorize chronic HBV (hepatitis B surface antigen positive or HBsAg+), past HBV (HBsAg-, hepatitis B core antibody positive or HBcAb+), resolved HBV (HBsAg-, HBcAb+, hepatitis B surface antibody positive or HBsAb+), likely prior vaccination (isolated HBsAb+), HBV negative (HBsAg-, HBcAb-), or unknown. Acute hepatitis B was defined by the appearance of HBsAg+ in the high risk period in patients who were pretreatment HBV negative. We assessed HBV antiviral treatment and the incidence of hepatitis, liver failure, and death during the high risk period. Cumulative hepatitis, liver failure, and death after anti-CD20 Ab initiation were compared by HBV disease categories and differences compared using the χ^2 test. Mean time to hepatitis peak alanine aminotransferase, liver failure, and death relative to anti-CD20 Ab administration and follow-up were also compared by HBV disease group.

RESULTS: Among 19304 VHA patients who received anti-CD20 Ab, 10224 (53%) had pretreatment HBsAg testing during the study period, with 49% and 43% tested for HBsAg and HBcAb, respectively within 6 mo pretreatment in 2014. Of those tested, 2% (167/10224) had chronic HBV, 4% (326/7903) past HBV, 5% (427/8110) resolved HBV, 8% (628/8110) likely prior HBV vaccination, and 76% (6022/7903) were HBV negative. In those with chronic HBV infection, $\leqslant$ 37% received HBV antiviral treatment during the high risk period while 21% to 23% of those with past or resolved HBV, respectively, received HBV antiviral treatment. During and 12 mo after anti-CD20 Ab, the rate of hepatitis was significantly greater in those HBV positive *vs* negative (P = 0.001). The mortality rate was 35%-40% in chronic or past hepatitis B and 26%-31% in hepatitis B negative. In those pretreatment HBV negative, 16 (0.3%) developed acute hepatitis B of 4947 tested during anti-CD20Ab treatment and follow-up.

CONCLUSION: While HBV testing of Veterans has increased prior to anti-CD20 Ab, few HBV+ patients received HBV antivirals, suggesting electronic health record algorithms may enhance health outcomes.

Key words: Hepatitis B; Hepatitis B reactivation; Anti-CD20 antibody; Rituximab; Lymphoma; Chemotherapy; Hepatitis B antivirals; Vaccination; Veteran

Core tip: Prior to anti-CD20 antibody (Ab) treatment in 2014, 61%-73% of 19304 Veterans had hepatitis B virus (HBV) tests. Of these, 11% tested were positive for hepatitis B surface antigen or core antibody and at risk for reactivation; $\leqslant$ 37% of these HBV+ patients received HBV antivirals during anti-CD20 Ab and follow-up. HBV+ patients had significantly higher hepatitis rates than HBV-. Among pretreatment HBV- patients, about 1 in 300 tested suffered acute hepatitis during anti-CD20 Ab and 12 mo follow-up. Electronic health record algorithms to increase HBV testing, antiviral use and vaccination will likely improve outcomes with anti-CD20 Ab treatment.

Hunt CM, Beste LA, Lowy E, Suzuki A, Moylan CA, Tillmann HL, Ioannou GN, Lim JK, Kelley MJ, Provenzale D. Veterans health administration hepatitis B testing and treatment with anti-CD20 antibody administration. *World J Gastroenterol* 2016; 22(19): 4732-4740 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4732.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4732

INTRODUCTION

In the United States, 4% of the population has

had hepatitis B viral (HBV) infection and 0.3% have chronic hepatitis B (with positive hepatitis B surface antigen)[1,2]. Following HBV infection, viral DNA persists in the liver - though its replication is suppressed by B- and T-cells or by HBV antivirals[3,4]. With immunosuppression, HBV can reactivate, even in patients with past or resolved infection. In untreated patients with chronic or prior HBV, nearly 40% of those receiving chemotherapy for hematological malignancies or solid tumors develop HBV reactivation[5]. HBV reactivation frequently interrupts chemotherapy and increases cancer mortality[6] by causing hepatitis (33%), liver failure (13%) and death (5%)[7]. Use of prophylactic HBV antivirals in patients with chronic or prior HBV infection largely prevents reactivation[1,7-11]. As most patients are asymptomatic and unaware of their HBV infection, hepatitis B serology before immunosuppression is the most effective means to identify the potential for reactivation[12]. Due to a higher HBV prevalence in lymphoma patients than the general population, HBV reactivation is a particular concern in lymphoma[13].

Anti-CD20 antibodies (Ab), such as rituximab, ofatumumab and obinutuzumab, are common treatments for non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), and rheumatoid arthritis. Anti-CD20 Ab act by depleting B-lymphocytes[14]. However, anti-CD20 Ab also decrease host immune suppression of HBV, potentially leading to viral reactivation - identified by increases in HBV DNA and alanine aminotransferase (ALT)[5]. Among patients with lymphoma and prior HBV infection receiving rituximab, 10%-60% exhibit HBV reactivation at a median of 3 mo after the last rituximab dose[8,15]. Despite 2007-8 guidance from the Centers for Disease Control and the American Association for the Study of Liver Disease[16] recommending HBV screening before immunosuppression, low screening rates persist nationally[4,17].

In 2013, the FDA reported 32 anti-CD20 Ab-related HBV reactivation fatalities occurring up to 12 mo post-therapy, in whom only 3 (9%) received prophylactic HBV antivirals during treatment and follow-up[10]. In 2013, the American Society for Clinical Oncology (ASCO) recommended universal HBV screening prior to anti-CD20 Ab; the 2014 ASCO Quality Oncology Practice Initiative reported nearly 70% HBV screening rates[10]. HBV screening and antiviral treatment decrease reactivation 10-fold and yield cost savings[18]. Additionally, antiviral treatment cost-effectively decreases lymphoma- and liver-related deaths in those with HBV infection[6].

With these improved outcomes with HBV antivirals, 2015 ASCO recommendations prior to anti-CD20 Ab include: (1) hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb) screening; (2) treating patients with chronic HBV with entecavir or tenofovir during anti-CD20 Ab and 6-12 mo following; and (3) use of either prophylactic or prompt on-demand HBV antivirals for HBV reactivation (identified by increased HBV DNA or ALT on every 3 mo testing) in those with prior HBV (HBsAg-, HBcAb+)[10]. While ASCO does not specify care of resolved (HBsAg-, HBcAb+, HBsAb+) HBV, prospective controlled lymphoma studies of anti-CD20 Ab report 20% HBV reactivation rates in resolved HBV without use of prophylactic HBV antivirals, resulting in chemotherapy interruptions, hepatitis, and reverse seroconversion (*i.e.*, HBsAg reappears)[1,11]. These events were prevented with prophylactic entecavir treatment[1]. Overall, prophylactic antivirals are associated with lower HBV reactivation, liver failure and death rates compared to on-demand antivirals[8,10].

The VHA is the largest single-system United States health care provider. Compared to the general United States population[2], Veterans exhibit a 2 to 3-fold higher prevalence of chronic HBV infection[19,20]. We aimed to identify all VA patients initiating anti-CD20 Ab (2002-2014) to assess the use of HBV serologic testing, vaccination, antivirals and the rate and timing of hepatitis B-associated complications during treatment.

MATERIALS AND METHODS

Study design and data source

We performed a retrospective cohort study using the VHA Corporate Data Warehouse (CDW), a national repository of VA electronic medical record data[19]. We ascertained patient demographics, inpatient and outpatient visits, laboratory results, procedures (including hepatitis B vaccination), vital signs, pharmacy data, and vital status. VA Vital Status mortality data is highly accurate, exhibiting 98% exact agreement with dates in the National Death Index[21].

Patient population

All patients initiating anti-CD20 Ab (2002-2014) were identified using VHA pharmacy data. Among these, those who were followed to death or for at least 12 mo after completing anti-CD20 Ab were analyzed in this study. The analysis was exempted by the Durham VAMC Institutional Review Board from review as it was performed for VHA quality improvement. Informed consent was not needed as only anonymized patient information was used in this national quality improvement analysis.

Hepatitis B-related variables and their definitions

Pretreatment hepatitis B vaccination at any prior time was obtained from CDW vaccination records. Pretreatment HBV testing was quantified at any preceding time, within the study period, and within 6 mo of anti-CD20 Ab initiation. However, we identified HBV disease categories by serologic testing during the study period only[22]. Most (about 90%) VHA

Table 1 Baseline characteristics and comorbidities and anti-CD20 antibody treatment indication *n* (%)

Baseline characteristics	
Males	18464 (96)
Mean age (range, SE)	66.6 yr (20.3-97.5, 0.0813)
Median, at risk (range)	478 d (365-4083)
Race	
White	14520 (76)
Black or African-American	2460 (12)
Hispanic or Latino	878 (5)
Native Hawaiian or Pacific Islander	171 (1)
American Indian or Alaska Native	148 (1)
Asian	71 (0)
Missing	1056 (5)
Indication for anti-CD20 antibody treatment	
Non-hodgkin's lymphoma	11384 (66.2)
Chronic lymphocytic leukemia	4,110 (23.9)
Rheumatoid arthritis	2,151 (12.5)
Wegener's granulomatosis	174 (1)
Microscopic polyangiitis	54 (0.3)
Baseline comorbidities	
Alcohol abuse	4286 (24.9)
Substance abuse	1485 (8.6)
Hepatitis C	1369 (8)
Cirrhosis	808 (4.7)
Decompensated liver disease	660 (3.8)
Hemodialysis-dependent renal failure	597 (3.5)
HIV	234 (1.4)
Sexually transmitted disease	25 (0.1)
Total number of patients	19304 (100)

HBV assays were qualitative (or categorical), and as normal ranges were not provided in CDW, numerous serology results were indeterminate. We divided the study population into six pretreatment HBV disease categories: definite chronic HBV infection (HBsAg+ for more than 6 mo regardless of HBV DNA), likely chronic HBV (single pretreatment HBsAg+), past HBV (HBsAg-, HBcAb+, HBsAb-)[10], resolved HBV (HBsAg-, HBcAb+, HBsAb+)[1,11], likely prior vaccination (isolated HBsAb+, HBsAg-, HBcAb-), HBV negative (HBsAg-, HBcAb-) or unknown (with no pretreatment HBV serology or those who could not be categorized). Reverse seroconversion was defined as the reappearance of HBsAg or HBeAg in patients with past or resolved HBV[10]. As earlier reported, the "high-risk period" was defined as the period of anti-CD20 Ab treatment and 12 mo follow-up[15]. In patients negative for HBV (HBsAg-, HBcAb-) before treatment, acute HBV was defined by the appearance of HBsAg+ in the high-risk period. Patients with acute, chronic, past or resolved HBV were categorized as HBV positive, while HBV negative or likely vaccinated patients were categorized as HBV negative.

During the high-risk period, HBV antiviral use (adefovir, entecavir, lamivudine, tenofovir, and telbivudine) was identified using the pharmacy data (yes *vs* no). HBV antiviral treatment was termed "prophylactic" when administered within 3 mo of anti-CD20 Ab initiation and "on demand" following this period. Due to very limited quantitative HBV DNA and HBeAg data, we were unable to identify HBV reactivation by published definitions[4,5,8,15]. The rates and timing of health outcomes in the high-risk period included hepatitis events, liver failure and death (overall, cancer-, liver-, or HBV-related). Outcomes were compared among the pretreatment HBV disease categories and by HBV antiviral use. Hepatitis events were defined as ALT > 2 × baseline (ALT immediately preceding anti-CD20 Ab) and ALT > 2 × upper limit normal (ULN) in the high-risk period[8], while liver failure was defined as hepatitis and an INR $\geqslant$ 1.5[23]. Information on death and cause of death in the high-risk period was retrieved from 2014 vital status information. Hepatitis B-associated death met the liver failure definition and had no other apparent cause of death. Liver-related death was identified by International Classification of Diseases, 9th Edition (ICD-9) prior to death[24], as was NHL/CLL cancer related death (ICD-9 codes 200, 202, and 204.12).

Other study variables

Age, gender, race, baseline comorbidities, and the anti-CD20 Ab indication were ascertained at the time of anti-CD20 Ab initiation. Baseline comorbidities were determined using ICD-9 codes related to cirrhosis, decompensated liver disease, hemodialysis-dependent renal failure, human immunodeficiency virus (HIV), sexually transmitted disease, and alcohol and substance abuse.

Statistical analysis

A biomedical statistician performed the statistical analyses and completed pre-submission statistical review. Baseline patient characteristics were tabulated. Statistical analyses were performed using Stata MP-64 version 13.1 (StataCorp LP, College Station, Texas), and differences were considered statistically significant when the *P*-value was less than 0.05. Cumulative hepatitis, liver failure, and death after anti-CD20 Ab initiation were compared by HBV disease categories (6 pretreatment HBV disease categories plus acute HBV: chronic, past, resolved, acute, negative, vaccinated, and unknown) and differences compared using the χ^2 test. Mean time to hepatitis peak ALT, liver failure, and death relative to anti-CD20 Ab administration and follow-up were also compared by HBV disease group.

RESULTS

Demographics

We identified 19304 patients who received anti-CD20 Ab in the VA from 2002-2014, of whom 14887 had at least 12 mo follow-up after anti-CD20 Ab. Most patients were older white males receiving anti-CD20 Ab with NHL (66%), CLL (24%), or rheumatoid arthritis (12%) (Table 1). Comorbid illnesses included alcohol or substance abuse, hepatitis C, cirrhosis, decompensated liver disease or hemodialysis-

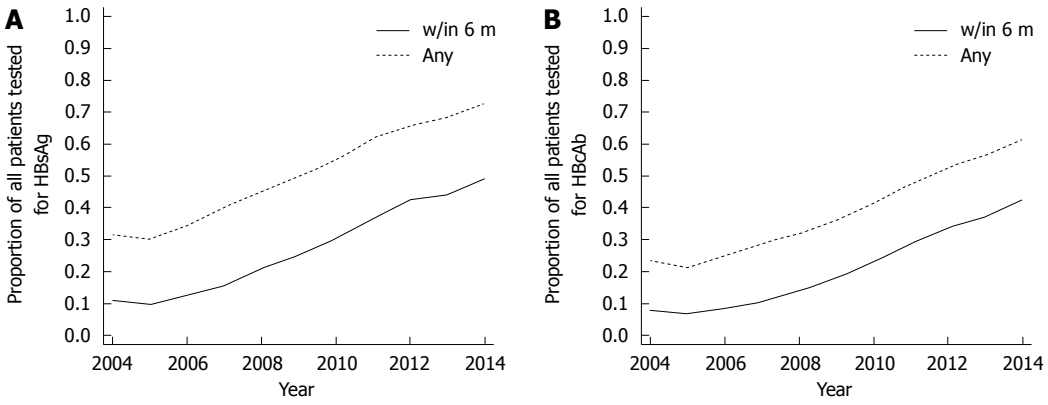

Figure 1 Proportion of all patients with pretreatment hepatitis B surface antigen (A) and hepatitis B core antibody (B) testing over time. Over the study period, pretreatment HBsAg testing within six months of anti-CD20 Ab initiation increased steadily, in parallel with pretreatment HBsAg testing obtained at any time. Over the study period, pretreatment HBcAb testing within six months of anti-CD20 Ab initiation steadily increased, in parallel with pretreatment HBcAb testing obtained at any prior time. HBsAg: Hepatitis B surface antigen; HBcAb: Hepatitis B core antibody.

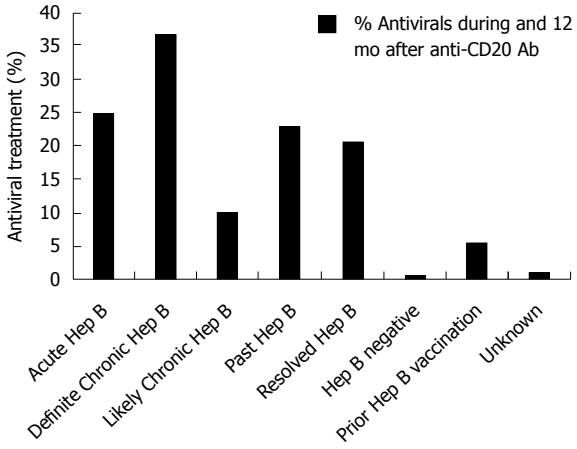

Figure 2 Hepatitis B antiviral treatment by hepatitis B category. Mean hepatitis B antiviral treatment use during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

dependent renal failure (Table 1).

Hepatitis B testing

Prior to anti-CD20 Ab treatment, 61%-73% had HBsAg and HBcAb tested at any time pretreatment in 2014 (Figure 1). During the study period, the rates of HBsAg and HBcAb testing increased more than two-fold (Figure 1) with overall pretreatment HBsAg and HBcAb measured in 53% (10224/19304) and 41% (7903/19304), respectively. In 2014, 43%-49% of patients had pretreatment HBsAg and HbcAb screening within 6 mo of anti-CD20 Ab initiation (Figure 1). During the high-risk period for reactivation, < 2% (261/14880) had HBV DNA measured.

Hepatitis B disease categories

In those tested, hepatitis B disease categories (7 categories including "unknown") were assessed as: definite chronic HBV in 40/10224 (0.4%), likely chronic HBV in 127/10224 (1.2%), past HBV in 326/7903 (4%), resolved HBV in 427/8110 (5%), HBV negative in 6002/7903 (76%), likely prior HBV vaccination in 628/8110 (7%), and acute HBV in 0.3% (16/4947) appearing during or after anti-CD20 Ab treatment. The remaining 11723/19304 (61% of overall) patients were termed "unknown," as missing serology or not otherwise categorized. Pretreatment HBV DNA was tested in 2% (403/19304) of patients, of whom 2% (9/403) were positive and 24% (97/403) were indeterminate. At pretreatment baseline, HBeAg was tested in 2% (474/19304), of whom 3% (12/474) were positive and 29% (139/474) were indeterminate. In all HBV categories, 17% or fewer received pretreatment HBV vaccination (as determined by vaccination records).

Antiviral treatment during high-risk period for HBV reactivation

Across all HBV disease categories, few patients receiving HBV antiviral treatment in the high-risk period had concomitant HIV infection (ranging from 1 in 59 to 2 in 9, or 2% to 22%). Overall HBV antiviral use throughout the high risk period ranged from 10%-37% in HBV positive patients at risk for reactivation (Figure 2); the highest rate of HBV antiviral use was 37% in those with definite chronic HBV. In the high-risk period, HBV positive patients exhibited low and variable rates of HBV antiviral treatment throughout the study period (data not shown), although most (80%) HBV antivirals were administered prophylactically (*i.e.*, started within 3 mo of anti-CD20 Ab initiation).

Acute hepatitis B

Among 16 pretreatment HBV negative patients acquiring acute HBV during the high-risk period, the mean peak ALT and bilirubin were 10 × ULN (+/- 13 × ULN) and 7 × ULN (+/- 10 × ULN), respectively (Figure 3). In the 25% (3/12) with acute HBV receiving HBV antivirals, the mean peak ALT was 19 × ULN (*vs* 7 × ULN in those not receiving antivirals). Those with

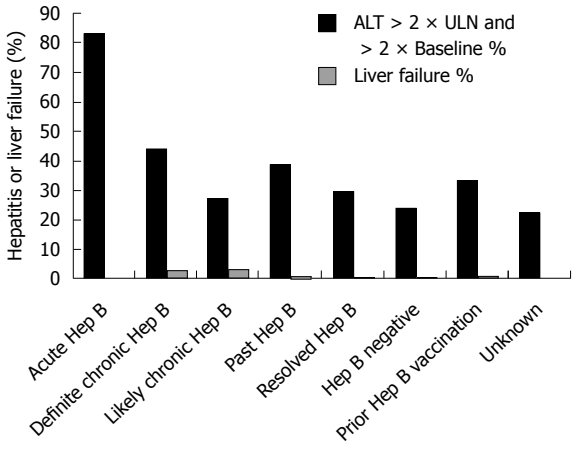

Figure 3 Incidence of hepatitis and liver failure by hepatitis B category. The incidence of hepatitis and liver failure during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

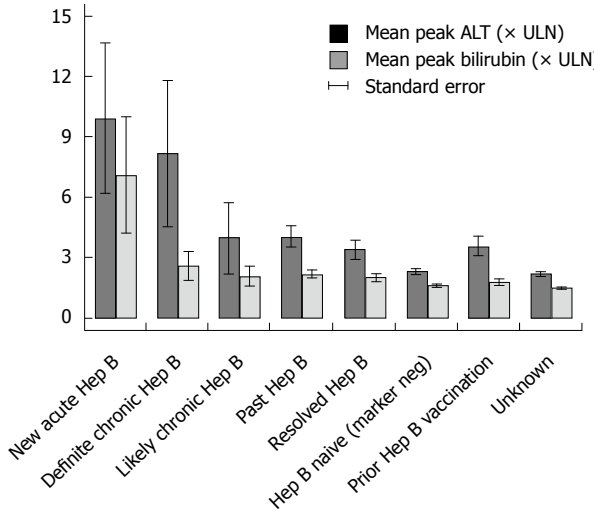

Figure 4 Mean peak ALT and bilirubin by hepatitis B category. The mean peak ALT and bilirubin of patients during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

acute HBV exhibited the highest rates of hepatitis [83%) 10/12] among all HBV positive patients, and experienced a 33% (4/12) all-cause mortality (Figure 4). Patients with acute HBV exhibited hepatitis and death at a mean time of 327 d or more following anti-CD20 Ab initiation.

Chronic hepatitis B

During the high-risk period, 37% (11/30) patients with definite chronic HBV received HBV antivirals and exhibited a mean peak ALT and bilirubin of 8 × ULN (+/- 19 × ULN) and 3 × ULN (+/- 4 × ULN), respectively. In contrast, 10% (9/88) with likely chronic HBV received antivirals and had a mean peak ALT 4 × ULN (+/- 16 × ULN) (Figure 3). Among chronic HBV positive patients, those with definite chronic HBV exhibited the highest rates of hepatitis [(43%) 13/30], liver failure [(3%) 1/30] and all-cause mortality [(40%) 12/30], while those with likely chronic HBV exhibited lower rates of

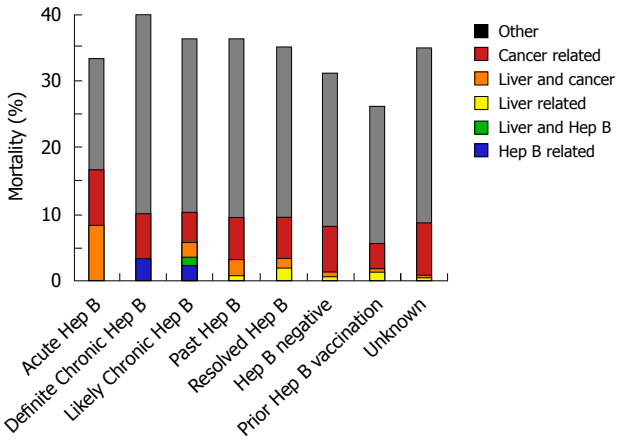

Figure 5 Incidence of overall, hepatitis-B associated, liver-related or cancer-related mortality by hepatitis B category. The overall, hepatitis-B associated, liver-related or cancer-related mortality incidence during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

hepatitis [(27%) 24/88], liver failure [(3%) 3/88] and overall mortality [(35%) 31/88] (Figures 4 and 5). Patients with chronic HBV experienced hepatitis and death at a mean time of more than 210 d after anti-CD20 Ab initiation.

Past and resolved HBV infection

Of patients with past and resolved HBV infection, 23% (59/256) and 21% (64/311) received HBV antivirals, respectively, and exhibited a mean peak ALT 3-4 × ULN (+/- 8 × ULN) (Figure 3). Among these patients, those with past HBV exhibited higher rates of hepatitis [(39%) 99/256], liver failure [(1%) 3/256] and overall mortality [(36%) 93/256], while those with resolved HBV exhibited lower rates of hepatitis [(30%) 93/311], liver failure [(0.6%) 2/311] and all-cause mortality [(35%) 109/311] (Figures 4 and 5). Patients with past or resolved HBV developed hepatitis and death at a mean time of 278 d or more following anti-CD20 Ab initiation.

HBV negative patients

In HBV negative patients, 7% (422/6022) had received prior HBV vaccination. HBV antiviral use was associated with concomitant HIV infection. HBV negative patients experienced a mean peak ALT of 2 × ULN (+/-8 × ULN) and the lowest rates of hepatitis [(24%) 994/4143] and liver failure [(0.6%) 27/4143] (Figure 4). These patients had a relatively low all-cause mortality [(31%) 1292/4143] (Figure 5).

Patients likely vaccinated against HBV infection

Patients likely vaccinated against hepatitis B (with isolated HBsAb+) exhibited 2-6-fold higher rates of baseline liver-related comorbidities (24% hepatitis C, 11% cirrhosis and 8% decompensated liver disease), relative to those of unknown HBV status (data not shown). During the high risk period, they had a mean

peak ALT of 4 × ULN (+/-10 × ULN), low rates of hepatitis [(34%) 140/416], liver failure [(1%) 5/416], and the lowest overall mortality [(26%) 109/416] (Figures 4 and 5).

Unknown HBV status

Patients with unknown HBV infection status (as serology missing or incomplete) exhibited the lowest rates of baseline comorbidity and pretreatment HBV vaccination rates [(2%) 252/11718], a mean peak ALT of 2 × ULN (+/-8 × ULN), low rates of hepatitis [(22%) 2163/9631], liver failure [(0.6%) 53/9631], and a moderate overall mortality of [(35%) 3363/9631] (Figures 4 and 5).

Hepatitis significantly higher in HBV positive patients

Patients with acute, chronic, past or resolved HBV infection were categorized as HBV positive, and are at risk of HBV reactivation due to the persistence of HBV DNA. When compared to HBV negative or likely vaccinated patients, the HBV positive patients exhibited significantly higher rates of hepatitis ($\chi^2 = 27.8$, $P = 0.001$), and nonsignificantly higher rates of liver failure and overall mortality. The small numbers of patients on HBV antiviral treatment precluded a planned analysis of health outcomes by HBV disease category in the presence or absence of antiviral treatment.

Relationship between hepatitis B vaccination and overall mortality

Patients with likely prior hepatitis B vaccination (isolated HBsAb+ pretreatment) had the lowest overall mortality rates (26% [108/416]) (Figure 5). In contrast, pre-treatment HBV negative patients who developed acute HBV during the high-risk period experienced a 33% (4/12) mortality rate.

DISCUSSION

In this first 12 year retrospective national VHA analysis, we evaluated HBV testing, vaccination, treatment and outcomes in nearly 20000 Veterans receiving anti-CD20 Ab treatment, largely for NHL or CLL. Rates of pretreatment HBV screening within 6 mo of anti-CD20 Ab initiation more than doubled over the study period. By 2014, most Veterans receiving anti-CD20 Ab had recent pretreatment HBsAg and HBcAb testing and the large majority had testing at any time, which compares favorably with the rates reported in ASCO quality oncology practices[10]. However, few patients susceptible to reactivation had HBV DNA testing during anti-CD20 Ab treatment and follow-up, limiting detection of HBV reactivation. Among those with pretreatment HBV testing, 1 in 9 were HBV positive and at risk for HBV reactivation - yet, only 21% received HBV antivirals during anti-CD20 Ab treatment and follow-up. As a result, HBV positive patients experienced a significantly higher rate of hepatitis than those HBV negative - with most events occurring within one year of treatment initiation. This data aligns with published data reporting the high risk period during anti-CD20 Ab treatment and 12 mo follow-up[15]. These hepatitis events, as well as related morbidity and costs, can be largely prevented with the use of safe, effective prophylactic antivirals in all HBV positive patients throughout the high-risk period of anti-CD20 Ab treatment and 12 mo follow-up[1,7,8,11].

Unexpectedly, we identified 16 cases of acute hepatitis B in the high-risk period arising in patients negative for HBsAg and HBcAb prior to anti-CD20 Ab initiation. These appear to be the first published reports of acute HBV arising *de novo* during anti-CD20 Ab therapy - likely as a result of the prolonged B cell suppression compromising host immune defense[3]. Hepatitis B vaccination substantively decreases the risk of acute HBV, even in high-risk adults[25,26]. Yet, in the current study, only 2% of the nearly 12000 "at risk" HBV unknown and 7% of the 6000 HBV negative patients had pretreatment hepatitis B vaccination. These rates are comparable to the 6% and 9% HBV vaccine immunity rates in Veterans and United States adults age 50 or older, respectively[2].

The strengths of this analysis include its large size, national scope, reliable pharmacy data, relatively high rate of HBV testing, identification of acute HBV risk, diverse indications for anti-CD20 therapy, and 12 mo follow-up of the large majority of patients after anti-CD20 Ab administration. Study limitations include the lack of VHA standardization of HBV serology resulting in some indeterminate results, and the predominantly qualitative HBV serologies. While HBV reactivation is generally identified by logarithmic increases in HBV DNA, reverse seroconversion (newly appearing HBeAg or HBsAg), or increases in ALT[27], the very limited quantified HBV DNA and HBeAg data required us to focus our evaluation on hepatitis - which occurs less frequently than HBV DNA increases in reactivation[4]. Additionally, the effect of HBV antivirals on health outcomes was limited by low antiviral treatment rates.

Automated clinical reminders and decision support have earlier been demonstrated to increase HBV screening and antiviral prophylaxis prior to immuno-suppressive therapy. For example, to increase HBV screening and antiviral prophylaxis in a Spanish medical center, computerized physician order entry prompts for HBV screening when ordering biologic therapies yielded > 90% screening rates, while appropriate consultation and prophylactic HBV antiviral treatment prevented HBV reactivation[28]. As computerized recommendations and follow-on treatment algorithms are highly effective in influencing physician behavior and prescribing[29], computerized decision support may decrease HBV-related disease with anti-CD20 Ab treatment in the VHA.

In conclusion, the VHA now screens most patients for HBV before anti-CD20 Ab treatment, yet seldom

measures HBV DNA during treatment and therefore, likely under-diagnoses HBV reactivation. Increasing VHA hepatitis B vaccination rates should diminish the risk of acute hepatitis B[26] and its complications during anti-CD20 Ab treatment and followup. In HBV positive patients, universal use of HBV antiviral treatment throughout anti-CD20 Ab treatment and 12 mo follow-up will likely decrease mortality and enhance quality of life.

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COMMENTS

Background

Among patients with lymphoma and prior hepatitis B virus (HBV) infection receiving rituximab, 10%-60% exhibit HBV reactivation at a median of 3 mo after the last rituximab dose. Pre-rituximab HBV testing and anti-viral treatment reduces HBV reactivation 10-fold and decreases lymphoma- and liver-related deaths in those with prior HBV infection.

Research frontiers

While the American Society for Clinical Oncology guidelines recommend HBV testing and treatment of patients with prior hepatitis B infection during and up to 12 mo following anti-CD20 antibody therapy, it is unclear how commonly these guidelines are followed in the United States Veterans Health Administration.

Innovations and breakthroughs

This 12 year retrospective cohort study analyzed 19304 Veterans in the United States Veterans Health Administration receiving anti-CD20 antibody therapy. The authors found that pre-treatment HBV testing increased over the study period, yet 37% or fewer received HBV antiviral treatment during anti-CD20 antibody treatment and 12 mo follow-up.

Applications

Results of this analysis can be shared with providers and used to develop electronic health record algorithms to enhance HBV testing and antiviral treatment with anti-CD20 antibody therapy and followup.

Peer-review

This retrospective study presented by Hunt *et al* demonstrates the necessity to screen patients for HBV before anti-CD20 Ab treatment, and most likely, prior to the administration of any immunosuppressive treatment; in order to determine if the patient will benefit from HBV vaccination or preventive antiviral treatment. This simple measure will reduce the number of HBV-related deaths occurring in a number of patients. It is an interesting and relevant study.

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ORIGINAL ARTICLE

Retrospective Study

Contrast-enhanced ultrasound of histologically proven hepatic epithelioid hemangioendothelioma

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Author contributions: Dietrich CF is accepting full responsibility for the conduct of the study, he and all others had access to the data, all had control of the decision to publish; Christoph F planned the study; Dietrich CF, Dong Y, D'Onofrio M, and Cantisani V drafted the manuscript; Dietrich CF and Dong Y performed the statistical analysis; each author made a statement that they approved the final draft submitted; all authors conducted the study, collected and/or interpreted data, and critically revised the manuscript for important intellectual content.

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Abstract

AIM: To analyze contrast-enhanced ultrasound (CEUS) features of histologically proven hepatic epithelioid hemangioendothelioma (HEHE) in comparison to other multilocular benign focal liver lesions (FLL).

METHODS: Twenty-five patients with histologically proven HEHE and 45 patients with histologically proven multilocular benign FLL were retrospectively reviewed. Four radiologists assessed the CEUS enhancement pattern in consensus.

RESULTS: HEHE manifested as a single (n = 3) or multinodular (n = 22) FLL. On CEUS, HEHE showed rim-like (18/25, 72%) or heterogeneous hyperenhancement (7/25, 28%) in the arterial phase and hypoenhancement (25/25, 100%) in the portal venous and late phases (PVLP), a sign of malignancy. Eighteen patients showed central unenhanced areas (18/25, 72%); in seven patients (7/25, 28%), more lesions were detected in the PVLP. In contrast, all patients with hemangioma and focal nodular hyperplasia showed hyperenhancement as the most distinctive feature (P < 0.01).

CONCLUSION: CEUS allows for characterization of unequivocal FLL. By analyzing the hypoenhancement in the PVLP, CEUS can determine the malignant nature of HEHE.

Key words: Guidelines; Recommendations; Liver tumor; Biopsy; Liver transplantation Contrast enhanced ultrasound

Core tip: In this retrospective study, a large cohort of very rare histologically proven hemangioendothelioma (HEHE) was evaluated. Contrast-enhanced ultrasound (CEUS) allowed for improved detection of multilocular HEHE. HEHE showed typical enhancement patterns on CEUS. Therefore, CEUS can help to determine the malignant nature of HEHE.

Dong Y, Wang WP, Cantisani V, D'Onofrio M, Ignee A, Mulazzani L, Saftoiu A, Sparchez Z, Sporea I, Dietrich CF. Contrast-enhanced ultrasound of histologically proven hepatic epithelioid hemangioendothelioma. *World J Gastroenterol* 2016; 22(19): 4741-4749 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4741.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4741

INTRODUCTION

Hepatic epithelioid hemangioendothelioma (HEHE) is a rare vascular neoplasm of endothelial origin with primary liver involvement and is characterized by the presence of epithelioid endothelial cells[1]. Weiss and Enzinger first reported 41 patients with this unique tumor in 1982[2]. This tumor is histologically characterized by an epithelial appearance and the endothelial nature of the tumor cells[3]. Currently no more than 200 patients with HEHE have been reported since its first description, and most of the studies were small series[4,5].

No definite etiopathogenetic factors, apart from an association with oral contraceptives, trauma, and exposure to vinyl chloride, have to date been ascribed to HEHE[1,6]. The tumor generally affects adults, with a strong female predominance and a peak incidence occurring between 30 and 40 years of age. The clinical manifestations and laboratory data of HEHE are nonspecific, usually presenting with general symptoms, such as right upper quadrant pain or weight loss. Some patients may present with liver failure, Budd-Chiari syndrome, or portal hypertension, while others may be asymptomatic. Its clinical course and prognosis are variable and unpredictable[7]. Due to its nonspecific clinical manifestations and prolonged clinical course, establishing diagnosis even with histopathological findings can often be challenging[8].

HEHE carries intermediate malignant potential, and transplantation may provide a long term cure[9]. Therefore, the recognition of the imaging features of this rare neoplasm may be helpful for the detection and further surgical treatment of this potentially curable disease. In addition, it is important to distinguish HEHE from other primary and secondary benign and malignant hepatic tumors, such as atypical (multilobulated) hemangioma and hemangiomatosis, hepatocellular adenoma and hepatocellular carcinoma, intrahepatic cholangiocarcinomas, lymphoma, and liver metastases[10]. Radiologists should be aware of its imaging findings and raise suspicion in the proper clinical setting[8,11].

Conventional ultrasound is the most commonly used imaging method for real time diagnosis of FLL. However, the most frequent imaging findings of multilocular HEHE are nonspecific[11]. Often, multiple HEHE on conventional ultrasound might be difficult to differentiate from other atypical multilocular FLLs[12,13]. As a result, the final diagnosis of HEHE depends on biopsy and histological findings[14,15].

Contrast enhanced ultrasound (CEUS) allows for the differentiation of most benign and malignant liver tumors in the portal venous and late phases (PVLP). This finding was summarized in the European Federation of Societies for Ultrasound in Medicine and Biology guidelines and recommendations for the use of CEUS in liver[16,17]. Benign FLLs are typically iso- or hyperenhancing in the PVLP; whereas malignant primary and secondary liver tumors almost always show hypoenhancement in the PVLP, since they do not contain the respective specific hepatic vessels. This hypoenhancement in the PVLP is decisive for determining if a lesion should be biopsied[18,19]. In

Table 1 Baseline characteristics of patients included in our study

Characteristic	HEHE (n = 25)	Hemangioma and FNH (n = 45)
Age (yr)		
mean ± SD	46 ± 14	46 ± 14
Range	24-78	23-74
Male/female	8/17	9/36
Number of FLL (single/multiple)	3/22	0/45
Histological results		
hepatic surgery	6	0
core needle biopsy	19	45

HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia; FLL: Focal liver lesions.

addition, CEUS findings of HEHE have not been well addressed. Therefore, the aim of our study is to analyze the CEUS features of histologically proven HEHE and to compare these features to those of other multilocular benign FLLs, including hemangiomas and focal nodular hyperplasia (FNH), since they are the most important for differential diagnosis. We assessed the clinical value of CEUS to define the malignant nature of the disease with hypoenhancement in the PVLP. To our best knowledge, this is the first report on the CEUS features of HEHE.

MATERIALS AND METHODS

Patients

Hemangioendothelioma: Between September 2004 and October 2015, 25 patients (eight male, 17 female, mean age 46 ± 14 years; range 24-78 years) were retrospectively analyzed. In this retrospective study, lesions were histologically proven by hepatic surgery (n = 6) or by 18-gauge core needle biopsy (n = 19).

Three patients had a single FLL, whereas 22 patients had multiple FLLs (Table 1). In patients with multiple FLLs, the selected lesions were those in which biopsies had been performed.

Multilocular hemangioma and FNH

Forty-five patients (nine male, 36 female, mean age 46 ± 14 years; range 23-74 years) with multilocular hemangioma and FNH were also retrospectively analyzed. All lesions were histologically proven by 18-gauge core needle biopsy.

Examination technique

Conventional ultrasound and CEUS were performed by five ultrasound systems: LOGIQ E9 (GE Healthcare, Milwaukee, WI, United States; C1-5 convex array probes, 1-5MHz), Acuson Sequoia (Siemens Healthcare, Erlangen, Germany, 3.5 MHz), Philips iU22 unit (Philips Healthcare, Bothell, WA, United States; C5-1 convex array probes, 1-5MHz), Technos MPX Scanner, and MyLab70 (Esaote, Genova, Italy; ca431 convex array probe 1-8 MHz).

CEUS was performed using contrast harmonic real time imaging at a low MI 0.05-0.30. Each examination lasted about 5 min after the bolus injection. The contrast agent used was SonoVue® (Bracco Imaging Spa, Milan, Italy). For each CEUS examination, a dose of 1.5-2.4 mL of SonoVue® was injected as a quick bolus *via* a 20 gauge intravenous catheter placed in the cubital vein, followed by 5-10 mL of 0.9% normal saline flush. Repeated injection of SonoVue® was performed when necessary.

To characterize the lesion, SonoVue® enhancement during the arterial phase (10-30 s), portal venous (20-120 s), and late vascular phases (120-300 s) were evaluated[17]. All examinations were digitally recorded.

Image analysis

All HEHE images were read by four independent radiologists (15, 17, 23, and 27 years of experience with abdominal ultrasound imaging) blinded to clinical and pathologic data in consensus. Criteria evaluated included number of lesions, maximum diameter, echogenicity (hyperechoic, hypoechoic, or isoechoic; homogeneous or heterogeneous; which were visually compared with the echogenicity of the surrounding liver parenchyma), shape (regular or lobulated), margin (ill- or well defined appearance), and color Doppler imaging features. Using CEUS, the pattern of contrast enhancement of the lesion in comparison to the surrounding liver parenchyma (hypoenhancing, hyperenhancing, isoenhancing), homogeneity of enhancement (homogeneous, heterogeneous), and additional features of enhancement during the arterial, portal venous, and late phases were noted as well, *e.g.,* rim-like or peripheral nodular enhancement, central or eccentric arterial enhancement).

CEUS features of 45 patients of histologically proven multilocular liver hemangioma and FNH were also retrospectively evaluated to compare the CEUS features for differential diagnosis. Digital cineloops were registered both during baseline and post contrast US scanning. All cineloops were digitally stored in a PC based workstation connected to the ultrasound systems.

Pathologic examination

The final pathologic diagnosis was based on hematoxylin-eosin stained sections and immunohistochemical staining results. The immunohistochemical staining included endothelial markers, such as CD 34, CD 31, and factor VIII-related antigen (FVIII Ag)[20].

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed with SPSS 17.0 software package (SPSS, Chicago, IL, United States). The χ^2 test was used to compare HEHE with liver hemangiomas and FNH in terms of enhancement pattern. For the features that played a statistically significant role in

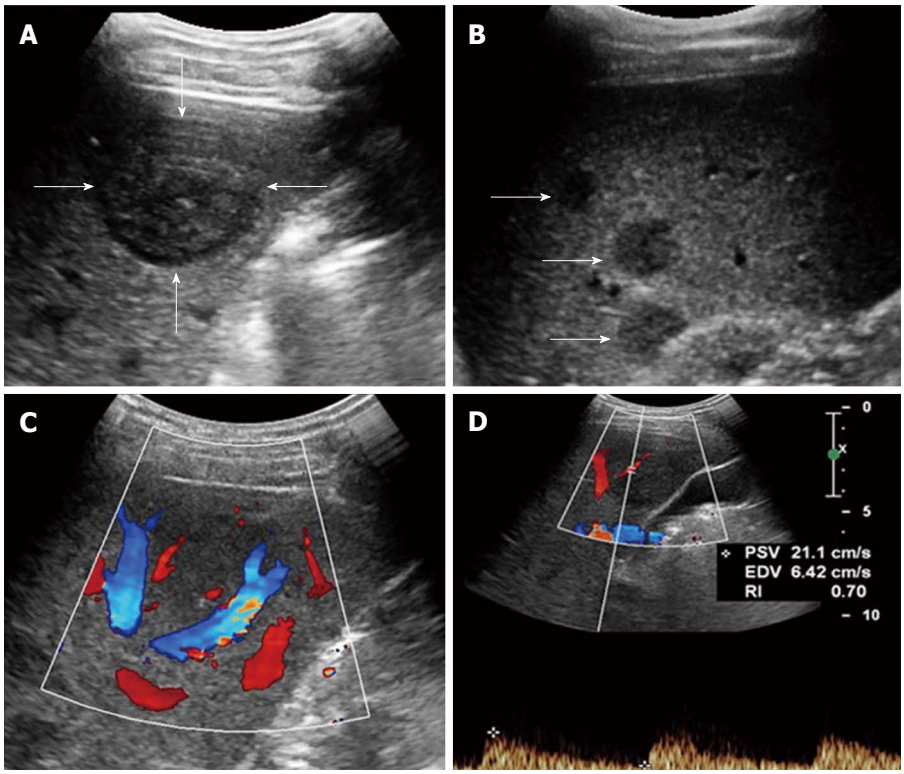

Figure 1 Multiple hepatic epithelioid hemangioendotheliomas in a 31 year female. A: Grayscale ultrasound showed a distinct hypoechoic focal liver lesion (FLL) (arrow); B: Multiple hypoechoic lesions (arrows) were also detected in this patient; C: Color Doppler imaging (CDFI) showed peripheral and intra-lesion color flow signals; D: The resistive index (RI) of color flow was 0.70.

the differentiation diagnosis, we calculated sensitivity and specificity. A difference was considered statistically significant with $P < 0.05$.

RESULTS

Clinical and general pathologic features

All patients were incidentally found to have hepatic lesions by conventional ultrasound screening. Conventional laboratory tests [including transaminases, bilirubin, and gamma-glutamyl transpeptidase (gGT)] were within normal limits or only slightly elevated in all patients. Alpha-fetoprotein, carcinoembryonic antigen, and cancer antigen 19-9 as well as hepatitis B surface antigen and hepatitis C virus were negative respective of normal in all patients.

Final pathologic diagnosis of HEHE showed the typical fibrosclerotic center and cellular periphery on hematoxylin-eosin staining. Immunohistochemically, tumors were positive for at least one endothelial marker, including CD 34 (n = 20), CD 31 (n = 20), or FVIII Ag (n = 11).

Features with conventional ultrasound in HEHE

HEHE manifested as single (3/25, 12%) or multiple FLLs (22/25, 88%) with ill-defined margins on grayscale ultrasound. The lesions were mainly hypoechoic (23/25, 92%) to adjacent liver parenchyma, but a heterogeneous echogenicity with hypo- or hyperechoic FLL was observed (2/25, 8%).

Color Doppler imaging detected branched intralesional vessels in 84% (21/25) of HEHE. The Doppler spectrum was measured in 13 patients. The mean value of resistive index (RI) was 0.64 ± 0.07 (Figure 1 and Table 2).

CEUS features

On CEUS, HEHE presented peripheral rim-like (18/25, 72%) (Figure 2) or heterogeneous hyperenhancement (7/25, 28%) at the arterial phase (Figure 3) and hypoenhancement (100%, 25/25) at PVLP (Figure 4). Central unenhanced areas were observed in 72% (18/25) of HEHE in the late phases. After CEUS, more lesions could be detected in seven patients of HEHE than with conventional ultrasound. Liver hemangioma typically demonstrated peripheral nodular contrast enhancement in all patients, whereas FNH showed central or eccentric arterial blood supply in the arterial phase. In addition, in all patients, both entities showed hyperenhancement in the PVLP, a sign of the benign nature of the lesion. Compared to multilocular liver hemangioma and FNH, characteristic CEUS features of HEHE were peripheral rim-like hyperenhancement in the arterial phase and quick washout in the PVLP with a central unenhanced area in the late phase ($P < 0.01$) (Table 3).

The sensitivity for peripheral rim-like hyperenhancement at the arterial phase was 72%; for

Table 2 Conventional ultrasound features of hepatic epithelioid haemangioendothelioma and hemangioma/Focal nodular hyperplasia *n* (%)

Characteristic	HEHE (*n* = 25)	Hemangioma/FNH (*n* = 45)
Number of nodules (single/multiple)	3/22	0/45
Size of nodules (mm)		
mean ± SD	41.5 ± 25.6	50.4 ± 25.7
range	12-120	20-138
Echogenicity of nodules		
Hyperechoic	2 (8)	19 (42.2)
Hypoechoic	23 (92)	9 (20.0)
Isoechoic	0	17 (37.8)
Homogenous/heterogeneous	9/16	15/30

HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia.

quick washout in the PVLP, it was 100%; for central unenhanced area at late phase, it was 72%; and for the combination of both, it was 85% (Table 3).

DISCUSSION

To the best of our knowledge, CEUS features of HEHE have not been well characterized. To date, only a few imaging studies have investigated HEHE, and most of them were limited patients series[4,15,21], and CEUS features of HEHE have been described only in a few patients[15,21,22]. In many patients, CEUS is the first and decisive imaging technique for detecting and characterizing liver tumors[23-25]. The use of ultrasound contrast agents improved detection and made it possible to assess the benign or malignant nature of liver tumors in most patients[13,26-28]. Previously, three forms of HEHE have been described: single nodular, multifocal nodular, and the diffuse type[1]. Consistent with our current study, most HEHE present as multiple FLL. After CEUS, more lesions could be detected in 7/25 (28%) patients[29]. As HEHE has ill-defined margins on grayscale ultrasound, CEUS may be helpful to detect more lesions with sharper and clearer margins.

In our current retrospective study, we discovered that CEUS reliably showed typical signs of HEHE in most patients with hyperenhancement in the arterial phase and hypoenhancement in the PVLP, which might be useful in determining whether a biopsy is necessary for suspected malignant lesions. In correlation with pathologic classification, histologically, HEHE possesses two distinctive characteristics, which are directly related to the echogenicity and enhancement pattern of HEHE on ultrasound images[1,20,30,31]. First, HEHE are composed of dendritic and epithelioid cells with intracytoplasmic lumina containing red blood cells. However, the peripheral proliferation remains active and forms numerous arterial-venous shunts, which

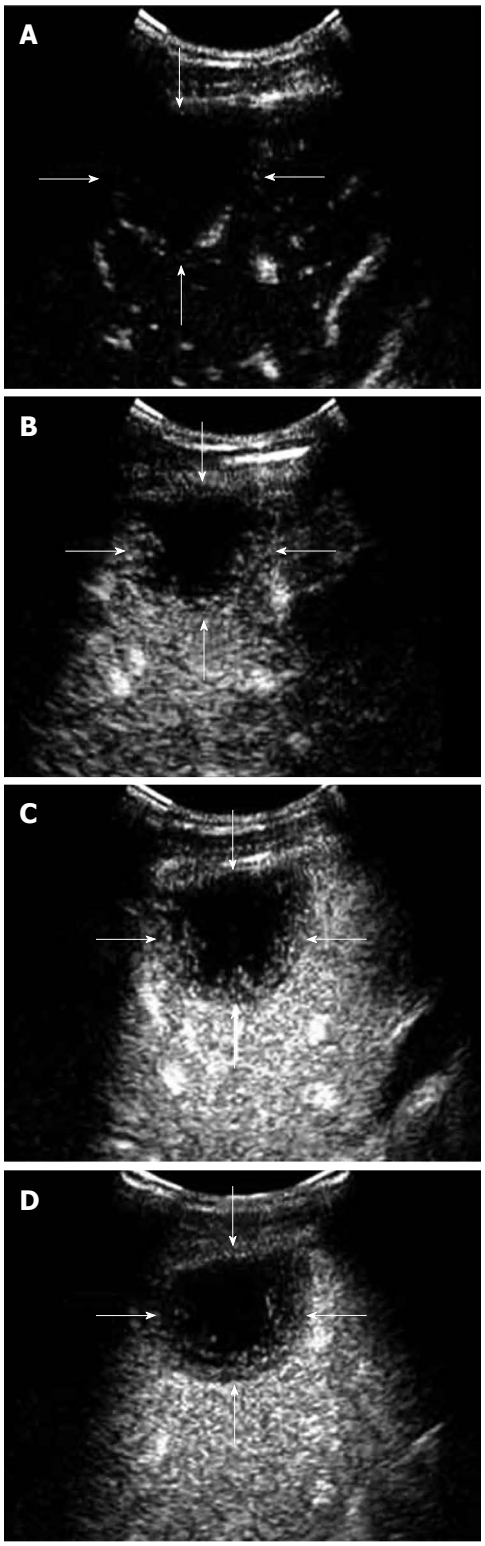

Figure 2 Contrast-enhanced ultrasound feature of hepaticepithelioid hemangioendothelioma in a 31 year female. A: Rim-like enhancement. In arterial phase (16 s after injection of SonoVue), peripheral rim-like enhancement was demonstrated; B: In peak enhancement (24 s after injection of SonoVue), the degree of the rim-like enhancement was equivalent to the liver parenchyma; C: In portal venous phase (45 s after injection of SonoVue), the lesion washed out quickly and showed hypoenhancement; D: In late phase (65 s after injection of SonoVue), the lesion remained hypoenhanced with central unenhanced area.

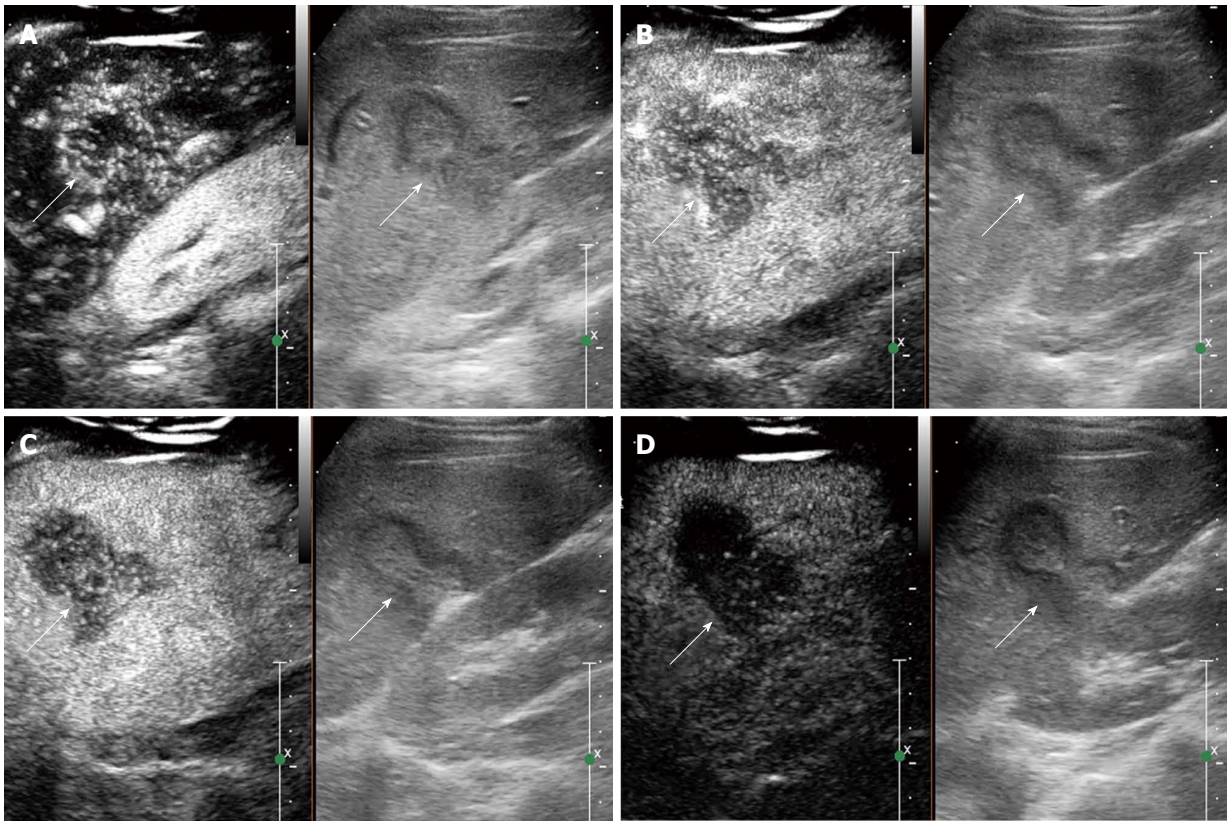

Figure 3 Contrast-enhanced ultrasound feature of hepatic epithelioid hemangioendothelioma in a 25 year female. A: Heterogeneous enhancement pattern. In the arterial phase (16 s after injection of SonoVue), the lesion showed heterogeneous enhancement; B: The enhancement gradually decreased (22 s after injection of SonoVue); C: In the portal venous phase (40 s after injection of SonoVue), the lesion washed out fast than the liver parenchyma and showed hypoenhancement. D: In the late phase (165 s after injection of SonoVue), the lesion remained hypoenhanced.

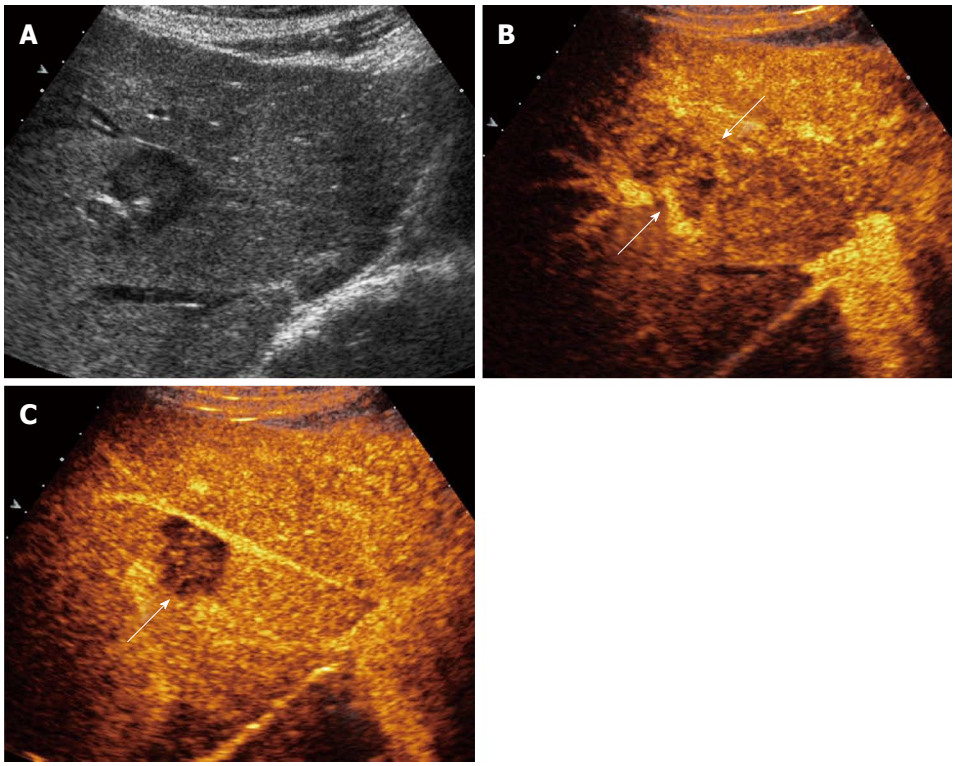

Figure 4 Contrast-enhanced ultrasound feature of hepatic epithelioid hemangioendothelioma in right lobe of liver. A: Grayscale ultrasound showed a hypoechoic focal liver lesions (FLL); B: In the arterial phase the lesion showed heterogeneous enhancement (22 s after injection of SonoVue); C: In the portal venous phase (53 s after injection of SonoVue), the lesion washed out fast and showed hypoenhancement.

Table 3 Contrast enhanced ultrasound imaging features of hepatic epithelioid haemangioendothelioma and multilocular hemangioma/ focal nodular hyperplasia *n* (%)

Characteristic	HEHE (*n* = 25 patients)	Hemangioma/FNH (*n* = 45 patients)
Arterial phase		
Rim like hyperenhancement	18 (72)	0
Heterogeneously hyperenhancement	7 (28)	6 (13.3)
Peripheral nodular enhancement	0	All hemangioma
Central arterial blood supply		All FNH
Portal-venous phase		
Hyperenhancement	0	100 (100.0%)
Hypoenhancement	25 (100)	0
Isoenhancement	0	0
Late phase		
Hyperenhancement	0	45 (100.0%)
Hypoenhancement	25 (100)	0
Isoenhancement	0	0
Sensitivity		
Rim like hyperenhancement	18/25 (72)	0
Hypoenhancement at portal venous phase	25/25 (100)	0
Central unenhanced area at late phase		
Yes	18 (72)	13 (28.9)
No	7 (28)	32 (71.1)

CEUS: Contrast enhanced ultrasound; HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia.

could account for the fast rim-like enhancement in the arterial phase and quick washout in the PVLP during CEUS[7]. Second, tumor cells and stroma of HEHE exist in variable proportions, and the central stromal portion of the lesion can vary from myxoid to densely fibrotic. With the growth of the tumor, the central stroma degenerate gradually and become sclerotic as the blood supply decreases[20]. In our results, hypoenhancement with central unenhanced area at PVLP of CEUS was mostly common in HEHE. Moreover, additional lesions were detected at CEUS, leading to improvements in liver staging.

Alomari *et al*[32] first described the lollipop sign as a new cross-sectional sign of HEHE on computed tomography (CT) and magnetic resonance imaging (MRI): a well-defined peripherally enhancing (or non-enhancing) lesion with an avascular core on enhanced images (the candy in the lollipop) and a histologically occluded vein (the stick). Concerning the CT imaging, focal calcifications were reported in 20% of patients; capsular retraction was in 10%-25% of patients[20]. The lesions demonstrated peripheral rim-like hyperenhancement in the arterial phase with even stronger enhancement in the portal venous phase by contrast enhanced MRI. Central areas of reduced signal may correspond to areas of hemorrhage, coagulation necrosis, and calcification[7]. We showed that peripheral rim-like hyperenhancement in the arterial phase and hypoenhancement in the PVLP with central unenhanced areas could be detected in 72% HEHE patients. Therefore, the contrast enhanced image modalities demonstrate a similar enhancement pattern of this disease. CEUS can be considered at least equal to, and in some ways (real time observation, no radiation, less expensive) superior to, CT and MRI as a diagnostic tool[33].

Most of the HEHE lesions were multinodular (88%) and hypoechoic (92%) in our current study. As set out in the current literature and in textbooks, the origin of hypoechoic lesions is considerably more varied and confusing than other lesions[13,23]. All hypoechogenic lesions should be investigated using a contrast enhanced imaging technique[16,18]. Evaluation with CEUS in the PVLP is determinant in this context, and contrast medium hypoenhancement in the late phase is a decisive indication for liver biopsy[23].

HEHE has a variable clinical and biological course compared to benign endothelial tumors (hemangiomas) and malignant angiosarcomas with a slowly progressive phenotype. The tumor can even be difficult to diagnose based on biopsy specimens[34]. CEUS differentiation of different liver tumors is essential because of different therapeutic approaches[35]. HEHE should be differentiated from atypical multilocular liver hemangioma and FNH, because both of them could demonstrate as multilocular hypoechoic liver lesions. Although benign FLLs are commonly iso- or hyperenhancing in the PVLP, malignant primary and secondary liver tumors almost always show hypoenhancement in the PVLP[18,19]. Based on results of our retrospective analysis, we believe that peripheral rim-like hyperenhancement at the arterial phase and quick washout at the PVLP with central unenhanced area are hallmark features that suggest a diagnosis of possible HEHE. In contrast, both multilocular hemangiomas and FNH showed hyperenhancement and remained iso or hyperenhanced in PVLP.

Furthermore, in the clinical setting, factors helpful for the differential diagnosis of HEHE are a medical history without extrahepatic malignant tumor, patients with no symptoms, and laboratory tests[35].

In conclusion, CEUS imaging findings reliably compile typical signs of HEHE, allowing for effective differentiation with other multilocular hypoechoic hepatic lesions, including liver hemangioma and FNH. CEUS can help to improve the diagnostic confidence of HEHE, a rare hepatic tumor, and the liver staging of the disease to guide additional diagnostic work-up.

COMMENTS

Background

To our best knowledge, contrast-enhanced ultrasound (CEUS) features of hepatic epithelioid hemangioendothelioma (HEHE), a rare hepatic tumor, have not been well characterized. To date, only a few imaging studies have investigated HEHE, and most of them were limited patients series.

Research frontiers

This is the first report on the CEUS features of HEHE.

Innovations and breakthroughs

CEUS imaging findings reliably compile typical signs of HEHE and differentiate effectively it from other multilocular hypoechoic hepatic lesions, including liver hemangioma and focal nodular hyperplasia.

Applications

CEUS can help to improve the diagnostic confidence and liver staging of HEHE to guide additional diagnostic work-up.

Terminology

CEUS allows for the differentiation of most benign and malignant liver tumors in the portal venous and late phases.

Peer-review

The aim of this retrospective study was to analyze the CEUS features of histologically proven HEHE in comparison to other multilocular benign focal liver lesions, which might be important differential diagnosis, and to assess the clinical value of CEUS to define the malignant nature of HEHE with hypoenhancement in the portal-venous and late phase.

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ORIGINAL ARTICLE

Retrospective Study

Lymph node dissection in esophageal carcinoma: Minimally invasive esophagectomy *vs* open surgery

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Informed consent statement: All patients provided written informed consent for surgery. Because of the retrospective nature of the study, the requirement for patient consent for inclusion in the present study was waived.

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Abstract

AIM: To compare lymph node dissection results of minimally invasive esophagectomy (MIE) and open surgery for esophageal squamous cell carcinoma.

METHODS: We retrospectively reviewed data from patients who underwent MIE or open surgery for esophageal squamous cell carcinoma from January 2011 to September 2014. Number of lymph nodes resected, positive lymph node (pN+) rate, lymph node sampling (LNS) rate and lymph node metastatic (LNM) rate were evaluated.

RESULTS: Among 447 patients included, 123 underwent MIE and 324 underwent open surgery. The number of lymph nodes resected did not significantly differ between the MIE and open surgery groups (21.1 ± 4.3 *vs* 20.4 ± 3.8, respectively, $P = 0.0944$). The pN+ rate of stage T3 esophageal squamous cell carcinoma in the open surgery group was higher than that in the MIE group (16.3% *vs* 11.4%, $P = 0.031$), but no differences was observed for stages T1 and T2 esophageal squamous cell carcinoma. The LNS rate at left para-recurrent laryngeal nerve (RLN) site was significantly higher for open surgery than for MIE (80.2% *vs* 43.9%, $P < 0.001$), but no differences were noted at other sites. The LNM rate at left para-RLN site in the open surgery group was significantly higher than that in the MIE group, regardless of pathologic T stage.

CONCLUSION: For stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable to that achieved

by open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

Key words: Esophageal cancer; Lymph node; Minimally invasive; Surgery

Core tip: Previous studies have not reported in detail whether minimally invasive esophagectomy (MIE) can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. Our study found that for stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable with that after open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

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INTRODUCTION

Esophageal carcinomas are a group of malignant tumors with poor prognoses. Among esophageal carcinomas, squamous cell carcinoma has a particularly poor prognosis, primarily because of extensive lymph node metastasis in three anatomical regions: the neck, mediastinum, and upper abdomen[1-8]. From 1980s, Japanese surgeons started to investigate three-field lymph node dissection to improve the prognosis of esophageal squamous cell carcinoma. In several studies, the 5-year survival was reported to be improved by approximately 10%. However, three-field lymph node dissection has not been widely accepted because of its complicated procedure and high risk of postoperative complications[9-12].

Minimally invasive esophagectomy (MIE) has been into a rapid development period. Its safety and efficacy to improve patients' life quality have been demonstrated in previous reports[13-18].

However, previous studies have not reported in detail whether MIE can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study attempts to retrospectively review the data from patients with esophageal squamous cell carcinoma who were treated at Shanghai Chest Hospital and compare the lymph node dissection results of MIE and open surgery.

MATERIALS AND METHODS

A total of 1343 patients who underwent surgeries to treat esophageal carcinoma at Shanghai Chest Hospital from January 2011 to September 2014 were retrospectively analyzed. The inclusion criteria were as follows: (1) diagnosed with squamous cell carcinoma; (2) underwent either open surgery or MIE; (3) received thoraco-abdominal two-field lymph node dissection; (4) *via* right-side thoracotomy; and (5) the esophageal-gastric anastomosis site was either at the thoracic apex or neck. All the surgeons involved in this study were experienced in both open and thoracoscopic esophagectomy and followed the same principle and technical requirement of lymph node dissection.

To better evaluate the efficacy of the surgeries for dissecting lymph nodes in different anatomical sites under thoracoscopy and laparoscopy, the mediastinal and abdominal lymph node metastasis regions were regrouped for this study (Table 1).

Preoperative evaluation

All patients received enhanced chest and abdominal computed tomography (CT) examinations, cervical CT scan examination or ultrasonic examination, and upper gastrointestinal endoscopic examination before operation. Any tumor involved middle and upper thoracic esophagus was examined by bronchoscopy. Positron emission tomography (PET) or PET-CT was used only in those patients who were willing to pay themselves and with possible distal metastasis. Primary tumor and mediastinal lymph node staging by the endoscopic ultrasound (EUS) examination was performed in all patients, except any patient who had such a narrow esophagus that a gastrointestinal endoscope could not pass through. Cranial magnetic resonance imaging (MRI) and bone emission CT scan were used selectively. The preoperative diagnosis of lymph node metastasis was based on radiology or EUS, endobronchial ultrasound or ultrasound-guided fine-needle aspiration biopsy. Patients who were diagnosed with cT_3 and cN_2 would receive inductive treatment after informed consent was obtained.

Surgical technique

The tri-incisional approach (McKeown) was adopted as the surgical approach for MIE. The esophageal-gastric anastomosis was performed at the neck.

Table 1 Group of lymph nodes

Region	Group
Upper mediastinal region	Right para-recurrent laryngeal nerve (RLN)
	Left para-RLN
	Upper para-esophagus
Carinal and hilar region	Subcarinal
	Left and right para-bronchi
Middle-low para-esophageal region	Middle and low para-esophagus
	Supra-diaphragm
Intraperitoneal region	Para-cardia
	Lesser gastric curvature
	Para-celiac artery and left gastric artery

Patients were in the left lateral recumbent position and leaned forward 30° while the esophagus in the thoracic cavity was freed, and the lymph nodes in the thoracic cavity were dissected under artificial pneumothorax. Afterward, patients were in the supine position while patients' stomachs were freed, and the lymph nodes in the upper abdominen were dissected under laparoscopy. A small midline abdominal incision (8 cm) was made below the xiphoid process to allow completion of the tailoring of the tubular stomach, which was uplifted to the neck to be anastomosed to the esophagus via the substernal or posterior mediastinal pathway. The McKeown or Ivor Lewis approach was used for the open surgery, and the thoraco-abdominal two-field lymph node dissection was required for both approaches.

Evaluation indices included the number of lymph nodes resected, lymph node metastatic (LNM) rate and positive lymph node (pN+) rate in different T stages, and the lymph node sampling (LNS) rate and LNM rate at different sites in the two groups.

Statistical analysis

All statistical analyses were performed with SPSS version 20 (IBM Corp., Armonk, NY). Continuous variables are expressed as mean ± SD. Comparisons of categorical variables were done using χ^2 or Fisher's exact test, and those of continuous variables were done using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

A total of 447 patients who met the inclusion criteria were included in this study. Of all the included patients, 324 underwent open surgery (226 males and 98 females with a mean age of 60.3 years), and 123 underwent MIE (97 males and 26 females). There were no significant differences in baseline characteristics, including gender, height, weight, smoking history, the American Society of Anesthesiologists (ASA) score and the rate of complete resection (Table 2). The postoperative pathological diagnostic results revealed

Table 2 Characteristics of the open surgery and minimally invasive esophagectomy groups

	Open surgery (*n* = 324)	MIE (*n* = 123)	*P* value
Age (yr)	60.3 ± 7.8	60.1 ± 6.3	0.782[1]
Gender			0.055[2]
Male	226	97	
Female	98	26	
ASA grade			0.820[2]
I	15	6	
II	248	97	
III	61	20	
Height (cm)	170.2 ± 4.8	171.1 ± 4.9	0.585[1]
Weight (kg)	65.2 ± 9.8	66.8 ± 8.1	0.152[1]
Smoker			0.912[2]
Never	123	46	
Current or former	201	77	
pT stage			
Tis	3	4	0.077
T1	20	36	< 0.001
T2	46	72	< 0.001
T3	225	11	< 0.001
T4	30	0	< 0.001
Resection rate			0.479[2]
R0	313	121	
R1	6	2	
R2	5	0	
Inductive chemo/radio therapy	40%	14%	0.788[2]
pTis/T1/T2	0	0	
pT3	15	6	0.912[2]
pT4	25	0	0.002
LNM rate			
pTis	0	0/4	
pT1	6 (30)	10/36, 27.8%	0.860[2]
pT2	20 (43.4)	29/72, 40.3%	0.731[2]
pT3	125 (55.6)	5/11, 45.5%	0.511[2]
pT4	22 (73.3)	0	
Surgical approach			< 0.001
Macheen	225	123	
Ivor-Lewis	99	0	

[1]The *t* test; [2]Fisher's exact test. LNM rate = number of patients with positive lymph nodes/number of total patients. LNM: Lymph node metastatic; MIE: Minimally invasive esophagectomy; ASA: American Society of Anesthesiologists.

that there were no significant differences in the LNM rates between the open surgery group and MIE group in different T stages. Only 6 patients received inductive chemo/radio-therapy in the MIE group, and those patients' postoperative pathological stages were all T3. Forty patients received inductive chemo/radio-therapy in the open surgery group, of whom 15 were in stage pT3 and 25 in stage pT4. The ratio of the number of patients who received inductive chemo/radio-therapy to the total number of patients in the open surgery group was higher than the ratio in the MIE group; however, there was no significant difference between the two groups.

Table 3 lists the lymph node dissection results for open surgery and MIE in different T stages. The number of lymph node resected did not significantly differ between the MIE and open surgery groups

Table 3 Comparison of the number of lymph node dissections and the rate of positive lymph nodes according to pathological T stage *n* (%)

	Number of lymph node dissections		*P* value[1]	Rate of positive lymph nodes		*P* value[2]	χ^2
	Open	MIE		Open	MIE		
pTis	18.3 ± 1.5	19.8 ± 1.3	0.2150	0	0		
pT1	19.3 ± 4.1	21.4 ± 3.8	0.0593	21 (5.0)	50 (5.8)	0.579	0.308
pT2	20.2 ± 3.2	22.1 ± 6.6	0.0715	98 (10.1)	190 (11.0)	0.494	0.468
pT3	20.3 ± 5.8	23.2 ± 4.1	0.1030	733 (16.3)	32 (11.4)	0.031	4.626
pT4	21.5 ± 3.6	0		130 (17.8)	0		
Total	20.4 ± 3.8	21.1 ± 4.3	0.0944	982 (14.8)	272 (9.5)	< 0.001	49.222

[1]The *t* test; [2]The χ^2 test. The rate of positive lymph nodes (pN+) = number of metastatic lymph nodes/number of removed lymph nodes. MIE: Minimally invasive esophagectomy.

Table 4 Comparison of lymph node sampling rates according to pathological T stage (Including Tis, T1-T4)

		LNS rates		*P* value[1]	χ^2
		Open (*n* = 324)	MIE (*n* = 123)		
Upper mediastinum	R-RLN	262	100	0.916	0.011
	L-RLN	260	54	< 0.001	56.345
	Upper para-esophagus	272	102	0.794	0.068
Subcarinal and parabronchial	Subcarinal	307	112	0.150	2.074
	Left and right parabronchial	310	120	0.353	0.863
Mid and low para-esophagus and diaphragm	Mid and lower para-esophagus	299	110	0.334	0.933
	Diaphragm	128	46	0.683	0.167
Intraperitoneal	Para-cardial	310	116	0.541	0.374
	Lesser gastric curvature	275	105	0.897	0.017
	Left gastric artery	272	106	0.560	0.339

[1]The χ^2 test. LNS rates = number of patients undergoing lymph node sampling/number of total patients. LNS: Lymph node sampling; RLN: Recurrent laryngeal nerve; MIE: Minimally invasive esophagectomy.

(20.4 ± 3.8 *vs* 21.1 ± 4.3, respectively, $P = 0.0944$). There were no significant differences in the pN+ rates between the pT1 stage patients in the two groups or between the pT2 stage patients in the two groups. However, the pN+ rate of the pT3 patients in the open surgery group was significantly higher than the pN+ rate of the patients in the MIE group (16.3% *vs* 11.4%, $P = 0.031$, $\chi^2 = 4.626$). In addition, the overall pN+ rate of the patients in the open surgery group was higher than that in the MIE group (14.8% *vs* 9.5%, $P < 0.001$, $\chi^2 = 49.222$).

Lymph node dissection results in different anatomical regions (the upper mediastinum, left and right para-RLNs, carina and hilus, lower mediastinum, peritoneum, *etc.*) are summarized in Table 4. Comparison of the LNS rates of the two groups revealed that the LNS rate at the left para-RLN site in the patients in the open surgery group was significantly higher than that in the patients of the MIE group (80.2% *vs* 43.9%, $P < 0.001$, $\chi^2 = 56.345$); there were no significant differences in the LNS rates at other sites between the two groups. Table 5 lists the results of the LNM rates of the two groups. The LNM rates in the upper mediastinal region (including the left and right para-RLNs and upper para-esophagus), lesser gastric curvature and middle and lower para-esophagus of the patients in the open surgery group were significantly higher than those in the MIE group. However, the results of stages pTis, T1 and T2 patients indicated that only the LNM rate at the left para-RLN site in patients in the open surgery group was significantly higher than the rate in the MIE group (10.1% *vs* 3.6%, $P = 0.045$); there were no significant differences in the LNM rates at other sites.

DISCUSSION

Esophageal carcinomas rank 7th on the list of fatal tumors and 4th on the list of fatal tumors among male patients. Esophageal squamous cell carcinoma, one type of esophageal carcinoma, is prevalent among Asian populations and has an incidence rate of more than 90%. Different from the conservative approaches that are often adopted in Western countries for treating esophageal carcinomas, radical surgical resection combined with systematic lymph node dissection has always been used as a significant approach for treating esophageal carcinomas in Asian countries, for example in Japan. Although neoadjuvant therapy has been increasingly accepted, surgeries remain the most valuable approach for treating esophageal carcinomas. Lymph node metastasis along the long axis of the esophagus can get to the neck in the upward direction and to the level of the celiac trunk in the downward direction. Given the previous studies, radical tumor

Table 5 Comparison of lymph node metastatic rates according to pathological T stage

		pTis, T1-4		*P* value	χ^2	pTis, T1-2		*P* value	χ^2
		Open (*n* = 324)	MIE (*n* = 123)			Open (*n* = 69)	MIE (*n* = 112)		
Upper mediastinum	R-RLN	71	10	0.001	11.416	6	9	0.876	0.024
	L-RLN	30	4	0.032	4.578	7	4	0.045[2]	
	Upper para-esophagus	40	2	0.001	12.034	1	2	0.999[2]	
Subcarinal and parabronchial	Subcarinal	36	11	0.505	0.445	5	8	0.979	0.001
	Left and right parabronchial	30	11	0.918	0.011	5	9	0.847	0.037
Mid and low para-esophagus and diaphragm	Mid and lower para-esophagus	59	10	0.008	6.939	6	9	0.876	0.024
	Diaphragm	3	1	0.910	0.013	1	1	0.999[1]	
Intraperitoneal	Para-cardial	42	13	0.426	0.634	8	12	0.854	0.034
	Lesser gastric curvature	15	0			3	0	0.042[1]	
	Left gastric artery	51	13	0.163	1.944	8	13	0.998	0.001

[1]The Fisher's exact test; [2]The χ^2 test. LNM rate = number of patients with positive lymph nodes/number of total patients. LNM: Lymph node metastatic; RLN: Recurrent laryngeal nerve; MIE: Minimally invasive esophagectomy.

resection and lymph node dissection (as extensive as possible) may be used to improve the prognosis of a patient with a low tumor load, particularly with a number of metastatic lymph nodes within N2.

The MIE technique has become increasingly popular, and this has been particularly remarkable in China[14]. Currently, there are no universally accepted criteria that determine which patients can receive MIE treatment. Whether MIE can be performed often depends on the experience of the surgeon. Many studies have focused on investigating whether MIE has the same safety level and capabilities for controlling tumors and improving the long-term prognosis and quality of life of patients as open surgery[13,14,16,17,19-23]. An important European randomized controlled trial demonstrated that MIE can better protect the pulmonary function of patients and can improve patients' long-term quality of life[24]. However, the exact oncological surgical results of MIE were not described in detail; the results only showed that the LNS rate of the patients in the MIE group was higher than that of the patients in the open surgery group. However, thorough lymph node dissection is particularly important in treating esophageal squamous cell carcinoma. MIE is affected by such aspects as the position of the patient, the assistant exposing technique and the learning curve; however, many aspects merit more study. To address this issue, a detailed retrospective analysis was conducted in this study. Our research indicates that there were no significant differences in the number of lymph nodes resected and the pathologic LNM rates between the MIE group and the open surgery group in different T stages, demonstrating that MIE can achieve the comparable staging and prediction results with open surgery in terms of lymph node dissection. However, the pN+ rate of the stage T3 patients in the MIE group was significantly lower than that in the open surgery group (11.4% *vs* 16.3%, $P = 0.031$, $\chi^2 = 4.626$); there were no significant differences in the pN+ rates for stages T1 and T2 esophageal squamous cell carcinoma between the two groups. Such a phenomenon has several causes. First, the preoperative patient screening was biased – stages T1 and T2 patients with even lower N stages were more likely to be selected to undergo MIE treatment; thus, MIE could achieve the comparable lymph node dissection results with open surgery for stages T1 and T2 esophageal squamous cell carcinoma. Second, MIE did not reach the same *en bloc* lymph node dissection level as open surgery, therefore, the obtained numbers of positive lymph nodes of patients with advanced stages were relatively low, and hence, it is necessary to provide such high-risk patients with more positive preoperative induction and postoperative adjuvant treatments. Third, the lymph node dissection results of the stage T3 patients in the MIE group were inferior to the results of the open surgery group; however, because only 11 patients were included in the MIE group in this study, it is necessary to increase the sample size to more thoroughly evaluate the difference between MIE and open surgery in terms of the lymph node dissection results of stage T3 patients.

A comparison of lymph node dissection results at specific anatomical sites was also done. The outcome reflected the limitation of the surgical technique used in the patients in the MIE group in this study. The LNS rate at the left para-RLN site (the most difficult site for exposing lymph nodes) of the patients in the MIE group was only 43.9%, whereas this value in the patients in the open surgery group was as high as 80.2%, indicating a significant difference between the two groups. This study further analyzed the LNM rates at different anatomical regions. In terms of the overall LNM rate (including Tis and T1-4), the LNM rates in the upper mediastinal region of the patients in the MIE group were lower than the rates of the patients in the open surgery group. The patients in the MIE group were primarily stages T1 and T2 patients. To

eliminate the effect generated by the biased inclusion process, we analyzed the stages T1 and T2 patients in separate groups. It has been proved that the LNM rate at the left para-RLN site in the MIE group was significantly lower than that in the open surgery group, but there were no significant differences in the other regions. Hence, lymph node dissection at the left para-RLN site remains a key technique of MIE that requires improvement. Currently, the following techniques were adopted to rectify the aforementioned shortcomings: (1) a single-lumen endotracheal tube-aided blocker is used to reduce the tracheal tension to allow easier exposure of the left space of the trachea during surgery; (2) the left RLN is moved upward through the assistant traction of the esophagus to allow easier lymph node dissection anterior to the nerves; and (3) the auxiliary artificial pneumothorax is used to enlarge the mediastinal space. After using these techniques, the LNS rate at the left para-RLN site recently increased to above 90%, which is similar to the results of the previously mentioned study.

Limitations of the study

This study was a single-center retrospective study; during the medical case accumulation process, the initial learning curve may have affected the results. In addition, the sample size is not sufficiently large; in particular, there are few stage T3 patients (only 11 patients) in the MIE group. However, considering the fact that the current MIE technique remains applicable to stages T1 and T2 patients, this study nevertheless reflects the basic surgical oncological results of the current MIE technique. Furthermore, this study did not statistically analyze the patients' survival rates, therefore, the best evidence for the lymph node dissection effect in MIE is lacking. In the future, a multicenter prospective randomized controlled study with a large sample size is expected to be conducted to verify the lymph node dissection effect of MIE.

In summary, we conducted a retrospective comparative study of MIE and conventional open surgery for treating esophageal squamous cell carcinoma. The initial results indicate that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. However, the lymph node dissection at the left para-RLN site remains a major technical challenge for MIE.

COMMENTS

Background

Previous studies have not reported in detail whether minimally invasive esophagectomy (MIE) can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study attempts to retrospectively review the data from patients with esophageal squamous cell carcinoma who were treated at Shanghai Chest Hospital and compare the lymph node dissection results of MIE and open surgery.

Research frontiers

MIE has been into a rapid development period. Its safety and efficacy to improve patients' life quality have been demonstrated in previous reports. The main aim of this study was compare lymph node dissection results of minimally invasive esophagectomy and open surgery for esophageal squamous cell carcinoma.

Innovations and breakthroughs

The authors conducted a retrospective comparative study of MIE and conventional open surgery for treating esophageal squamous cell carcinoma. This study is a large cohort. The initial results indicate that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma.

Applications

This study proved that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. This is very important for the development of MIE.

Terminology

MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. However, the lymph node dissection at the left para-RLN site remains a major technical challenge for MIE.

Peer-review

Previous studies have not reported in detail whether MIE can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study found that for stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable with that of open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

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ORIGINAL ARTICLE

Observational Study

Chinese physicians' perceptions of fecal microbiota transplantation

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Informed consent statement: The need for informed consent in this study was waived by the Chinese PLA General Hospital Institutional Review Board because the study was a survey of physicians' perceptions using questionnaires; there was no risk to the participants, and no individual physician information was revealed under the condition of anonymity.

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Abstract

AIM: To explore Chinese physicians' perceptions towards fecal microbiota transplantation (FMT) and to provide information and an assessment of FMT development in China.

METHODS: A self-administered questionnaire was developed according to the FMT practice guidelines and was distributed to physicians in hospitals *via* Internet Research Electronic Data Capture (REDcap) software and electronic mails to assess their attitudes toward and knowledge of FMT. The questionnaire included a brief introduction of FMT that was followed by 20 questions. The participants were required to respond voluntarily, under the condition of anonymity and without compensation. Except for the fill-in-the-blank questions, all of the other questions were required in the REDcap data collection systems, and the emailed questionnaires were completed based on eligibility.

RESULTS: Up to December 9, 2014, 844 eligible questionnaires were received out of the 980 distributed questionnaires, with a response rate of 86.1%. Among the participants, 87.3% were from tertiary hospitals, and there were 647 (76.7%) gastroenterologists and 197 (23.3%) physicians in other departments (non-gastroenterologists). Gastroenterologists' awareness of FMT prior to the survey was much higher than non-gastroenterologists' (54.3 *vs* 16.5%, $P < 0.001$); however, acceptance of FMT was not statistically different (92.4 *vs* 87.1%, $P = 0.1603$). Major concerns of FMT included the following: acceptability to patients (79.2%), absence of guidelines (56.9%), and administration and ethics (46.5%). On the basis of understanding, the FMT indications preferred by physicians were recurrent *Clostridium difficile* infection (86.7%), inflammatory bowel disease combined with *Clostridium difficile* infection (78.6%), refractory ulcerative colitis (70.9%), ulcerative colitis (65.4%), Crohn's disease (59.4%), chronic constipation (43.7%), irritable bowel syndrome (39.1%), obesity (28.1%) and type 2 diabetes (23.9%). For donor selection, the majority of physicians preferred individuals with a similar gut flora environment to the recipients. 76.6% of physicians chose lower gastrointestinal tract as the administration approach. 69.2% of physicians considered FMT a safe treatment.

CONCLUSION: Chinese physicians have awareness and a high acceptance of FMT, especially gastroenterologists, which provides the grounds and conditions for the development of this novel treatment in China. Physicians' greatest concerns were patient acceptability and absence of guidelines.

Key words: Fecal microbiota transplantation; Chinese physicians; Gastroenterologists; Perception; Survey

Core tip: Perceptions and attitudes toward fecal microbiota transplantation (FMT) by physicians and patients play an important role in determining its acceptability. We investigated Chinese physicians' acceptance levels of FMT, their concerns about FMT, and their perspectives of FMT techniques. The few data about the perceptions of physicians toward FMT are all from Western countries; this is the first study of physicians' perceptions of FMT in an Asian country. Additionally, our study was representative with a large respondent number (844) and a large coverage area of China (22 out of 34 provinces); thus it can provide preliminary information for the development of FMT in China.

Ren RR, Sun G, Yang YS, Peng LH, Wang SF, Shi XH, Zhao JQ, Ban YL, Pan F, Wang XH, Lu W, Ren JL, Song Y, Wang JB, Lu QM, Bai WY, Wu XP, Wang ZK, Zhang XM, Chen Y. Chinese physicians' perceptions of fecal microbiota transplantation. *World J Gastroenterol* 2016; 22(19): 4757-4765 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4757.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4757

INTRODUCTION

Fecal microbiota transplantation (FMT) refers to the instillation of fecal suspension from a healthy person into the gastrointestinal (GI) tract of a patient to cure a certain disease by restoring the construction of intestinal flora. FMT is by no means a new concept. Fecal medicine was recorded 3000 years ago in the "Collection of 52 Prescriptions"[1,2], which was

described as the oldest traditional prescription book in China. Later, during the Eastern Han dynasty in the 2nd century A.D. in China, Zhang Zhongjing described the use of a human fecal suspension by mouth to treat food poisoning in "Jin Gui Yao Lüe" (Synopsis of Golden Chamber)[3]. To our knowledge, this was the first literary record of using human fecal liquid to treat diseases. Then, Ge Hong, Sun Simiao, Li Shizhen, *etc*., described a series of prescriptions using fecal suspensions or dry feces to treat abdominal diseases in their famed traditional Chinese medicine books[4-6]. The first description of FMT in Western countries was in 1958, when four patients with pseudomembranous colitis were cured using fecal enemas[7]. However, FMT did not gain public attention until recently and only after several studies reported that fecal suspension had astounding efficacy for recurrent *Clostridium difficile* infection (RCDI)[8,9]. Since then, FMT, an ancient medicine, has become a hot topic and interest has surged in recent years. Currently, more than 40 reports are available about treating RCDI with FMT, with similarly high reported efficacy. FMT was recommended by the American CDI guidelines in 2013 if there was a third recurrence after a pulsed vancomycin regimen[10]. As FMT may restore the dysbiosis of gut microbiota, it is also proposed in treating other GI diseases and non-GI diseases, which have been considered to be linked to the composition of gut microbiome, with associations described between intestinal flora, immune system, and active metabolites[11], such as in inflammatory bowel diseases (IBD), chronic constipation, type 2 diabetes mellitus, metabolic syndrome, and symptoms of Parkinson's disease[12-15]. However, using fecal suspension to treat diseases other than CDI is still speculative, even for IBD.

The perceptions and attitudes toward FMT held by physicians and patients play an important role in determining its acceptability. A few reports discuss patients' attitudes towards the acceptance of FMT[16,17]. Despite the unappealing nature of stool, 46% of patients with ulcerative colitis were willing to accept FMT as a treatment, and if it was recommended by their physicians, up to 94% of patients with recurrent CDI are ready to accept FMT[16]. One study reported that 97% of patients with RCDI who had undergone FMT once were willing to accept the treatment again, and an equal number of patients (53%) chose FMT as the treatment of first choice[17]. Nevertheless, minimal data exist regarding physicians' perception of this technique[18,19]. The acceptance of FMT in Asian countries remains unknown. Therefore, this survey was designed to evaluate Chinese physicians' perceptions, and especially their acceptance of FMT. We will compare the different views about FMT technology, to provide information and an assessment of the future development of FMT.

MATERIALS AND METHODS

The study was conducted from June 2014 to September 2014. A self-administered questionnaire was developed according to the practice guidelines and other literature on FMT[9,20] and was distributed to physicians *via* Internet Research Electronic Data Capture (REDcap) software[21] and emails. The participants were a convenience sample of physicians working in hospitals and practicing gastroenterology; other specialists, such as those physicians working in endocrinology, pediatrics, general surgery, and neurosurgery, were also included in the study. These physicians were recruited through gastroenterology associations and their subspecialty groups in different provinces.

The questionnaire included a brief introduction of FMT, followed by 20 questions, which were comprised of three sections: demographic information of the interviewees, their attitudes toward FMT, and FMT technique-associated questions (see Supplementary material). The participants were required to respond voluntarily and under the condition of anonymity and without compensation. Except for the fill-in-the-blank questions, all other questions were required in the REDcap system. The email questionnaires were completed according to eligibility.

Statistical analysis

Study data were collected and managed using REDCap tools hosted at the General Hospital of the Chinese PLA. REDCap was used to manage study data and perform the descriptive analysis. The data were also analyzed using Microsoft Excel and JMP 10.0.0 software. Continuous data are presented as the mean ± SD and analyzed by the ANOVA test. Categorical data are presented as percentages and were analyzed by the χ^2 test. Univariate analysis and multivariate logistic regression analysis were employed to identify the impact of various factors on physicians' preferences for FMT. Odds ratios (ORs) and 95% confidence intervals were calculated and a *P*-value less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the respondents

Up until December 9, 2014, 844 eligible questionnaires were received out of the 980 distributed questionnaires, with a response rate of 86.1%. Respondents were selected from six different regions of China, and the study included respondents from most areas of China (22 out of 34 provinces). There were 449 (53.2%) females and 395 (46.8%) males with an average age of 36.1 ± 9.2 years (age range: 19-81 years). The majority of respondents were gastroenterologists (76.7%, 647/844), and most of them were associated

Table 1 Characteristics of the survey respondents *n* (%)

Characteristic	*n* = 844
Age, mean ± SD (range)	36.1 ± 9.2 (19-81)
Gender, male	395 (46.8)
Region	
North West	211 (32.7)
North	152 (23.6)
East	100 (15.5)
North East	83 (12.9)
South West	68 (10.5)
South Central	31 (4.8)
Missing data	199
Education	
College degree	295 (35.0)
Postgraduate degree	341 (40.4)
Doctoral degree	188 (22.3)
Post-doctoral degree	19 (2.3)
Professional title	
Resident physician	291 (34.6)
Attending physician	210 (24.9)
Associated chief physician	198 (23.5)
Chief physician	143 (17.0)
Level of hospital	
Community hospital	15 (1.8)
Secondary hospital	88 (10.4)
Tertiary hospital	737 (87.3)
Profession	
Gastroenterologist	647 (76.7)
General surgeons	49 (5.8)
Endocrinologist	28 (3.3)
Others	120 (14.2)
Working time in gastroenterology (yr)	
< 2	295 (35.3)
3-5	111 (13.3)
6-10	106 (12.7)
10-20	188 (22.5)
> 20	135 (16.2)

Regions were classified according to the common geographical zones in China.

with tertiary hospitals (87.3%, 737/844). More than half of the physicians were qualified postgraduates or above, and almost half of the physicians held senior professional titles and had worked in gastroenterology for more than 6 years (Table 1).

Attitudes toward FMT

Among the physicians, 607 (71.9%) had heard of FMT prior to the survey, but only 45.6% (385/844) had an awareness or understanding of FMT (*i.e.*, "had knowledge of FMT principles and technology"). The primary advertising approach included conferences (60.3%, mainly domestic conferences), professional journals (54.8%) and communication with colleagues (42.1%). Gastroenterologists' prior awareness of FMT was much higher than non-gastroenterologists' (54.3 *vs* 16.5%, $P < 0.001$), they were more interested in FMT training (92.4 *vs* 81.4%, $P < 0.001$), and they showed a more positive attitude to the feasibility (74.5 *vs* 59.3%, $P < 0.001$) and potential (71.5 *vs* 53.9%, $P < 0.001$) of FMT. However, the acceptance of FMT was similarly high among gastroenterologists and non-gastroenterologists (92.4 *vs* 87.1%, $P = 0.1603$).

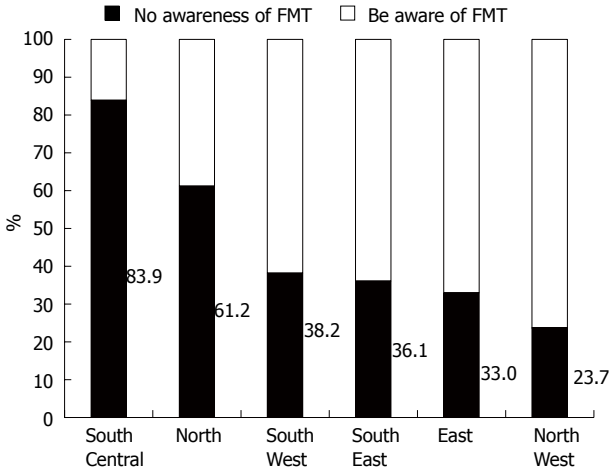

Figure 1 Physicians' awareness of fecal microbiota transplantation in different regions. FMT: Fecal microbiota transplantation.

Table 2 Multivariate analysis of the factors associated with fecal microbiota transplantation awareness

Variable	*P*-vaule	OR	95%CI
Age	0.160	1.391	0.878-2.203
Region	< 0.001[1]		
Region (North)	< 0.001[1]	0.288	0.163-0.508
Region (North East)	0.011[1]	0.385	0.185-0.800
Region (South Central)	0.058	3.005	0.963-9.376
Region (East)	0.089	0.555	0.282-1.093
Region (South West)	0.051	0.467	0.217-1.003
Educational background	< 0.001[1]	1.958	1.402-2.733
Professional title	0.010[1]	1.676	1.133-2.480
Level of hospital	0.069	1.759	0.958-3.228
Department	0.001[1]		
Department (gastroenterology)	< 0.001[1]	4.182	1.895-9.229
Department (general surgery)	0.104	2.429	0.834-7.073
Department (endocrinology)	0.903	0.919	0.235-3.584
Working time on gastroenterology	0.476	1.090	0.860-1.383

[1]$P < 0.05$. Age was divided into 4 groups: ⩽ 30 years, 30-40 years (including 40 years), 40-50 years (including 50 years), > 50 years.

In the univariate analysis, significant factors ($P < 0.05$) that influenced physicians' awareness of FMT included age, educational background, professional designation, level of hospital, region, department and working experience in gastroenterology. The multivariate logistic regression analysis confirmed that physicians with a higher education (OR = 1.958, 95%CI: 1.402-2.733, $P < 0.001$) and a higher professional title (OR = 1.676, 95%CI: 1.133-2.480, $P = 0.010$) were more likely to understand FMT, and gastroenterologists were more likely to comprehend FMT than physicians in other departments (OR = 4.182, 95%CI: 1.895-9.229, $P < 0.001$). Physicians in different regions had significantly different understandings of FMT ($P < 0.001$) (Figure 1 and Table 2).

The acceptance rate of the 385 physicians who had knowledge of FMT was 91.9%. Of these physicians, 59.5% (229/385) were willing to choose FMT ahead of

Table 3 Multivariate analysis of factors associated with fecal microbiota transplantation preference

Variable	*P*-vaule	OR	95%CI
Age	0.672	1.155	0.593-2.250
Region	0.007[1]		
Region (North)	0.838	1.101	0.437-2.773
Region (North East)	0.095	0.412	0.146-1.167
Region (South Central)	0.748	1.419	0.168-11.975
Region (East)	0.096	0.456	0.180-1.151
Region (South West)	0.006[1]	0.264	0.102-0.683
Educational background	0.945	1.016	0.657-1.570
Professional title	0.757	0.913	0.513-1.624
Level of hospital	0.041[1]	0.359	0.134-0.961
Department	0.910		
Department (gastroenterology)	0.510	1.291	0.604-2.760
Department (general surgery)	0.778	1.177	0.379-3.657
Department (endocrinology)	0.598	1.463	0.356-6.020
Working time on gastroenterology	0.683	0.933	0.670-1.299
Understanding of FMT	0.002[1]	3.265	1.555-6.855

[1]$P < 0.05$. FMT: Fecal microbiota transplantation.

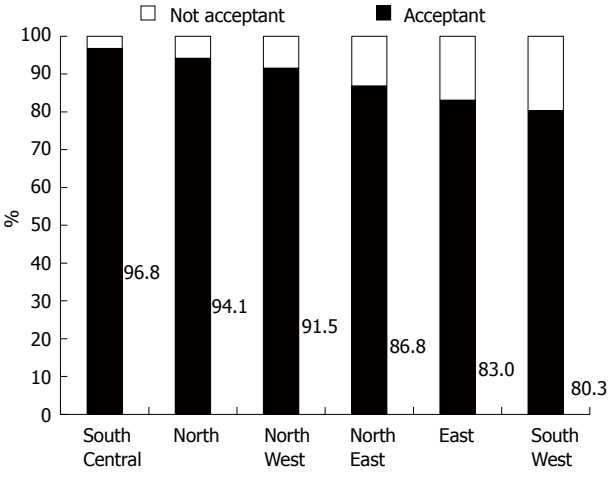

Figure 2 Physicians' acceptance of fecal microbiota transplantation in different regions.

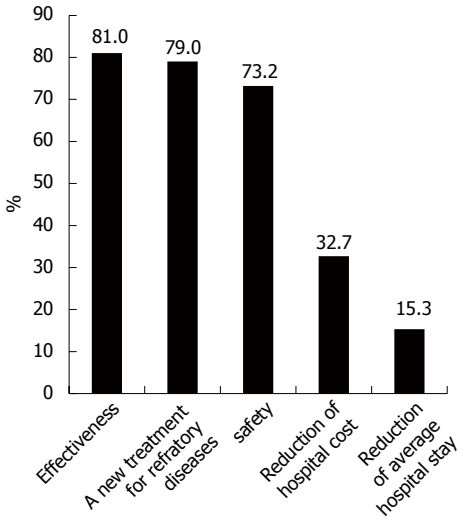

Figure 3 Physicians' concerns about choosing fecal microbiota transplantation as a treatment.

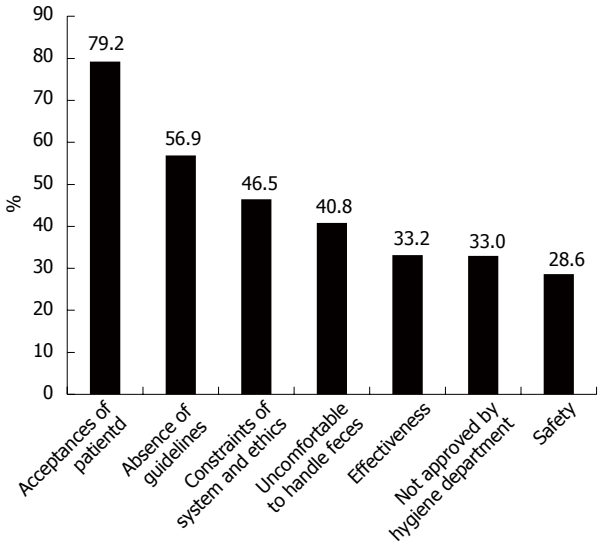

Figure 4 Barriers against clinical applications of fecal microbiota transplantation.

other treatments, and 80.8% (126/156) of physicians who declined FMT as the first treatment selected FMT as an alternative treatment.

A univariate analysis revealed that only geographic region can significantly influence physicians' acceptance ($P < 0.05$). Factoring the significant variables in a univariate analysis and those affecting the acceptance of FMT, such as age, educational background, professional title, hospital level, department, working time in gastroenterology and understandings of FMT into the multivariate logistic regression analysis, it was unexpectedly discovered that understandings of FMT, hospital level and region were all statistically significant (Table 3). Physicians with a greater comprehension of FMT were more likely to accept FMT (OR = 3.265, 95%CI: 1.555-6.855, $P = 0.002$). The higher the level of hospital physicians worked at, the less likely they were to accept FMT (OR = 0.359, 95%CI: 0.134-0.961, $P = 0.041$). The lowest acceptance of FMT (80.3%) was observed among physicians working in Southwest China, followed by those in the East (83.0%). Acceptance rate of physicians was above 85% in all other regions ($P = 0.007$) (Figure 2).

The three most frequent reasons for choosing FMT were as follows: efficacy (81.0%), a new treatment option for refractory diseases (79.0%) and safety (73.2%) (Figure 3). Primary barriers for the clinical application of FMT included patients' acceptance (79.2%), absence of guidelines (56.9%) and systemic and ethical constraints (46.5%) (Figure 4).

Perspectives on FMT technique-associated questions

Although we provided a brief description of FMT in the questionnaire, there were some questions about the details of FMT procedures. Therefore, it might not have been reasonable to ask physicians who had

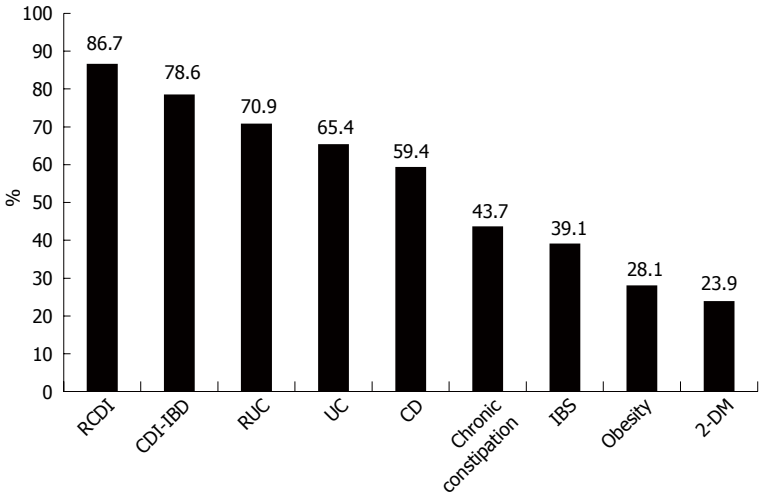

Figure 5 Fecal microbiota transplantation indications. RCDI: Refractory *Clostridium difficile* infection; UC: Ulcerative colitis; CD: Crohn's Disease; RUC: Refractory ulcerative colitis; CDI-IBD: Inflammatory bowel disease with *Clostridium difficile* infection; IBS: Irritable bowel syndrome; 2-DM: Type 2 diabetes mellitus.

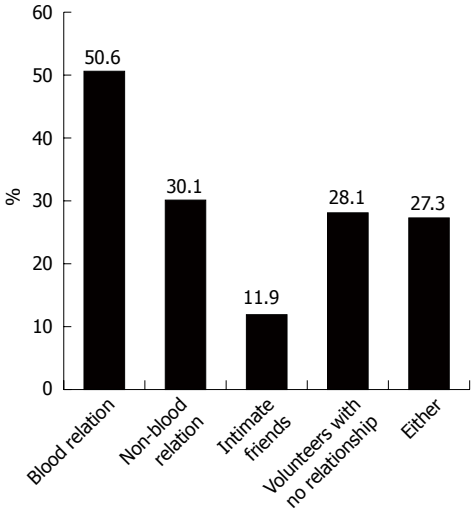

Figure 6 Selection of donors.

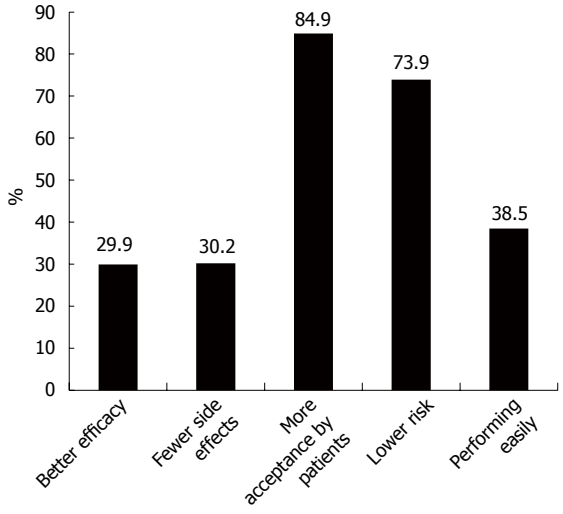

Figure 7 Reasons for lower gastrointestinal tract selection.

no awareness of FMT to analyze FMT technology. To disclose the physicians' true perceptions of FMT procedures, we excluded physicians who had no knowledge of FMT in the following analysis.

Indications: The majority of physicians (86.7%) selected recurrent RCDI, followed by other diseases such as inflammatory bowel disease with CDI, refractory ulcerative colitis, ulcerative colitis, and Crohn's disease (Figure 5).

Donor selection: Most participants preferred someone who had a similar microbiota environment to the recipient, including blood relatives (50.6%), non-blood relatives (30.1%) and intimate friends (11.9%) (Figure 6). Only 28.1% of participants selected volunteers with no relationship, and 27.3% held the view that either of the above was an option contingent on the health of the donor; 29.7% of physicians were more inclined to prefer children donors, 35.4% selected adults, and 34.9% preferred both.

Selection of the administration route: Overall, 76.6% of the respondents preferred the lower GI tract as the route of administration, with the primary reasons being that patients would more likely accept this route (84.9%) and that it had lower risk (73.9%) (Figure 7). Only 13.9% of the physicians selected the upper GI tract, and others (7.1%) thought that both approaches were acceptable. With regard to the site for performing FMT, nearly half of the physicians (44.9%) preferred the Endoscopy Center, and only 21.3% preferred wards.

Risk of FMT: Most participants (69.2%) held the opinion that FMT has a low risk with transient abdominal symptoms such as diarrhea, and 14.4% of physicians thought that FMT had a high and even lethal risk (Figure 8). The vast majority of these respondents thought that disease history (93.5%), stool and blood

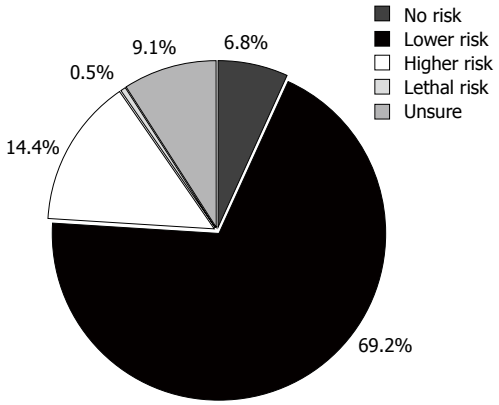

Figure 8 Physicians' perceptions of fecal microbiota transplantation risk.

examinations (92.7% and 90.9%) were all necessary considerations before qualifying as a donor.

DISCUSSION

The evolution of FMT has been rapid and certain. Physicians' and patients' awareness and perceptions of FMT are critical factors in determining FMT popularity. Our study was the first of its kind to investigate physicians' perceptions of FMT in an Asian country. Although there were only 844 physicians in our survey, which is a small proportion of the entire Chinese physician population (more than 200 millions), this survey covered most areas of China (22 out of 34 provinces) and was representative to some extent. This investigation will, undoubtedly provide information of FMT development in China and hopefully in other Asian countries.

Our investigation found high levels of FMT perception, as the vast majority of physicians had heard of FMT prior to this survey and nearly half of understood it well. Among these physicians, gastroenterologists had a better awareness and a more favorable attitude toward the development of this novel method than non-gastroenterologists, which was expected. All the physicians had a very high level of acceptance of FMT and a high interest in FMT training. In our study, geographical region was an important factor affecting physician perceptions of FMT. The significant geographical differences may be related to the differences in the economy, the frequency of information communication, and the uneven distribution of medical resources. Northwest China is less developed than other areas, and it has fewer medical resources and a slower spread of new knowledge and technology.

Chinese physicians' responses regarding the acceptance of FMT were somewhat astonishing. The high acceptance rate may be related to knowledge of Chinese traditional medicine in which FMT had originated. In this study, for the first time, the attitude of physicians toward FMT as an acceptable treatment was directly assessed. The results revealed that although human beings have a natural aversion to fecal material, the overwhelming majority of physicians were willing to accept FMT as a treatment method. A multivariate analysis revealed that increased awareness of FMT among physicians will enhance the likelihood of its acceptance. Conversely, the technique was less likely to be accepted by physicians working in higher level hospitals. It is possible that the higher level hospitals were more rigorous and cautious in the administration and implementation of new technologies.

Physicians accepted FMT as a treatment modality mainly on account of its effectiveness and safety, and they considered it an optional therapy for refractory diseases. This result was consistent with clinical studies, which reported that FMT was effective and safe in some diseases that were refractory to standard therapy or had shown frequent recurrence. Currently, there are few data about physicians' attitudes about FMT. In one investigation, 65% (83/135) of physicians had neither offered nor referred a patient for FMT, with the most common reasons being lack of appropriate clinical indication (33%), patients' acceptance or otherwise (24%) and institutional or logistical barriers (23%)[18]. In our investigation, the primary concern of Chinese physicians was the patients' acceptance, followed by the absence of guidelines and system and ethical constraints, similar to physicians overseas. This result suggests that the standardization and extension of FMT are imperative.

In addition to recurrent CDI, physicians showed interest in the use of FMT for many other diseases. Several studies have confirmed the astounding efficacy of FMT in the treatment of RCDI. Studies on IBD, IBS, and chronic constipation treatment with FMT followed suit. Further, FMT has a potential therapeutic value in non-GI diseases associated with gut flora, such as obesity, metabolic syndrome and chronic fatigue syndrome, which is based on preliminary case reports or animal experiments[22]. The results of our survey on the selection of potential FMT indications were consistent with these studies, although additional rigorous studies are needed to determine the efficacy of FMT for these diseases.

Until now, there is no evidence that stool material from related donors was better than that from unrelated donors. One argument for the use of related donors is that they are presumed to have shared gut flora exposures; however, they are also more likely to test for infectious disease markers than unrelated volunteer donors[23]. A long-term multicenter follow-up study showed that CDI cure rates were not influenced by the donor-recipient relationship[24], which provided grounds for the commercialization of frozen fecal microbiota and the development of FMT. Nevertheless, donors with different genders, ages, diets or lifestyles may have varying effects on the efficacy of FMT, which should be confirmed by further studies.

FMT is often delivered *via* the lower GI route, including *via* colonoscopy and retention enema, and/or

via the upper GI route, such as by gastroduodenoscopy, a nasoenteric tube and oral pills. To date, the optimal approach is still unclear, and approximately 75% of cases with RCDI are administered *via* the lower GI tract, and 25% *via* the upper GI tract[25]. A systematic review reported that FMT administered by colonoscopy had a higher cure rate (91%) than other routes for RCDI[26]. However, a recent RCT demonstrated a remarkable cure rate using the nasoenteric tube compared to colonoscopy[27]. Our results revealed that the vast majority of physicians (76.6%) preferred the lower GI tract with the primary argument that it may be easily accepted by patients psychologically. Another reason for the selection of the lower GI tract was that it may theoretically have a lower risk with easier colonization *in situ*, compared with the upper routes through which the small intestinal bacterial may overgrow and whether the stool suspension can reach the entire colon is unknown.

In terms of risk, although the majority of physicians in our survey considered FMT safe, an overwhelming majority of physicians suggested rigorous screening of donors to lessen the risk, including collection of a detailed disease history, and stool and blood examinations. Transient abdominal discomfort such as bloating, diarrhea and abdominal cramps have been observed after FMT and often disappeared within two days after treatment[24,25]. However, limited long-term safety data exist. Reports of concurrent infections after FMT treatment exist. Elizabeth *et al*[28] described a patient with refractory ulcerative colitis who acquired *cytomegalovirus* infection after FMT, which revealed a potential risk of FMT, although it was not confirmed whether the virus was directly from the donor. Cases involving *norovirus*[29], *S.typhi*, and *Blastocystishominis* infections have been reported. In our research center, despite rigorous screening, a patient developed an infection with two opportunistic pathogens, *Proteusmirabilis* and *Candidaalbicans* following FMT[30]. We still have limited knowledge of the impact of FMT on the intestinal flora and subsequent secondary infections after it. Therefore, the clinical utility of FMT must follow a strict and standardized protocol. It is recommended that patients undergo FMT in a hospital instead of at home. A standard protocol to screen donors is imperative.

In summary, this study is the largest survey of physicians' perceptions of FMT and it is the first time that physicians' perception of the indications, donors, and other technology associated with FMT have been evaluated in an Asian country. The keen interest, high acceptance and good understanding of FMT provide the grounds and conditions for the development of this novel treatment in China. The need to establish a standard procedure and protocol cannot be overstated.

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COMMENTS

Background

While there has been growing interest in fecal microbiota transplantation (FMT), it is still in early phases worldwide. Physicians' and patients' perceptions and attitudes toward FMT play an important role in determining its acceptability. This article explores Chinese physicians' perceptions towards FMT to provide information and an assessment of FMT development in China.

Research frontiers

There are a few reports discussing patients' attitudes towards the acceptance of FMT. Nevertheless, few studies exist regarding physicians' perceptions of this technique; all of these studies were conducted in Western countries. The acceptance of FMT in Asian countries remains unknown.

Innovations and breakthroughs

This is the first study to acquire physicians' perceptions of FMT in an Asian country. This study was representative with a large respondent number (844 eligible questionnaires were collected) and a vast coverage area of China (22 out of 34 provinces); thus, it can provide preliminary information for the FMT development in China. Additionally, the authors reviewed the literature and traced the history of human fecal medicine back 3000 years to the "Collection of 52 Prescriptions", and they found that the first use of human fecal suspension by mouth occurred 2nd century.

Applications

The keen interest and high acceptance of FMT provide the grounds and conditions for the development of this novel treatment in China. Nevertheless, guidelines and strict protocols are necessary to implement this technique.

Terminology

FMT refers to the instillation of fecal suspension from a healthy person into the gastrointestinal tract of a patient to cure a certain disease by restoring the construction of the intestinal flora.

Peer-review

The strongest point of this manuscript is being the first of its kind in China and other Asian countries. The idea is original and interesting, exploring the knowledge and attitudes regarding fecal microbiota transplantation (a very hot topic in gastroenterology nowadays) among Chinese physicians. The results give some ideas regarding how FMT might impact on clinical practice in the foreseeable future and provide important findings.

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META-ANALYSIS

Hybrid, sequential and concomitant therapies for *Helicobacter pylori* eradication: A systematic review and meta-analysis

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Abstract

AIM: To compare hybrid therapy (HT) with traditional sequential therapy (ST) and concomitant therapy (CT) for *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: We performed an electronic search of PubMed, Embase, and the CENTRAL database. Randomized controlled trials (RCTs) of HT were included in the meta-analysis. The primary outcome was the eradication rate of *H. pylori*. The secondary outcomes included the compliance rate and adverse event rate. Effect estimates were pooled using the random-effects model.

RESULTS: Twelve studies were included. Pooled results showed no significant differences in eradication rate between HT and ST in per-protocol (PP) analysis (RR = 1.03, 95%CI: 0.94-1.12, P = 0.59) or in intention-to-treat (ITT) analysis (RR = 1.00, 95%CI: 0.89-1.12, P = 0.94). HT and ST showed similarly high compliance rate (96% *vs* 98%, P = 0.55) and acceptable adverse event rate (30.3% *vs* 28.2%, P = 0.63). No significant results were seen in the eradication rate between HT and CT in PP analysis (RR = 1.01, 95%CI: 0.96-1.05, P = 0.76) or in ITT analysis (RR = 0.99, 95%CI: 0.95-1.03, P = 0.47). HT displayed a slightly higher compliance rate than CT (95.8% *vs* 93.2%, P < 0.05). The adverse event rates of HT and CT were similar (39.5% *vs* 44.2%, P = 0.24).

CONCLUSION: Compared with ST or CT, HT yields a similar eradication rate, high compliance rate, and acceptable safety profiles.

Key words: Hybrid therapy; Sequential therapy; Concomitant therapy; *Helicobacter pylori*; Meta-analysis

Core tip: This meta-analysis of randomized controlled trials compared the novel hybrid therapy with sequential and concomitant therapy in the treatment of *Helicobacter pylori*. The eradication rate, compliance rate and the adverse event rate were investigated as the main outcomes and were compared. Overall, similar results were shown regarding these outcomes by hybrid and sequential therapy, and by hybrid and concomitant therapy. Hybrid therapy could be an effective and safe alternative to sequential or concomitant therapy.

Song ZQ, Zhou LY. Hybrid, sequential and concomitant therapies for *Helicobacter pylori* eradication: A systematic review and meta-analysis. *World J Gastroenterol* 2016; 22(19): 4766-4775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4766.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4766

INTRODUCTION

Approximately 50% of the global population are infected with *Helicobacter pylori* (*H. pylori*). The presence of *H. pylori* in the stomach is directly associated with a series of gastric diseases, including chronic gastritis, peptic ulcer, and gastric cancer[1]. Triple therapy, consisting of one proton pump inhibitor (PPI), amoxicillin, and clarithromycin, has been established as the standard first-line treatment for *H. pylori* eradication since the 1997 Maastricht Conference[2]. However, the eradication rates have decreased to unacceptable levels (less than 80%) in many countries[3]. Growing resistance of *H. pylori* strains to clarithromycin and metronidazole is the major cause of treatment failure[4,5].

Worldwide efforts led to the development of new regimens to improve the eradication rate. Sequential therapy is one of the latest innovations, which was introduced by Zullo *et al*[6] in 2003. It entails the use of a PPI and amoxicillin for the first 5-7 d, followed by 5-7 d of PPI-clarithromycin-metronidazole (or tinidazole)[2,3]. With less clarithromycin resistance[3], the sequential regimen was more effective than standard triple therapy for *H. pylori* eradication[7,8]. However, some researchers argued that the benefit of sequential therapy only resulted from additional antibiotic therapy. Thus, it has been postulated that the four components of sequential therapy could be administered concurrently as concomitant therapy comprising PPI-clarithromycin-amoxicillin-metronidazole over several days[9]. The latest guideline recommends sequential and concomitant therapies as alternative first-line treatment in areas with a high rate of clarithromycin resistance[2].

Hybrid therapy entails administration of amoxicillin and a PPI for 5-7 d, followed by a PPI, amoxicillin, metronidazole, and clarithromycin for 5-7 d[10]. The recent randomized clinical trials (RCTs) of hybrid therapy showed conflicting results. Two studies showed that hybrid therapy outperformed sequential therapy in *H. pylori* eradication[11]. However, similar eradication rates were presented by other studies[12-14]. Furthermore, the duration of sequential or concomitant therapy was inconsistent between the studies. Therefore, we conducted this meta-analysis to evaluate the efficacy of hybrid therapy. We compared the efficacy, compliance, and safety of this new therapy with sequential or concomitant therapy.

MATERIALS AND METHODS

Search strategy

This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) Statement[15]. Two reviewers independently performed systematic literature search of PubMed, Embase, and the Cochrane Central Register of Controlled Trials (CENTRAL) from their inception through October 2015. The search strategy is shown in Table 1. We used the following keywords or MESH Terms: "*helicobacter pylori*" or "*H. pylori*", "hybrid" or "sequential-concomitant". The language was limited to English. We also manually searched the references of eligible studies in case of any omission.

Inclusion criteria

Studies meeting the following inclusion criteria were included in the meta-analysis: (1) comparison of hybrid therapy (proton-pump inhibitors and amoxicillin for 5 to 7 d, followed by proton-pump inhibitors, amoxicillin, clarithromycin, and metronidazole for another 5 to 7 d) with other treatment regimens (sequential therapy, concomitant therapy, or triple therapy) in patients with *H. pylori* infection, or comparing different durations of hybrid therapy; (2) randomized controlled trials (RCTs); (3) *H. pylori* infection was diagnosed with rapid urease test, 13C-urea breath test, histology, or culture; and (4) comparison of the eradication rate, compliance, and/or adverse events. The *H. pylori* eradication was assessed by UBT at least 4 wk after treatment.

Data extraction and quality assessment

Two authors independently abstracted the data using a standardized form. The following data were collected from each study: author and year, study design, country, sample size, gender, comparison arms, diagnosis of *H. pylori,* eradication of *H. pylori*, and follow-up. The quality of the included study was evaluated by the Jadad scale, which assessed the study quality by randomization (2 points), blinding (2

Table 1 Characteristics of included studies involving hybrid therapy

Ref.	Region	Design	No. of patients	Age, mean or range, yr	Men, %	Hybrid group	Control group	Confirmation of infection	Confirmation of eradication	Follow-up	Jadad score
Hsu *et al*[10] (2011)	Taiwan	Single-arm	117	54	50	E 40 mg + A 1g, bid, 7d; E 40 mg + A 1g + C 500 mg + M 500 mg, bid, 7d	NA	RUT, UBT, and histology	UBT	8w	NA
Sardarian *et al*[25] (2012)	Iran	RCT	420	43	48	P 40 mg + A 1g, bid, 7d; P 40 mg + A 1 g + C 500mg + T 500 mg, bid, 7d	Sequential therapy (P 40 mg + A 1g, bid, 5d; P 40 mg + C 500mg + T 500 mg, bid, 5d)	RUT and/or histology	UBT	8w	3
Molina-Infante *et al*[27] (2013)	Spain, Italy	RCT	343	18-87	49	O 40 mg + A 1g bid, 7d; O 20 mg +A 1 g + C 500 mg + N 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + N 500 mg, bid, 14d)	UBT or any two of RUT, histology, or culture	UBT	8w	3
Zullo *et al*[6] (2013)	Italy	RCT	270	49	41	O 40 mg + A 1 g bid, 7d; O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 5d); sequential therapy (O 20 mg + A 1 g, bid, 5d; O 20 mg + C 500mg + T 500 mg, bid, 5d)	RUT and histology	UBT	6w	3
Oh *et al*[13] (2014)	Korea	RCT	184	57	37	R 20mg + A 1g bid, 7d; R 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	RUT or histology	UBT	6w	3
De Francesco *et al*[12] (2014)	Italy	RCT	440	47	42	O 20 mg + A 1g bid, 7d; O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + M 500 mg + T 500 mg, bid, 5d or 14d); sequential therapy (R 20 mg + A 1 g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	RUT+histology	UBT	6-8w	2
Wu *et al*[23] (2014)	Taiwan	RCT	220	53	49	E 20 mg + A 1g bid, 3d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Hybrid therapy (E 20 mg + A 1g bid, 5d/7d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d)	RUT, UBT, histology, or culture	UBT or triple negative (RUT + histology + culture)	8w	3
Cuadrado-Lavin *et al*[28] (2015)	Spain	RCT	300	44	38	O 20 mg + A 1 g bid, 5d; O 20 mg + A 1 g + C 500 mg + M 500 mg, bid,5d	Concomitant therapy (O 20 mg + A 1g + C 500 mg + M 500 mg, bid, 10d)	RUT, UBT, or histology	UBT	4w	3
Heo *et al*[29] (2015)	Korea	RCT	422	57	59	E 20 mg + A 1g bid, 5d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 5d	Concomitant therapy (E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 10d)	Any two of UBT, histology, or RUT	UBT	4w	3
Hwang *et al*[26] (2015)	Korea	RCT	284	59	46	R 20 mg + A 1g bid, 7d; R 20 mg + A 1g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	UBT, histology, or RUT	UBT	4w	3
Chen *et al*[11] (2015)	Taiwan	RCT	175	53	37	R 20 mg + A 1g bid, 7d; R 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 5d; R 20 mg + C 500 mg + M 500 mg, bid, 5d)	RUT + histology, culture	RUT + histology or UBT	8w	2
Metanat *et al*[24] (2015)	Iran	RCT	270	46	44	P 40 mg + A 1g, bid, 5d; P 40 mg + A 1 g + C 500 mg + T 500 mg, bid, 5d	Sequential therapy (P 40 mg + A 1 g, bid, 7d; P 40 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d)	RUT, histology	UBT	8w	2

A: Amoxicillin; C: Clarithromycin; E: Esomeprazole; M: Metronidazole; Mo: Moxifloxacin; N: Nitroimidazole; O: Omeprazole; P: Pantoprazole; R: Rabeprazole; RUT: Rapid urease test; T: Tinidazole; UBT: 13C-urea breath test.

points), and attrition information (1 point)[16].

Statistical analysis

The effect size was calculated as the relative risk (RR) and the 95% confidential interval (CI) for each dichotomous outcome. The meta-analysis was conducted using the STATA software (StataCorp LP, College Station, TX, United States). The eradication rate, compliance rate and side effects rate were pooled by the Comprehensive Meta-Analysis statistical package (CMA Version 2.2, Biostat, Englewood, NJ, United States). The random-effects model using the DerSimonian and Laird method was employed for pooling the data because of suspected heterogeneity[17]. The heterogeneity was evaluated by the Cochran's Q statistic (statistical significance defined as $P < 0.05$), and the I^2 statistic (significant heterogeneity defined as $I^2 > 50\%$)[18]. Intention-to-treat (ITT) analysis was preferred to a per-protocol (PP) approach. The non-

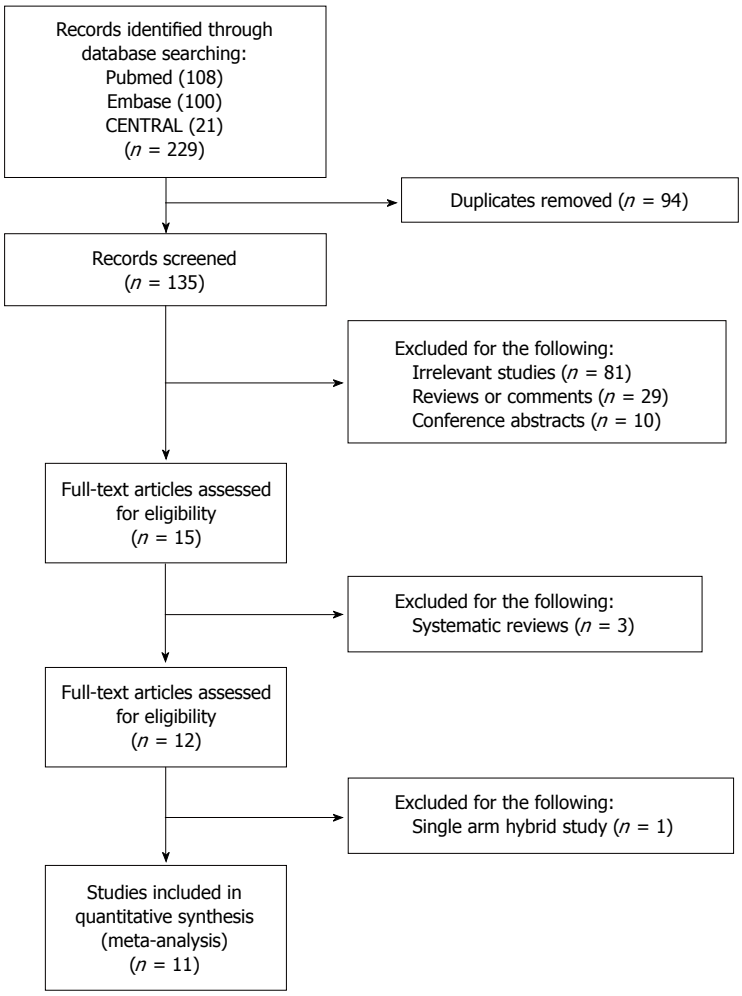

Figure 1 Study selection process of the meta-analysis.

compliant patients or withdrawals were included in the ITT analysis to minimize bias[19]. Sensitivity analysis was performed by excluding the studies one by one. Subgroup analyses were conducted by stratifying the duration of therapy. The publication bias was assessed by the Egger's test and the funnel plot. $P < 0.05$ was considered statistically significant.

RESULTS

Study selection

Our initial search identified 229 publications in total, including 108 articles from PubMed, 100 from Embase, and 21 from the CENTRAL database. Ninety-four duplicate publications were excluded. We discarded 81 irrelevant studies, 31 reviews or comments, and 10 conference abstracts. Fifteen records were eligible for full-text evaluation, of which one was a single-arm hybrid therapy study[20], and three were systematic reviews[4,21,22]. In the final meta-analysis, two studies compared different durations of hybrid therapy[23,24]. Six studies compared hybrid therapy with sequential therapy[11-14,25,26], and 5 studies compared hybrid therapy with concomitant therapy[12,14,27-29]. The selection process is shown in Figure 1. The characteristics of included studies are shown in Table 1. In the quality assessment, the blinding item was least fulfilled as no study used placebo or declared blinding to treatment regimen for patients or researchers. Except for three RCTs[11,12,24], most RCTs described the method of randomization. All studies clearly presented the follow-up data and conducted ITT analysis.

Overall eradication rate of hybrid therapy

The eradication rate was reported in 12 studies. In PP analysis, the overall eradication rate was 91.2% (88.5%-93.4%), with significant heterogeneity (I^2 = 63.9%, $P < 0.05$). In subgroup analyses, the pooled rate was 91.1% (87.4%-93.8%) for 10 studies using the 14-d regimen, and 90.3% (84.6%-94.1%) for 4 studies using the 10-d regimen (Figure 2A). In ITT analysis, the pooled eradication rate was 85.2% (82.1%-87.8%). For 10-d regimen (4 records) and 14-d regimen (10 records), the pooled rate was 82.2% (75.7%-87.2%) and 86.5% (82.6%-89.7%), respectively.

Different durations of hybrid therapy

Only two RCTs compared the hybrid therapies lasting 10 and 14 d, respectively[23,24]. In PP analysis, the 14-d

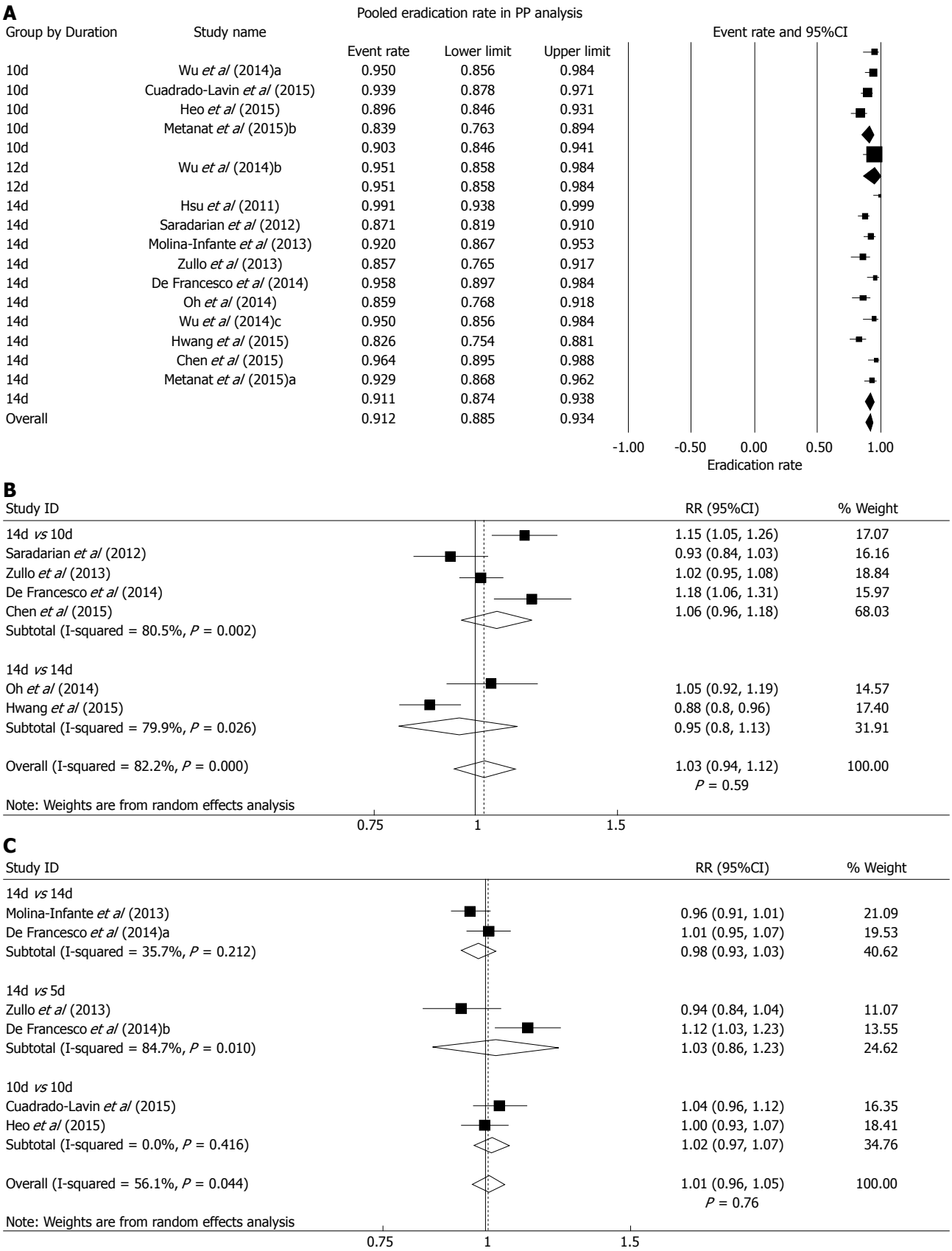

Figure 2 Per-protocol analysis. Forest plot showing the overall eradication rate of *Helicobacter pylori* (*H. pylori*) using hybrid therapy based on data from PP analysis. Subgroup analyses were conducted based on different durations of hybrid regimen. B: Forest plot comparing hybrid with sequential therapy in *H. pylori* eradication using data from PP analysis. Subgroup analyses were conducted based on different durations of sequential regimen. C: Forest plot comparing hybrid with concomitant therapy in *H. pylori* eradication using the data from PP analysis. Subgroup analyses were conducted based on different durations of concomitant regimen.

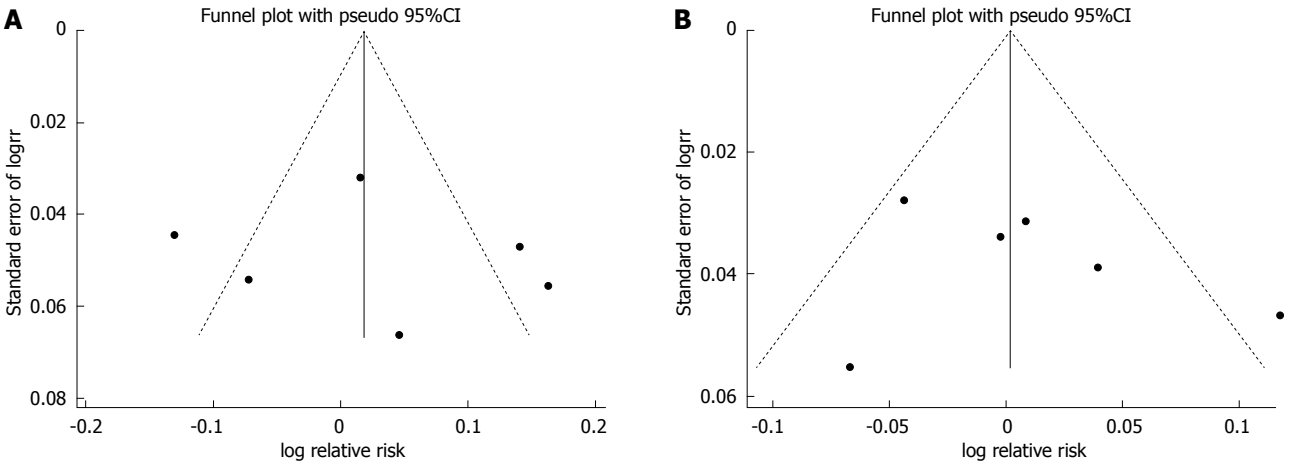

Figure 3 Publication bias. A: Funnel plot of studies comparing hybrid with sequential therapy; B: Funnel plot of studies comparing hybrid with concomitant therapy.

Table 2 Summary of meta-analyses: hybrid therapy *vs* sequential and concomitant therapy

Outcomes	Studies, *n*	Hybrid group	Control group	RR (95%CI)	I^2	*P* value for heterogeneity
Hybrid *vs* sequential						
Eradication rate (PP)	6	88.6%	87.8%	1.03 (0.94-1.12)	82.2%	< 0.05
Eradication rate (ITT)	6	84.3%	85.1%	1.00 (0.89-1.12)	85.2%	< 0.05
Compliance rate	5	96.0%	98.0%	0.99 (0.96-1.02)	50.4%	> 0.05
Side effect rate	6	30.3%	28.2%	1.05 (0.86-1.02)	37.8%	> 0.05
Hybrid *vs* concomitant						
Eradication rate (PP)	5	91.3%	92.4%	1.01 (0.96-1.05)	56.1%	< 0.05
Eradication rate (ITT)	5	84.8%	86.7%	0.99 (0.95-1.03)	0	> 0.05
Compliance rate	4	95.8%	93.2%	1.03 (1.00-1.05)[1]	0	> 0.05
Side effect rate	4	39.5%	44.2%	0.93 (0.82-1.05)	0	> 0.05

[1]Statistically significant results. ITT: Intention-to-treat; PP: Per-protocol.

regimen did not show significantly higher eradication rate compared with 10-d regimen (RR = 1.04, 95%CI: 0.92-1.18, $P > 0.05$). Significant heterogeneity was presented (I^2 = 73.4%, $P = 0.05$). In ITT analysis, no significant superiority was found for the 14-d regimen compared with the 10-d regimen (RR = 1.08, 95%CI: 0.99-1.19, $P > 0.05$), without heterogeneity (I^2 = 0%, $P > 0.05$).

Hybrid therapy vs sequential therapy

Eradication rate: Six studies were available[11-14,25,26]. Two Korean RCTs[13,26], and 2 Italian RCTs[12,14], were conducted by the same groups, during different study periods. In PP analysis, the eradication rate was 88.6% (95%CI: 83.6%-92.3%) for hybrid therapy and 87.8% (95%CI: 79.9%-92.9%) for sequential therapy. No statistically significant difference was found between the hybrid and sequential therapies, with significant heterogeneity (RR = 1.03, 95%CI: 0.94-1.12, $P = 0.59$; I^2 = 82.2%, $P < 0.05$) (Figure 2B). In ITT analysis, the eradication rate was 84.3% (95%CI: 79.3%-88.2%) for hybrid therapy and 85.1% (95%CI: 78.4%-89.9%) for sequential therapy. No significant differences were seen with hybrid therapy compared with sequential therapy (RR = 1.00, 95%CI: 0.89-1.12, $P = 0.94$). Significant heterogeneity was found (I^2 = 85.2%, $P < 0.05$) (Table 2).

Sensitivity analyses were carried out by excluding the studies one by one. Notably, no significant change was shown for PP or ITT results. Regarding sequential therapy, 4 studies used the 10-d regimen[11,12,14,25], and 2 studies used the 14-d regimen[13,26]. Based on the different durations, subgroup analysis of PP data did not find statistically significant changes for the 10-d regimen (RR = 1.06, 95%CI: 0.96-1.18) or for the 14-d regimen (RR = 0.95, 95%CI: 0.80-1.13) (Figure 2B). Similarly, subgroup analysis of ITT data revealed no significant alteration for the 10-d regimen (RR = 1.03, 95%CI: 0.88-1.20) or for the 14-d regimen (RR = 0.93, 95%CI: 0.79-1.09).

Compliance: Five studies evaluated the compliance[11,13,14,25,26]. Both therapies displayed a high compliance rate [96% (95%CI: 93%-98%)] for hybrid therapy, and 98% (95%CI: 95%-99%) for sequential therapy. No significant difference was observed (RR = 0.99, 95%CI: 0.96-1.02, $P = 0.55$; I^2 = 50.4%, $P > 0.05$) (Table 2).

Side effects: The overall adverse effect rate was 30.3% (95%CI: 20.9%-41.6%) for the hybrid therapy, and 28.2% (95%CI: 15.7%-45.4%) for the sequential

therapy. The hybrid therapy did not show significantly lower incidence of adverse effect (RR = 1.05, 95%CI: 0.86-1.02, P = 0.63). No significant heterogeneity was observed (I^2 = 37.8%, P > 0.05) (Table 2).

Hybrid therapy vs concomitant therapy

Eradication rate: Five studies were available[12,14,27-29]. In PP analysis, the eradication rate of hybrid and concomitant regimen was 91.3% (95%CI: 87.7%-93.9%) and 92.4% (95%CI: 89.2%-94.7%), respectively. In ITT analysis, the eradication rate of hybrid and concomitant regimen was 84.8% (95%CI: 78.9%-89.2%) and 86.7% (95%CI: 80.7%-91.0%), respectively. In PP analysis, no statistically significant difference was observed between hybrid therapy and concomitant therapy (RR = 1.01, 95%CI: 0.96-1.05, P = 0.76; I^2 = 56.1%, P < 0.05) (Figure 2C). In ITT analysis, no significant difference was found between the two regimens, and no heterogeneity was observed (RR = 0.99, 95%CI: 0.95-1.03, P = 0.47; I^2 = 0%, P > 0.05) (Table 2).

In sensitivity analysis by excluding studies one by one, no significant change was seen in PP or ITT analysis. For concomitant therapy, two studies presented results of the 14-d regimen[12,27], 2 of the 10-d regimen[28,29], and 2 of the 5-d regimen[12,14]. Subgroup analyses based on different durations of concomitant therapy revealed no significant difference.

Compliance: Four studies were relevant[14,27-29]. The compliance rate was 95.8% (95%CI: 93.2%-97.4%) for hybrid therapy, and 93.2% (95%CI: 89.7%-95.6%) for concomitant therapy. Patients receiving hybrid therapy showed significantly higher rate of compliance when compared with concomitant therapy (RR = 1.03, 95%CI: 1.00-1.05, P < 0.05). No heterogeneity was revealed (I^2 = 0%, P > 0.05) (Table 2).

Side effects: Four studies were included[12,14,27,28]. The overall side effect rate was 39.5% (95%CI: 21.7%-60.7%) for hybrid therapy, and was 44.2% (95%CI: 26.7%-63.2%) for concomitant therapy. No significant difference was seen between hybrid therapy and concomitant therapy (RR = 0.93, 95%CI: 0.82-1.05, P = 0.24). No heterogeneity was observed (I^2 = 0%, P > 0.05) (Table 2).

Publication bias

Publication bias was representatively evaluated for PP data. For hybrid *vs* sequential therapy, the funnel plot was symmetrical, with a non-significant result in Egger's test (P = 0.74) (Figure 3A). In hybrid *vs* concomitant therapy, the funnel plot was symmetrical (Figure 3B). No statistical significance was revealed by Egger's test (P = 0.48).

DISCUSSION

Eradication rate plays a pivotal role in evaluating the success of *H. pylori* treatment. The efficacy of *H. pylori* eradication was graded as follows: (1) excellent (> 95%); (2) good (90-95%); (3) fair (85-89%); (4) bad (81%-84%); and (5) unacceptable (< 80%)[30]. In ITT and PP analyses, therapeutic significance was achieved when the eradication rates exceeded 80% and 90%, respectively[26]. In this meta-analysis, hybrid therapy yielded a good eradication rate (91%) in PP analysis, and fair (85%) in ITT analysis, both exhibiting significant therapeutic values. The pooled data showed similar treatment success (an eradication rate closer to 90%) with hybrid, sequential, and concomitant therapies against *H. pylori.* Hybrid therapy had good compliance to medications, which was similar to sequential therapy and slightly better than concomitant therapy. The differences in adverse event rates were small between hybrid, sequential, and concomitant therapies. All the three therapies showed acceptable safety profile. The 10-d hybrid regimen did not show significant inferiority with respect to the eradication rate. Meta-analyses have shown that the eradication outcome was duration dependent[9]. However, the differences in eradication rate across all subgroups stratified by duration were minimal.

Currently, in the absence of any new drugs against *H. pylori,* different combination regimens, including sequential, concomitant, and hybrid therapies, have been investigated extensively. Hybrid therapy evolved from sequential therapy and concomitant therapy. Compared with sequential therapy, hybrid therapy extended the duration of amoxicillin. Prolonging the duration of traditional triple therapy from 7 to 10-14 d improved the eradication success rate by approximately 5%[2]. The prescription of PPI and amoxicillin was similar for concomitant and hybrid therapies. However, clarithromycin and metronidazole were used over a shorter duration of hybrid therapy. The adverse effects of metronidazole included nausea and regurgitation. Furthermore, both metronidazole and clarithromycin may cause bitter tastes[29]. With decreased pill burden, hybrid therapy was superior in cost-effectiveness over concomitant therapy.

The participants included in the RCTs were residents of Taiwan, Iran, Italy, Spain, and Korea, which represent regions with a high prevalence of antibiotic-resistant *H. pylori* strains[5,11]. Worldwide increase of *H. pylori* resistance to antibiotics, especially clarithromycin and metronidazole, is the most important determinant of eradication failure in traditional triple therapy[31]. For sequential therapy, the eradication rate of clarithromycin-resistant and metronidazole-resistant strains was 72.8% and 86.4%, respectively. However, the rate decreased to just 37% for dual-resistant strains[32]. Concomitant regimen outperformed sequential regimen in areas with a high incidence of clarithromycin and/or metronidazole resistance[33,34]. However, eradication was expected to fail (< 90%) when the prevalence of dual clarithromycin-metronidazole resistant strains was > 15%[34]. Compared with concomitant therapy, hybrid

therapy initially prescribed amoxicillin, which may prevent the occurrence of secondary clarithromycin resistance[35,36]. Compared with sequential therapy, hybrid regimen extended the duration of amoxicillin exposure. Hybrid therapy combined the advantages of sequential and concomitant therapy. Unfortunately, very few studies conducted antimicrobial susceptibility testing before hybrid treatment. Chen *et al*[11] showed that sequential therapy resulted in a 71.4% (5/7) eradication rate in patients harboring strains with dual resistance. Hybrid therapy yielded a 100% (4/4) eradication rate. Molina-Infante *et al*[34] revealed that for clarithromycin-resistant and dual-resistant strains, the concomitant regimen resulted in a 100% (8/8 and 3/3, respectively) eradication rate. By constrast, hybrid therapy only achieved a rate of 75% (6/8) and 33% (1/3), respectively. Nevertheless, the very small number of patients with resistant strains precluded definite conclusions.

Our meta-analysis represented the most comprehensive review of hybrid therapy and an update of two similar meta-analyses[21,22]. Notably, five studies have only recently been published, which were not included in previous meta-analyses[11,24,26,28,29]. The number of studies for meta-analysis doubled that of the previous studies, generating more robust conclusions, albeit with similar non-significant results between different regimens. Additionally, it was the first time that hybrid therapy was compared with different durations of sequential or concomitant therapy. The overall eradication rate with durations of hybrid therapy was demonstrated.

This study had several limitations. The number of included trials was still small, and the sample size was not large enough for the majority of studies. For example, although we did not detect the impact of different durations of sequential or concomitant therapy, the results should be extrapolated with caution as only few studies were included. Most RCTs did not report blinding to treatment regimen. Lack of blinding may influence compliance and the reporting of side effects. The quality of included studies was low. The majority of studies did not conduct susceptibility tests to determine antibiotic resistance[12,25,28]. In fact, we have tried to assess the eradication efficacy in resistant strains. However, we had insufficient related data and very small sample sizes of resistant patients. A number of confounding factors may play a role in determining the *H. pylori* eradication rates. Except for the disparity between different regions regarding the prevalence of resistant strains, the rates were influenced by genetic differences in the PPIs metabolism, degree of gastritis, administration of probiotics, and the nature of the underlying disease[37]. Additionally, different types of PPIs and nitroimidazole medications, and varying duration of follow-up may potentially generate small amounts of bias[27,28]. Participation in an RCT enhanced the patient compliance, and the compliance gap between hybrid therapy and other treatment regimens might be wider in clinical practice[29].

In conclusion, hybrid therapy yielded good eradication efficacy for *H. pylori* in regions with a high prevalence of antibiotic-resistant strains. Hybrid regimens achieved equivalent eradication rates compared with sequential or concomitant therapy. The compliance and adverse events were not different between hybrid, sequential or concomitant therapies. The 14-d and 10-d hybrid therapy showed similar eradication rates. Further studies are urgently required to clarify important differences in eradication of *H. pylori* in the setting of varying patterns of antibiotic resistance.

COMMENTS

Background

Previous trials reported inconsistent results regarding the efficacy, compliance rate and adverse events following the use of hybrid therapy when compared with traditional sequential therapy and concomitant therapy for the eradication of *H. pylori*.

Research frontiers

The emerging resistance of *H. pylori* strains is the major cause of treatment failure. Hybrid therapy represented a renewal of sequential therapy and concomitant therapy, and the efficacy and safety of hybrid therapy need to be investigated.

Innovations and breakthroughs

Our meta-analysis represented the most comprehensive review of hybrid therapy and an update of two similar meta-analyses. The authors for the first time, compared hybrid therapy following different durations of sequential or concomitant therapy. They also compared the different durations of hybrid therapy, and demonstrated the overall eradication rate of hybrid therapy.

Applications

Hybrid therapy showed a good eradication rate, high compliance rate, and acceptable safety profiles compared with traditional sequential therapy and concomitant therapy. These findings may represent a future strategy for the treatment of patients with *H. pylori* infection.

Peer-review

The study is a meta-analysis comparing hybrid therapy with traditional sequential therapy and concomitant therapy against *H. pylori* infection. The present manuscript included 5 additional studies published in 2015 and therefore strengthens the outcomes of previous meta-analyses.

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CASE REPORT

Endoscopic resection of sparganosis presenting as colon submucosal tumor: A case report

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Abstract

Human sparganosis is a rare parasitic disease caused by infection with the tapeworm *Sparganum*, the migrating plerocercoid (second stage) larva of *Spirometra* species. Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but involvement of other sites such as the brain, eye, peritoneopleural cavity, urinary track, scrotum, and abdominal viscera has also been documented. Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea. Rectal sparganosis is an uncommon disease but should be considered in the differential diagnosis of unusual and suspicious rectal submucosal tumors. We report a case of rectal sparganosis presenting as rectal submucosal tumor. We performed endoscopic submucosal dissection of the rectal submucosal tumor. The sparganosis was confirmed based on the presence of calcospherules in the submucosal layer on histological examination. Moreover, the result of the immunoglobulin G antibody test for sparganosis was positive but became negative after endoscopic submucosal dissection. Though rare, rectal sparganosis should be considered in the differential diagnosis of rectal submucosal tumor-like lesions. This case suggests that physicians should make effort to exclude sparganosis through careful diagnostic approaches, including detailed history taking and serological tests for parasites. In this report, we aimed to highlight the clinical presentation of *Sparganum* infection as a rectal submucosal tumor.

Key words: Rectum; Submucosal tumor; Sparganosis; *Sparganum*; Parasite disease

Core tip: This rare case exhibited the rectal sparganosis presenting as rectal submucosal tumor. This is the first case of rectal sparganosis presenting as submucosal tumor that are treated with endoscopic submucosal dissection. Though rare, this case suggests that sparganosis should be considered in the differential diagnosis of submucosal tumor-like lesions in gastrointestinal track.

Kim JK, Baek DH, Lee BE, Kim GH, Song GA, Park DY. Endoscopic resection of sparganosis presenting as colon submucosal tumor: A case report. *World J Gastroenterol* 2016; 22(19): 4776-4780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4776.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4776

INTRODUCTION

Human sparganosis is a rare infectious disease caused by *Sparganum*, a plerocercoid tape worm larva of the genus *Spirometra*, as first described by Manson in 1882[1]. Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea[2-4]. In humans, it is accidentally acquired by ingestion of larva-containing water or by eating raw snakes and frogs. Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but involvement of other sites such as the brain, eye, peritoneopleural cavity, urinary track, scrotum, and abdominal viscera has also been documented[5,6]. The larvae are commonly found in the chest, abdomen, urogenital organs, extremities, central nervous system, and orbital region[5]. The rectum is a distinctly rare site of the infection. The rectum is a distinctly rare site of the infection. Clinical manifestations of sparganosis are diverse, including headache, non-specific discomfort, vague pain, palpable mass, or even no symptoms[7]. The most common clinical manifestation is a migrating subcutaneous mass. Clinically and radiologically, the mass may mimic a neoplasm. Thus, it is difficult to diagnose preoperatively in most cases[2]. We report an interesting rare case of rectal sparganosis presenting as a submucosal tumor (SMT) treated with endoscopic submucosal dissection (ESD). This report aimed to highlight the existence and clinical presentation of *Sparganum* infection, which is considered to be rare in South Korea. Moreover, this case report reveals an atypical picture where the larval form was not identified on gross examination of a resected specimen and the diagnosis was confirmed based on histological examination and enzyme-linked immunosorbent assay (ELISA) results.

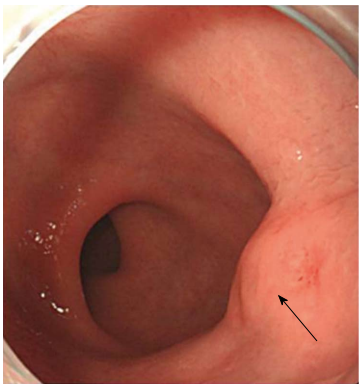

Figure 1 Endoscopic features of the rectal submucosal tumor. A prominently elevated submucosal tumor-like lesion covered with normal mucosa 5 cm above the anal verge (black arrow).

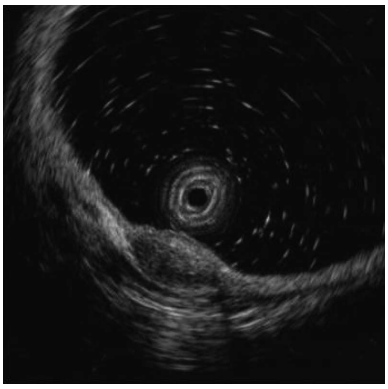

Figure 2 Endoscopic ultrasonographic features of the rectal submucosal tumor. An approximately 1.0 cm × 0.5 cm, hypoechoic lesion in the deep mucosa and submucosal layer.

CASE REPORT

In October 2013, a 38-year-old Korean male was referred to gastroenterology team for evaluation of rectal SMT. His medical history was uneventful. His family history was unremarkable. In addition, review of system and physical examination results were unremarkable. The results of the laboratory tests, including complete blood count, liver function test, renal function test, and coagulation factor assay, were normal. Moreover, no eosinophilia was observed, and tumor markers and anti-HIV antibody levels were normal. Colonoscopy revealed a round SMT covered with normal mucosa, located 5 cm from the anal verge (Figure 1). Endoscopic ultrasonography (EUS) revealed a well-demarcated hypoechoic mass 1.0 cm × 0.5 cm in size chiefly located in the deep mucosa and submucosal layer (Figure 2). For complete and total resection of the lesion and accurate histological diagnosis, we performed an ESD (Figure 3A and B). Histological examination of the resected specimen revealed chronic granulomatous inflammation with foreign bodies associated with acute suppurative inflammation (Figure 4A). A high-resolution image showed some calcospherules in the submucosal

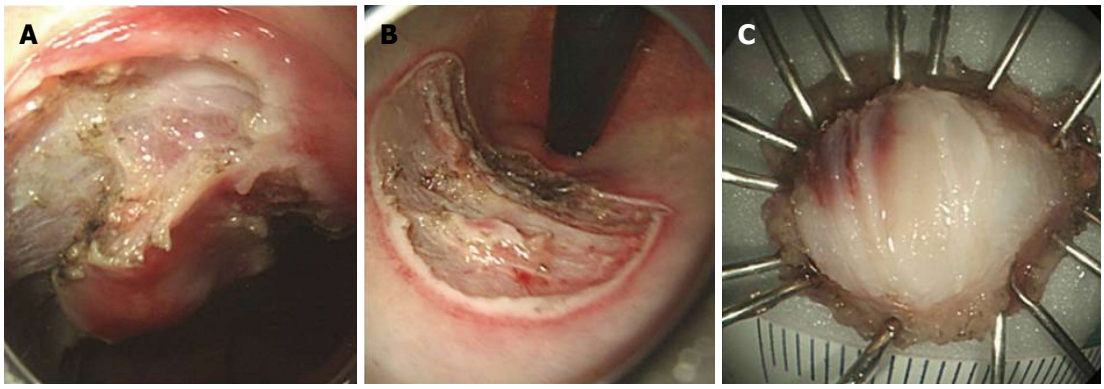

Figure 3 Rectal endoscopic submucosal dissection. A: During submucosal dissection; B: Endoscopic features after endoscopic submucosal dissection (ESD); C: Gross features of the specimen from ESD.

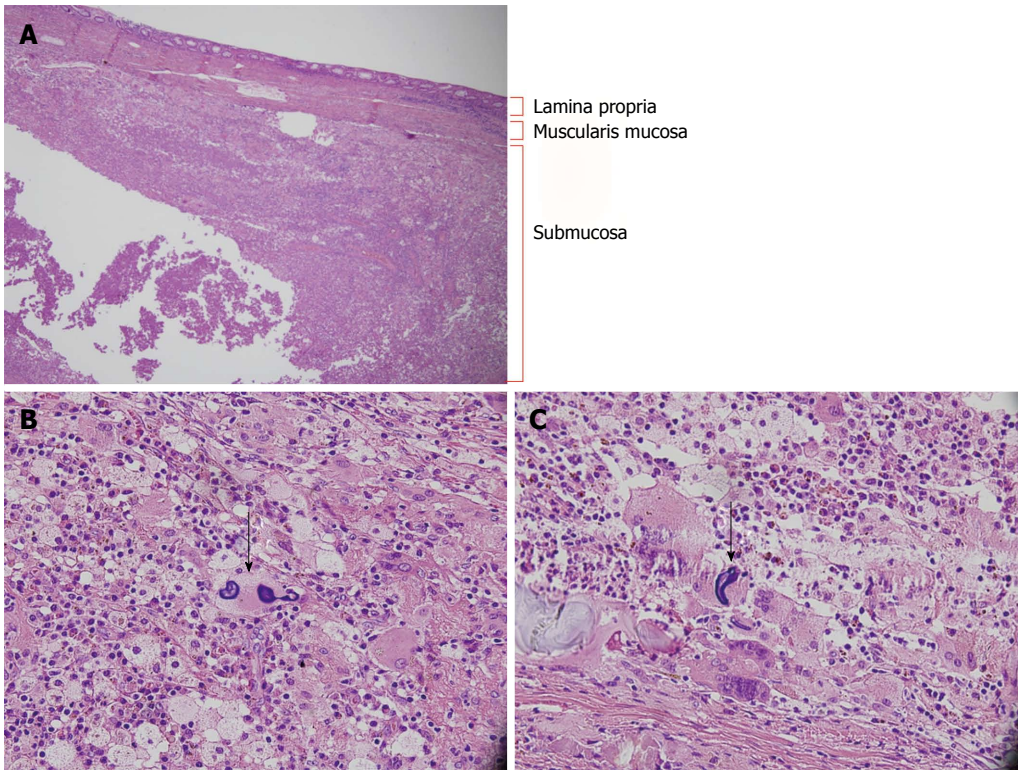

Figure 4 Pathological features. A: Suppurative inflammation and chronic granulomatous inflammation caused by foreign bodies mainly located in the submucosal layer (Hematoxylin-eosin stain, original magnification × 40); B and C: Calcospherules in the submucosal layer (Hematoxylin-eosin stain, original magnification × 100).

layer (Figure 4B and C). The calcospherules are characteristic structures that reveal previous parasitic infection. Subsequently, immunoglobulin G (IgG) antibody tests for cysticercosis, paragonimiasis, clonorchiasis, and sparganosis were performed, and the results were positive only for sparganosis. Although the gross examination of the excised tissue did not reveal any larval forms, the diagnosis was confirmed based on histological examination and ELISA results. We reevaluated the patient's medical history. Particularly noteworthy is the fact that he had enjoyed eating organic vegetables. We suggest that his dietary habit was the route of the infection. No additional therapy such as antiparasitic medication or surgery was administered. Thirteen months after the ESD, follow-up colonoscopy revealed only the presence of a scar at the site of the ESD, and chest radiography and abdominal computed tomography did not reveal any additional abnormal findings. Moreover, the result of the IgG antibody test for sparganosis became negative after the ESD. He was doing well at 24 mo of follow-up.

DISCUSSION

To our knowledge, this is the first case of sparganosis presenting as rectal SMT. Human sparganosis is a rare infectious disease caused by *Sparganum*, a

plerocercoid tapeworm larva of the genus *Spirometra.* Spargana larvae can be found in any part of the human body and have a preference for subcutaneous involvement and migration. In the extramammary organs, sparganosis most frequently manifests as a migrating subcutaneous nodule and presents clinically with vague or indeterminate symptoms.

Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea. The most common route of infection is *via* ingestion of contaminated water containing procercoids, which can penetrate into the intestine and migrate to the muscle or subcutaneous tissue. The second route is from ingestion of raw or partially cooked frogs, snakes, fish, and chickens. The third route of infection may be from poultice applications infested with cyclops containing procercoids and are utilized for open wounds or the eyes[8]. Economic development and advancement in sanitation have influenced the routes, sites, and latent period of the infection. In our case, the patient denied eating raw meat or fish, including snakes and frogs. In addition, he had never traveled in Southeast Asia such as China, and Japan. Instead, he had enjoyed eating organic vegetables during his lifetime. We assume that the route of his infection was larvae from organic vegetables.

Sparganosis as a usual practice is diagnosed after the surgical removal of the worm from the site of inflammation. Although the larvae are decimated, the characteristic pathological findings of the chronic granulomatous inflammation with central necrosis and calcospherules can be helpful for diagnosis. However, in cases for which the feasibility of surgery is limited, surrogate diagnostic methods such as ELISA for *sparganum* in relation to a relevant history of exposure can be used[9]. ELISA performed for *sparganum*-specific IgG is highly sensitive and specific. Serum ELISA has 85.7% sensitivity and 95.7% specificity[6]. Negative ELISA results after surgical removal predict successful treatment of human sparganosis, but persistent positive reaction strongly suggests incomplete removal of the worm[8]. In our case where the gross examination of the excised tissue did not reveal any larval forms, the diagnosis was confirmed based on the histological examination and ELISA results. The negative ELISA result for sparganosis after ESD also strongly supported our diagnosis.

The definitive treatment of sparganosis is the surgical removal of larvae. However, when it cannot be removed surgically such as in multiorgan infection, drug therapy with praziquantel can be administered. However, many case reports showed that drug therapy with praziquantel did not have a favorable outcome and had high recurrence rates[10]. In our case, we did not administer praziquantel after ESD, the patient was doing well without recurrence for 24 mo after the ESD.

In conclusion, this is the first case of rectal sparganosis presenting as SMT. Furthermore, ESD performed to remove this parasite lesion is the first case. Though rare, rectal sparganosis should be considered in the differential diagnosis of rectal SMT-like lesions. This case suggests that physicians should make effort to exclude sparganosis through careful diagnostic approaches, including detailed history taking and serological tests for parasites. Although the ESD is a rather uncommon way in order to treat sparganosis, ESD can be considered for both diagnostic and therapeutic purposes like our case. In cases in which the rectal sparganosis is confined to the deep mucosa and/or submucosa on EUS and other sites are free of the disease, endoscopic resection with close follow-up should be considered as an alternative treatment modality.

COMMENTS

Case characteristics

38-year-old man with no significant medical history underwent colonoscopy for screening of the lower digestive tract. A rectal submucosal tumor was detected on 5 cm from the anal verge.

Clinical diagnosis

The patient had no symptom.

Differential diagnosis

Endoscopic ultrasonography (EUS) revealed a hypoechoic mass chiefly located in the deep mucosa and submucosal layer, which could be seen in the context of rectal neuroendocrine tumor, rectal tonsil, lymphoma, or chronic granulomatous inflammation.

Laboratory diagnosis

Laboratory data were within normal range with the exception of positive immunoglobulin G (IgG) antibody tests for sparganosis.

Imaging diagnosis

Colonoscopy revealed a round submucosal tumor covered with normal mucosa, located 5 cm from the anal verge. Endoscopic ultrasonography revealed a well-demarcated hypoechoic mass 1.0 cm × 0.5 cm in size chiefly located in the deep mucosa and submucosal layer.

Pathological diagnosis

The pathologic features of the resected specimen revealed chronic granulomatous inflammation with foreign bodies associated with acute suppurative inflammation and some calcospherules in high-resolution image.

Treatment

The patient underwent rectal endoscopic submucosal dissection for both diagnostic and therapeutic purpose.

Related reports

Human sparganosis is a rare infectious disease caused by *Sparganum*, a plerocercoid tapeworm larva of the genus *Spirometra.* Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but rectal involvement presenting as submucosal tumor has not been documented.

Term explanation

A submucosal tumor is defined as any intramural growth under the mucosa, where etiology cannot readily be determined by endoscopy. EUS can be of help to make a diagnosis.

Experiences and lessons

Physicians should consider sparganosis in the differential diagnosis of rectal submucosal tumor-like lesions. And detailed history taking and serological tests for parasites can also be helpful in diagnosis.

Peer-review

This article is very interesting from the point of the parasitic disease because rectal sparganosis is an uncommon and rare. Furthermore, endoscopic submucosal dissection performed to remove this parasite lesion was probably the first case.

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CASE REPORT

Successful management of adult lymphoma-associated intussusception by laparoscopic reduction and appendectomy

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Author contributions: Hsu SD performed the surgery and provided the conception; Huang CY helped in interpreting the pathology; Chen YL helped in image interpretation; Lin YY and Tsuei YW helped edit the manuscript; and Yang TW collected data and wrote this manuscript.

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Informed consent statement: This case report is delivered for academic communication only, not for other purposes; In this case report, the images do not disclose the patient's personal information; Consent was acquired from the patient for publication of this case report.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Abstract

Although primary gastrointestinal lymphoma is a rare malignancy, it can cause an intussusception in adults and can be a clinically challenging condition to manage. Intussusception could progress to life-threatening complications if left untreated or could delay chemotherapy if inappropriate surgical management is used. We report a 31-year-old man diagnosed with human immunodeficiency virus who was being treated with antiretroviral therapy. He presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk. Abdominal computed tomography revealed a well-defined homogeneous mass in the mesenteric root region, together with a long segmental wall thickening in the ileum with ileocolic-type intussusception, which was suspected to be caused by a lymphoma. The intussusception was successfully laparoscopically reduced, and the tumor involvement of the appendix was confirmed

by appendectomy with intraoperative frozen section. Systemic chemotherapy was immediately initiated after surgery without the need for bowel resection.

Key words: Intussusception; Adult; Intestinal lymphoma; Appendectomy

Core tip: In general, surgical management of adult with an intussusception mandates the resection of the involved bowel segment. However, the surgical resection of an intussusception that is caused by intestinal lymphoma is controversial because the intestinal involvement is generally diffuse. Concerning the diffuse invasive characteristics of gastrointestinal lymphomas, laparoscopic reduction of intussusceptions and appendectomy with intraoperative frozen section were both performed that enabled us to intraoperatively identify the tumor involvement of the resected appendix. By avoiding bowel resection, systemic chemotherapy could be initiated early after surgery.

Yang TW, Lin YY, Tsuei YW, Chen YL, Huang CY, Hsu SD. Successful management of adult lymphoma-associated intussusception by laparoscopic reduction and appendectomy. *World J Gastroenterol* 2016; 22(19): 4781-4785 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4781.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4781

INTRODUCTION

Although an intussusception is common in the pediatric population, it is a rare[1-5] but clinically challenging condition in adults, accounting for 1%-5% of mechanical bowel obstruction cases[6,7]. If left untreated, an intussusception could progress to tissue necrosis, bowel perforation, or peritonitis. In adults, it is typically because of the presence of a pathological lead point within the bowel, which is malignant in over half of all cases[6,7]. Most surgeons agree that adults with an intussusception require resection via surgery because most of such cases have lesions intraluminally. Nevertheless, the optimal resected range and if the intussusception should be reduced or not remain a controversy[8]. In this study, we present an unusual case of an adult with ileocolic intussusception that is caused by an intestinal lymphoma. He was successfully treated with laparoscopic surgery without bowel resection.

CASE REPORT

The patient was a 31-year-old man with human immunodeficiency virus (HIV) who was being treated with antiretroviral therapy. He presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk. On arrival, his blood pressure was 126/66 mmHg; pulse rate, 92 beats per minute; and body temperature, 36.3 ℃. Physical examination disclosed tympanic sounds on percussion and mild periumbilical tenderness on palpation. Upper gastrointestinal (GI) panendoscopy was performed but revealed no specific findings; however, abdominal sonography subsequently identified a mass over the periumbilical region. Therefore, a contrast-enhanced computed tomography (CT) of the abdomen was performed, which revealed a well-defined, homogeneous mass, measuring approximately 6 cm × 4 cm × 7 cm, in the mesenteric root region (Figure 1, asterisk). The lymphoma was clinically highly suspected on the basis of imaging finding. In addition, there was evidence of a long segmental wall thickening in the terminal ileum with an ileocolic-type intussusception, and a lymphoma in the terminal ileum was also suspected (Figure 1, arrow).

The patient underwent diagnostic laparoscopy, which revealed tumors with diffuse involvement that included the terminal ileum and proximal colon. On

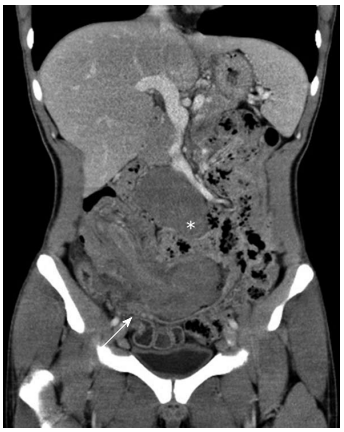

Figure 1 Contrast-enhanced computed tomography showed a well-defined homogeneous mass (asterisk) in the mesenteric root region, together with a long segmental wall thickening in the ileum with ileocolic-type intussusception (arrow).

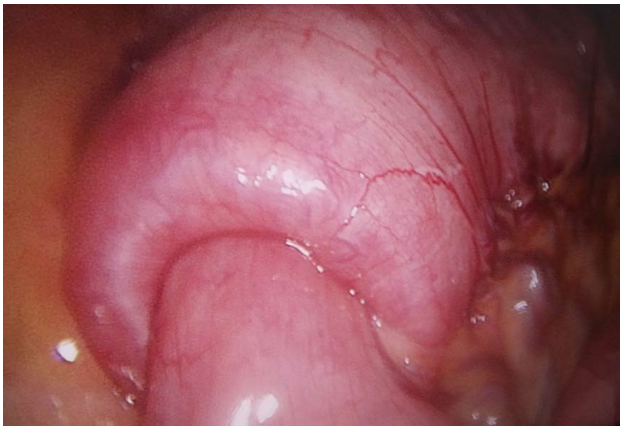

Figure 2 Invagination of the terminal ileum into the proximal colon.

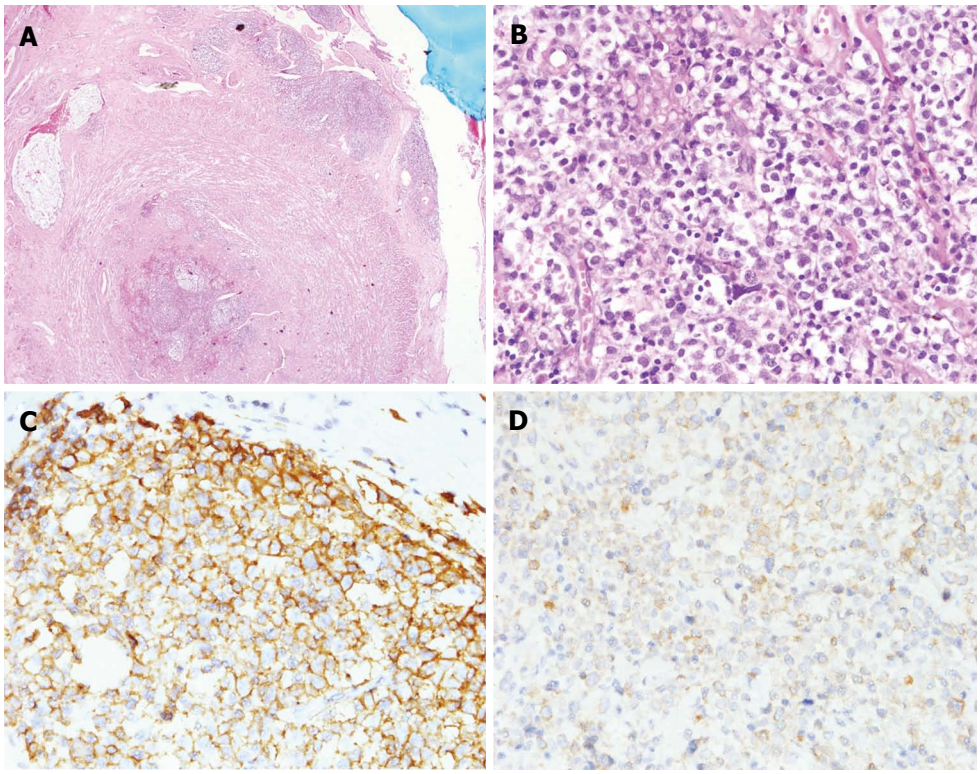

Figure 3 Histological and immunohistological examination of the specimens showing diffuse large B-cell non-Hodgkin's lymphoma. A: Magnification × 20; HE staining. Tumor cell infiltrates can be observed in the serosal layer of the appendix; B: Magnification × 400, HE staining, polymorphic large B cells; C: Magnification × 400, CD20(+); D: Magnification × 400, Bcl-2(+). Bcl-2: B-cell lymphoma 2; HE: Hematoxylin and eosin.

closer inspection, the terminal ileum and appendix were both invaginating into the proximal colon, and the tip of the appendix was still visible outside the invagination. Both of the invaginated ileum and proximal colon were relatively healthy, with no ischemic changes (Figure 2). We used non-traumatic graspers to retract the ileum and residual appendix and successfully reduced the intussusception. The appendix appeared red and swollen under laparoscopy.

Tumor cell infiltration in the serosal layer of the appendix was confirmed by an immediate frozen section of the appendix, and the final pathology result indicated a diffuse large B-cell lymphoma (Figure 3). The patient received systemic chemotherapy on postoperative day 12, and enteral feeding was well tolerated. The patient was subsequently discharged with good bowel movements and without any complication. He was referred to hematology for further chemotherapy treatment.

DISCUSSION

The optimal management of an intussusception remains controversial in adults. Surgical management usually involves the resection of the affected bowel to determine the underlying cause, which is malignant in more than half of all cases[9]. In contrast, chemotherapy is often the preferred treatment for primary intestinal lymphomas; surgery plays a limited role because the diffuse involvement would necessitate complete resection that might be complicated with short bowel syndrome[10-13]. Therefore, it is recommended that resection be reserved for patients with acute complications, such as obstruction, hemorrhage, abscess, or perforation, with no evidence supporting the uses of preventive surgery[14,15].

After Kaposi sarcoma, lymphomas are the second most malignancy that is observed in patients with HIV[16], and the most common type are diffuse large B-cell non-Hodgkin lymphoma[17]. GI tract involvement of lymphoma is common in patients with HIV[18], including the appendix, which is considered a part of the gut-associated lymphoid tissue. Leukemic and lymphomatous tumor involvement of the appendix can be primary or secondary[19]. Typical CT findings of the intestinal lymphoma include a enormous mass, extenseive infiltration fat planes preservation, multiple site involvement, and associated bulky lymphadenopathy[20,21]. Other differential diagnoses similar to these CT findings include carcinoids, adenocarcinomas, sarcoma (*e.g.*, gastrointestinal stromal tumor), and leiomyomatosis peritonealis disseminata. In our case, there was a well-defined homogeneous mass in the mesenteric root region and a long segmental wall thickening in the terminal ileum on contrast-enhanced CT of the abdomen. Intestinal lymphoma was presumed on the basis of this imaging, and tumor involvement of the appendix

was suspected because of its proximity to the terminal ileum. During surgery, the use of a frozen section of the resected appendix helped us quickly confirm that there was tumor-cell infiltration of the appendix. Given these findings, surgical management was limited to laparoscopic reduction of the ileocolic intussusception and appendectomy. Therefore, possible complications from diffuse large bowel resection could be avoided. Had the frozen section of the resected appendix not revealed tumor involvement, we might have considered performing a tumor biopsy instead.

Most intussusceptions are of the ileocolic type and occur because of a leading point that is located in the ileocolic region[1]. Because of this, we used traction of the appendix with surgical forceps to make the ileocolic intussusception easier to reduce and help perform the laparoscopic appendectomy.

Although adjuvant chemotherapy is typically initiated within 6-8 wk after surgery, several recent meta-analyses have confirmed that delayed administration of adjuvant chemotherapy is associated with significantly reduced overall survival[22,23]. Laparoscopic surgery is typical considered as a less-injurious surgery that enables earlier initiation of adjuvant chemotherapy[24,25]. Our patient was able to initiate systemic chemotherapy without too much delay because the surgery was limited to laparoscopic reduction of the intussusception and appendectomy.

In conclusion, diffuse large B-cell lymphomas may involve multiple sites in the GI tract and can cause intussusception in patients with HIV. To minimize any surgery-related complications and to improve prognosis, surgeons could consider laparoscopic reduction and appendectomy with intraoperative frozen section to identify tumor invasion. This approach facilitates earlier initiation of postoperative chemotherapy and could improve patient outcomes.

COMMENTS

Case characteristics

A 31-year-old human immunodeficiency virus (HIV)-infected man who was being treated with antiretroviral therapy, presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk.

Clinical diagnosis

Nausea, vomiting, and intermittent abdominal cramping pain with mild periumbilical tenderness.

Differential diagnosis

Gastroesophageal reflux disease, peptic ulcer disease, irritable bowel syndrome, small or larbe bowel obstruction, cholelithiasis or pancreatitis.

Laboratory diagnosis

All laboratory tests were within normal limits.

Imaging diagnosis

Computed tomography (CT) of the abdomen revealed a 6 cm x 4 cm x 7 cm well-defined mass in the mesenteric root region, and a long segmental wall thickening in the terminal ileum with an ileocolic-type intussusception.

Pathological diagnosis

Diffuse large B-cell lymphoma.

Treatment

Laparoscopic reduction and appendectomy with postoperative systemic chemotherapy.

Related reports

Intussusception in patients with HIV is often associated with lymphoma, and other causes including Kaposi sarcoma and opportunistic infection had been reported.

Term explanation

After Kaposi sarcoma, non-Hodgkin lymphomas are the second most malignancy that is observed in patients with HIV, and the most common type are diffuse large B-cell lymphoma.

Experiences and lessons

For adult lymphoma-associated intussusception with diffuse intestinal involvement, laparoscopic reduction and appendectomy with intraoperative frozen section could be an alternative to make a definite diagnosis and avoid bowel resection.

Peer-review

CT findings in our case are not specific to lymphoma and could evoke other differential diagnosis.

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von Willebrand factor antigen as a therapeutic target of portal hypertension in cirrhosis

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Abstract

Increased thrombotic potential within the liver sinusoids due to local endothelial production of von Willebrand factor antigen macromolecules could represent an additional therapeutic target of portal hypertension in patients with cirrhosis. In this case, anti-inflammatory and antithrombotic drugs could modulate portal pressure by preventing the formation of intrahepatic platelet-induced microthrombi.

Key words: von Willebrand factor antigen; Endothelial dysfunction; Treatment; Portal hypertension

Core tip: The purpose of this letter to the Editor is to comment on the potential contribution of increased intrahepatic levels of von Willebrand factor as an additional mechanism that could be related to increased portal pressure in patients with cirrhosis and propose drugs which could decrease portal pressure on the basis of von Willebrand factor's production or effects.

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TO THE EDITOR

We read with great interest the article by Garbuzenko[1] on the pharmacotherapy of cirrhosis associated portal hypertension (PH) on the basis of its pathogenetic mechanisms. We fully agree that the major advances that have been made the recent years in our understanding of the pathophysiology of PH need to be translated into novel therapeutic strategies for the reversal of increased portal pressure. In his review, the author highlighted intrahepatic endothelial dysfunction

(ED) and endotoxinemia associated with bacterial translocation (BT) as important targets of future treatment of cirrhosis associated PH. Indeed, a large body of evidence suggests that sinusoidal ED is a key mediator of the pathogenesis of increased intrahepatic vascular resistance *via* a number of mechanisms which synergistically result in decreased hepatic nitric oxide (NO) production[2,3]. On the other hand, BT-related exposure to bacterial products and activation of cytokine cascade, which increase along with the severity of cirrhosis, are thought to play a dual causal role in PH by inducing downstream effects on intrahepatic NO synthesis[3,4] while, in contrast, stimulate NO production in the splanchnic arterial bed with a subsequent increase in portal venous inflow[5].

Apart from NO, the platelet adhesive protein von Willebrand factor antigen (vWF-Ag) has been proposed as a valuable indicator of ED in patients with cirrhosis[6,7]. vWF-Ag is produced and released as ultralarge multimers by activated endothelial cells in several vascular ED disorders[8,9], including inflammatory states[10]. Interestingly, vWF immunostaining is usually positive in large vessels but negative in the sinusoidal endothelial cells in the normal state[11]. On the occurrence of cirrhosis the sinusoidal endothelial cell becomes positive for vWF[12,13], presumably in association with the capillarization of hepatic sinusoids[14]. Based on accumulating data, it can be suggested that vWF-Ag may be a factor which initially links BT-related inflammation and intrahepatic ED, and subsequently predisposes to portal microthombosis with possible clinical implications in future therapeutic approaches to PH.

Circulating vWF-Ag levels have been found to be markedly elevated in patients with cirrhosis. Similarly to BT-related inflammation, plasma levels of vWF-Ag are significantly correlated with the severity of liver disease and PH[7,13,15]. A previous report by Ferro *et al*[7] demonstrated that endotoxemia is strongly correlated with plasma levels of vWF-Ag in the setting cirrhosis. It is also known that on the occurrence of superimposed systemic inflammation in patients with cirrhosis, plasma levels of vWF-Ag increase according to the degree of inflammatory response[16]. In this regard, endotoxin in a dose-dependent manner[7], and inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-8, have been shown to stimulate the release of vWF-Ag from activated endothelial cells[17,18]. Further, the administration of nonabsorbable antibiotics in patients with cirrhosis caused a significant decrease of vWF-Ag plasma levels concomitantly with the decrease of endotoxemia[7]. vWF-Ag is cleaved by the protease ADAMTS13, which is mainly synthesized in the liver[19], into smaller forms which are less potent than the macromolecules in mediating platelet adhesion and aggregation[20]. The inflammatory cytokines TNF-α, IL-4, and IL-8 have been found to suppress ADAMTS13 synthesis in hepatic stellate cells and endothelial cells[18,21], which may contribute to the reduced levels of ADAMTS13 reported in cirrhosis[22].

It can therefore be suggested that increasing BT-mediated inflammatory responses as liver disease progresses predispose to accumulation of vWF-Ag multimers within the liver microcirculation thus enhancing platelet adhesion and aggregation to the sinusoidal endothelium despite the thrombocytopenic conditions of cirrhosis. This could lead to intrahepatic formation of platelet-induced microthrombi, progressive occlusion of portal microvasculature, and intensification of PH. BT-related release of inflammatory cytokines, such as TNF-α and IL-1, could potentiate the prothombotic state produced by vWF-Ag macromolecules within the cirrhotic liver by downregulating hepatic synthesis of protein C[23]. Intrahepatic microthrombi have been demonstrated in patients with cirrhosis and have been associated with accelerated liver fibrogenesis[24], which could further increase portal pressure. Microvascular occlusion of portal vein branches by platelet-rich thrombi due to inflammation stimulated elevation of vWF-Ag levels and decrease in ADAMTS13 activity has also been implicated in the pathogenesis of non-cirrhotic intrahepatic PH[25].

From a clinical point of view, higher concentrations of vWF-Ag levels in plasma[7,13,15] and in liver tissue[13] have been related to more severe PH and increased incidence of decompensation in patients with cirrhosis. Further, we have recently demonstrated in these patients that high levels of thrombin-antithrombin complexes, as a marker of hypercoagulability, was independently associated with major PH-related events, such as new-onset ascites and variceal bleeding, which could be related to the presence of thrombogenic mechanisms operative within the cirrhotic liver[26].

Consequently, available data suggest that increased thrombotic potential within the liver sinusoids due to high concentrations of vWF-Ag macromolecules could represent an additional therapeutic target of PH in patients with cirrhosis. In this case, anti-inflammatory and antithrombotic drugs could modulate portal pressure by preventing the formation of intrahepatic platelet-induced microthrombi.

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