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EDITORIAL

Helminths as an alternative therapy for intestinal diseases

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Abstract

Animal models and clinical studies have shown that helminth infections exert immunomodulatory activity, altering intestinal permeability and providing a potential beneficial action on autoimmune and inflammatory disorders in human beings, such as inflammatory bowel disease (IBD) and celiac disease. This is consistent with the theory that intestinal microbiota is responsible for shaping human immunological responses. With the arrival of the immunobiologic era and the use of antibodies, we propose a distinctive pathway for treating patients with IBD and celiac disease. We have some evidence about the safety and tolerability of helminth use, but evidence about their impact on disease activity is lacking. Using worms to treat diseases could be a possible way to lower treatment costs, since the era of immunobiologic agents is responsible for a significant rise in expenses. Some questions remain to be investigated regarding the use of helminths in intestinal disease, such as the importance of the specific species of helminths used, appropriate dosing regimens, optimal timing of treatment, the role of host genetics, diet, environment, and the elucidation of the exact mechanisms of action. One promising approach is the use of helminth-derived anti-inflammatory molecules as drugs. Yet there are still many challenges with this method, especially with regard to safety. Studies on intestinal permeability point to *Strongyloides stercoralis* as a useful nematode for these purposes.

Key words: Helminths; Strongyloidiasis; Immunology; Inflammation; Inflammatory bowel diseases; Intestinal diseases; Intestinal permeability; Celiac disease

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Core tip: Inflammatory bowel disease and celiac disease are immune-mediated pathologies that remain

a treatment challenge for gastroenterologists. Despite the recent introduction of novel therapies, notably biological agents and newer management strategies, there are still many patients who do not respond, or have a poor response to current treatments. Helminth therapy seems a promising pathway to newer drugs, because it has been proven to alter intestinal permeability, altering the host's immune response to a Type 2 cytokine-mediated response in animal models and pre-clinical studies. This editorial aims to stimulate further research in this field, hoping for better care for our patients.

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INTRODUCTION

The intestinal epithelium functions as an important part of the digestion and absorption of fluids and nutrients. It also plays an immunologic role because it protects the host from environmental pathogens and antigens[1]. When this barrier is altered, it leads to ease of antigen entry and subsequently to immune stimulation and inflammation[2].

Many intestinal diseases have immunogenic components that alter intestinal permeability. Some pathologic processes increase intestinal permeability, such as inflammatory bowel disease (IBD)[3-11], atopic eczema[12], celiac disease, dermatitis herpetiformis[13], cystic fibrosis[14,15], alcohol consumption[16], use of nonsteroidal anti-inflammatory drugs[17-20], and acute infectious diarrhea[12,21]. In contrast, some infections, such as those caused by *Blastocystis hominis*[22], can decrease intestinal permeability and alter the barrier function of the epithelium. These may thus provide an alternative pathway for treating patients by deviating the host's immunologic response.

Intestinal permeability can be measured in many ways. One is *via* measuring urinary clearance of radioactive chromium-51 labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA). When urinary clearance of ^{51}Cr-EDTA is decreased, this indicates decreased intestinal permeability. Werneck-Silva *et al*[23] demonstrated that infection with *Strongyloides stercoralis* can diminish intestinal permeability compared to healthy volunteers (P = 0.0001). Intestinal infection with *S. stercoralis* led to abnormalities in mucus secretion and intestinal motility, as well as possible loss of macromolecules. *S. stercoralis* is a soil-transmitted helminth and is one of the most common parasites that affects patients living in tropical areas[24]. It infects 100 to 200 million people worldwide[25]. It predominantly compromises the mucosa of the duodenum and upper jejunum, although the whole intestinal wall or more extensive segments of the intestine can be involved, especially in immunocompromised patients[26-28].

DISCUSSION

Hygiene hypothesis

The hygiene hypothesis was initially described in the 1970s. It suggests that the higher incidence of allergic diseases in predominantly urban white communities, compared to those rural and indigenous, is due to the less frequent viral, bacterial and helminth infections[29]. A decade later, Strachan proposed that reduced exposure to infections in early childhood, owing to a combination of diminished family size, improved living standards and higher levels of personal hygiene, might result in an increased risk of allergic disease later in life[30]. In addition to allergic disease[31-33], it is believed that the recent increase in other autoimmune and inflammatory disorders, especially in developed countries, could be explained by a similar hypothesis. Many factors may be involved, such as changes in intestinal microbiota during childhood[34].

Helminth infections, in specific intestinal worms, are a particular area of research interest, since they can modulate the host response, inducing immunologic tolerance. Aoyama *et al*[35] have demonstrated an inverse relationship between autoimmune liver diseases, such as primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis, and *S. stercoralis* infection. Recent studies point to the negative effects of deworming, since the helminths are able to not only downregulate specific immune responses, but also to modulate autoimmune and allergic inflammatory responses, contributing to metabolic homeostasis[36]. The study on the use of helminths and their products as anti-inflammatory treatments is a growing field.

Helminth-induced immune responses

Parasitic helminths evolved with the mammalian immune system, promoting their own survival by altering host immune responses[37]. The immune response induced by these worms is dependent on a Type 2 cytokine response, involving the secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13, accompanied by the activation of intestinal mast cells[38], eosinophils, goblet cells, enterocyte proliferation and intestinal contractility[39]. Granuloma formation then occurs, isolating the eggs and larvae, and inducing tissue repair[40]. Other accessory pathways are activated, including the upregulation of regulatory T cell and IL-10 and/or transforming growth factor beta levels, leading

to a predominantly anti-inflammatory response. It has been shown that IL-10- deficient-mice with helminth infections have higher mortality and/or morbidity[41].

The role of CD4+ T cells in expressing Th1, Th2 and Th17 cytokines in human infection with *S. stercoralis* is better explored by Anuradha *et al*[42], who demonstrated a decrease in functional Th1 and Th17 cells and an increase in functional Th2 cells, compared to uninfected individuals. The regulation of Th1, Th2 and Th17 cells was predominantly dependent on IL-10, while the regulation of Th2 but not Th1 or Th17 cells was also dependent on TGFβ. Anuradha *et al*[43] also examined the circulating levels of cytokines in infected individuals (n = 32) compared to those uninfected, discovering significantly lower circulating levels of pro-inflammatory cytokines (gamma interferon, tumor necrosis factor alpha and IL-1) and significantly higher levels of anti-inflammatory cytokines (IL-4, IL-5, IL-9, IL-10, IL-13, IL-27, IL-37, and TGF-β). In addition, treatment of infection led to an opposite immunological response in the two studies. The question is whether these anti-inflammatory properties could be used in intestinal disorders with a predominant Type 1 cytokine response.

Helminth therapy for intestinal inflammation

The ability of helminth infections to alter and/or to suppress immune responses and intestinal inflammation could be useful in IBD[37]. To date, only two species of helminths have been used as clinical treatment: *Trichuris suis*, the pig whipworm, and *Necator americanus*, the human hookworm.

The first is acquired by ingestion of ova and colonization of the caecum and proximal colon of the human gut by worms, which only lasts a few weeks. The second infection develops after percutaneous administration of larvae that migrate to the small intestine, where they survive by feeding on blood from the mucosa. *T. suis*, due to the species-specificity and the lack of chronic infection, requires repeated treatments, although it poses lesser health issues. In the case of *N. americanus*, the long lasting infection means greater risk of anemia and gastrointestinal symptoms, which could be deleterious side effects[37]. To date, there are no studies of *S. stercoralis* for treating intestinal inflammation.

Evidence of helminth therapy in inflammatory bowel disease

Approximately 15 years ago, the first clinical studies of helminth therapy for intestinal disease in humans utilized embryonated viable eggs of *T. suis* in the treatment of ulcerative colitis (UC) and Crohn's disease (CD). These studies showed safety, tolerance and a significant disease remission when oral administration of viable and embryonated eggs was performed repeatedly[44,45]. A placebo–controlled, double-blind, randomized trial in UC patients significantly improved the disease activity index and showed no side effects, although the remission rate was not different than placebo[46]. Another double-blind, placebo-controlled, randomized study (NCT01434693) reported that a single dose of *T. suis* ova (TSO) up to 7500 ova was well tolerated and did not result in short- or long-term treatment-related side effects in CD patients[47].

A brief review of Clinicaltrials.gov reveals three interventional studies of TSO in CD and two in UC. In CD, the studies were sponsored by Coronado Biosciences, which changed its name to Fortress Biotech, and by Dr. Falk Pharma GmbH. TRUST-1 (NCT01576471), a Phase 2 clinical trial evaluating 250 North American patients with moderate-to-severe disease did not improve the disease activity index or remission rates, although a nonsignificant improvement was noted in patients with a more severe disease score.

TRUST-2 (NCT01279577), a double-blind, placebo-controlled, randomized trial of 252 European adults with mildly-to-moderately active ileo-colonic, uncomplicated CD, documented that the administration of fortnightly doses of 250, 2500, or 7500 TSO/15 mL suspension/day over 12 wk, with a four-week follow-up, was safe, with no serious adverse drug reactions. There was a dose-dependent immunological response, but no TSO dose showed a clinically relevant effect over placebo for the induction of clinical remission (CD Activity Index < 150) or response[48].

In UC, the first study was sponsored by the New York University School of Medicine, and the second by the National Institute of Allergy and Infectious Diseases. Both were terminated due to a small sample size and because it was not possible to draw meaningful conclusions. MUCUS (NCT01433471), a randomized, double-blind, placebo-controlled crossover study, was conceived to examine mucosal immunity after therapy with 2500 eggs by mouth every 2 wk for 12 wk. Primary outcomes were designed to better understand the mechanism of action of TSO on the intestinal mucosa and secondary outcomes were to bring about changes in the Mayo Score and in the Simple Clinical Colitis Activity Index.

A second, more controversial approach, was the use of *N. americanus*. Croese *et al*[49] showed that 7 of 9 patients with CD infected with 25-50 larvae followed over 20 wk experienced an improved CD activity index, while the other 2 worsened. There were no search results for interventional studies regarding the use of *Strongyloides, Ascaris, Ancylostoma, Wuchereria, Onchocerca, Toxocara* or *Enterobius* in CD or UC on Clinicaltrials.gov.

Evidence of helminth therapy in celiac disease

There are few studies examining the use of helminths

in celiac disease, most of which with small samples. McSorley *et al*[50] and Daveson *et al*[51] examined 20 celiac patients followed by wheat challenge after 20 wk exposed to 5-10 larvae of *N. americanus*, compared to placebo, at Princess Alexandra Hospital, in Brisbane, Australia. The dose was well tolerated and analysis showed reduced gamma interferon and interleukin-17A in duodenal biopsies. No difference in symptoms was observed.

Another Australian clinical trial, NaCeD study (NCT016619330), evaluated the desensitization and gluten tolerance of 12 diet-managed celiac patients. They were previously infected with *N. americanus* and exposed to small incremental doses of gluten, in the form of pasta, over 12 wk. The mucosal histopathology before and after gluten challenge was examined. There were no significant differences in terms of duodenal villus height and crypt depth ratio and intraepithelial lymphocyte count.

Another clinical trial (NCT02754609) was registered in 2016 by James Cook University in Queensland, Australia, on Clinicaltrials.gov. This trial aims to be a phase 1b multicenter, multinational, randomized, double-blind, placebo-controlled clinical trial with a single-blind arm and an open label extension phase. The objective is to evaluate the safety and predictability of escalating gluten consumption to activate celiac disease. The cohort with diet-managed disease will be treated with placebo or with low- and medium-dose hookworm inocula. The primary outcome is to measure the difference in duodenal villus height and crypt depth ratio between baseline (week 2) and week 42.

There were no search results for interventional studies about *Trichuris, Strongyloides, Ascaris, Ancylostoma, Wuchereria, Onchocerca, Toxocara* or *Enterobius* use in the treatment of celiac disease on Clinicaltrials.gov.

Other uses for helminth therapy

There are other studies examining the role of helminth therapy in allergy, atopy and asthma, with conflicting results. The biggest problem seems to be that these studies proved that preventing allergic reactivity is possible, but only a handful have reported the ability to impact an already established process. In addition to allergy, a number of clinical trials are currently registered for the use of TSO in patients with multiple sclerosis[52], psoriasis, autism and rheumatoid arthritis[37], and for the use of *N. americanus* in patients with multiple sclerosis[35].

Helminth products as possible new drugs

Helminths are complex organisms that have a variety of immunomodulatory substances, such as lipids, carbohydrates and proteins, and jointly defined excretory-secretory products (ES). The identification of helminth products that can be used as biologicals in place of whole parasites is an engaging area of research. The ES-62 glycoprotein from the filarial nematode *Acanthocheilonema vitae* is one of the most studied compounds and is capable of promoting a Th2 response, inhibiting Th1 and Th17. Animal studies have demonstrated the ability of various ES products to inhibit intestinal inflammation in colitis models. These studies suggest a potential way for discovering new drugs for IBD. Concerns about antigenicity and safety need to be clarified prior to clinical testing[53].

Most published studies focus on the use of nematodes and their products in the treatment of intestinal disease. Unlike most studies, there is an ongoing multicenter phase 2 clinical trial, in the recruiting phase, sponsored by the University Hospital, Lille, France, named ACROHNEM (NCT02281916). It is designed to assess safety and tolerability of P28GST (protein 28 Kd glutathion S transferase), aiming to control inflammation in moderate CD, before or after intestinal resection surgery. P28GST is a parasite enzyme molecule from *Schistosoma* with potent immunogenic and anti-oxidant properties. Based on the experimental evidence of its anti-inflammatory properties, investigators hypothesized that the administration of P28GST could protect against recurrence after intestinal resection surgery in CD. To carry out this study, 24 moderate CD patients will be enrolled. Patients with moderate CD will be included after intestinal resection surgery. Drug therapy will consist of three injections of 100 μg of P28GST for 3 mo (one injection per month). The main objective of this study is to assess safety and tolerability in a 1-year follow-up. Secondary objectives are to control immunologic and inflammatory blood and tissue markers and evaluate clinical recurrence as assessed by CDAI (CD Activity Index).

CONCLUSION

The intestinal microbiota is responsible for shaping the human immune system, and the composition of the microbiome can alter and deviate specific host immune responses. Although much has been written about bacteria, we cannot forget that other organisms, such as the helminths, may possibly play an important role in maintaining a "healthy intestinal community"[54].

Mouse models[55] and human cross-sectional studies have shown that chronic helminth infections exert immunomodulatory activity and are able to regulate the host immune response, providing a potential beneficial action on autoimmune and inflammatory disorders in humans, such as IBD, celiac disease, asthma, atopy, allergy, multiple sclerosis, psoriasis, autism and rheumatoid arthritis[37].

We have some evidence about the safety and

tolerance of helminth use, but evidence about the impact on various intestinal diseases is lacking. We need more clinical studies with larger samples, longer follow-ups and standardized doses of helminths and helminth products. Some questions remain to be investigated regarding the use of helminths in intestinal disease, such as the importance of the particular species of helminths used; appropriate dosing regimens (low or high); optimal timing of treatment (before the onset of disease, in acute or chronic disease, or at younger ages); the role of host genetics, diet and environment, and elucidation of the exact mechanisms of protective effect.

In regard to the species of helminth, we believe that the majority of the studies had negative results because of the use of *T. suis*. This pig whipworm induces a less intense and persistent inflammatory response, although Williams *et al*[56] verified that *T. suis* can mature to adult size and reproduce in humans. That is why we see *S. stercoralis* as a more potentially useful nematode, as it has proven to significantly diminish the intestinal permeability in humans[23], altering the interleukin profile in a more systemic way[42]. The prolonged interaction between *S. stercoralis* and its host induces a greater immunomodulatory action. Regarding the appropriate dose and duration of treatment, we have little comprehension of how much and how long is required to exert a significant and beneficial effect; therefore, safety concerns limit the dose that can be applied.

One important challenge is the high polymorphism of the human species, which reacts in a spectral manner to helminth infection. The genetic profile of each individual alters this response. In this context, the identification of helminth-derived anti-inflammatory molecular mediators may be a better and promising approach, since it replicates the benefits without the detriments[57]. There are many challenges with this method, such as the selection of a substance with a good safety profile and low antigenicity that is easily produced and that has a significant impact on clinical trials.

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REVIEW

Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis

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Abstract

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are complex diseases that result from the chronic dysregulated immune response in the gastrointestinal tract. The exact etiology is not fully understood, but it is accepted that it occurs when an inappropriate aggressive inflammatory response in a genetically susceptible host due to inciting environmental factors occurs. To investigate the pathogenesis and etiology of human IBD, various animal models of IBD have been developed that provided indispensable insights into the histopathological and morphological changes as well as factors associated with the pathogenesis of IBD and evaluation of therapeutic options in the last few decades. The most widely used experimental model employs dextran sodium sulfate (DSS) to induce epithelial damage. The DSS colitis model in IBD research has advantages over other various chemically induced experimental models due to its rapidity, simplicity, reproducibility and controllability. In this manuscript, we review the newer publicized advances of research in murine colitis models that focus upon the disruption of the barrier function of the intestine, effects of mucin on the development of colitis, alterations found in microbial balance and resultant changes in the metabolome specifically in

the DSS colitis murine model and its relation to the pathogenesis of IBD.

Key words: Dextran sodium sulfate; Experimental colitis; Inflammatory bowel disease; Pathogenesis; Intestinal barrier

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Core tip: In the last few decades the proliferation of research in experimental colitis models of inflammatory bowel diseases (IBD) has had profound effects in our understanding of human IBD pathophysiology as well as to exploit potential therapeutic avenues outside of immunologic therapy. The dextran sodium sulfate colitis model, through its rapidity, simplicity, reproducibility and controllability has been instrumental in our understanding of intestinal barrier function through the dysregulation of mucin, interaction with the intestinal microbiome and metabolome.

Eichele DD, Kharbanda KK. Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. *World J Gastroenterol* 2017; 23(33): 6016-6029 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6016.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6016

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine caused by a dysregulated immune response. Crohn's disease (CD) and ulcerative colitis (UC) are the principal types of IBD. Both usually involve severe diarrhea, pain, fatigue and weight loss. IBD can be debilitating and sometimes leads to life-threatening complications. A convenient and time honored approach to study the pathogenesis and complexity of human IBD has been the development of a variety of animal models. These animal models have provided meaningful and indispensable insights into the histopathological and morphological changes in the intestinal tract related to the pathogenesis of human IBD[1]. While no single model has proven to sufficiently represent the complexity of the clinical and histopathological characteristics of human disease, the collective data obtained from these various animal models have provided a more detailed understanding of the underlying principles of human IBD pathogenesis[2,3]. These models have become an indispensable tool to elucidate the histopathological, immunological and morphological changes in the intestinal tract and potential therapeutic targets. These various models can be grouped into categories broadly defined as spontaneous colitis, chemically inducible colitis, genetically modified and adoptive transfer models[1,4-7].

The most widely used mouse model of colitis employs dextran sodium sulfate (DSS), a chemical colitogen with anticoagulant properties, to induce epithelial damage. The DSS colitis model lends itself to IBD research due to its rapidity, simplicity, reproducibility and controllability. Acute, chronic and relapsing models of intestinal inflammation can be achieved by modifying the concentration of DSS and the frequency of administration[8]. There are excellent and exhaustive reviews focused on the immunological aspects of experimental animal models in inflammatory bowel disease and we would recommend the reader to refer to these articles for the specifics in immunology[2,9]. In this review, we aim to provide an updated and concise review of the less publicized aspects of research in murine colitis models that focus upon the barrier function of the intestine in one specific chemically induced experimental colitis model, the DSS colitis murine model.

HUMAN IBD PATHOPHYSIOLOGY: MECHANISTIC INSIGHTS

IBD is a disorder of chronic intestinal inflammation without an exact etiology. The leading hypothesis on IBD pathogenesis states that it is an inappropriate and overly aggressive inflammatory response to enteric microbes in a genetically susceptible host with environmental factors precipitating the onset or reactivation of disease[10,11]. Epidemiologic data suggest these environmental factors include antibiotic use, microbial exposure and possibly dietary components[12-15]. Host genetics, luminal microbiome and its associated antigens and immune response, all have been implicated in playing important roles in IBD pathogenesis (Figure 1).

Genetics

Advances in IBD genetics have indicated modifications in genes regulating mucosal barrier integrity and function, innate immune response and microbial homeostasis[11]. Thus far, four genes have been associated with increased susceptibility to CD and one gene mutation has been associated with ulcerative colitis[11]. The most widely known and studied gene implicated in IBD is the gene for *CARD15*, formerly known as *NOD2*, which is responsible for luminal bacterial recognition. The leucine-rich repeat region of *CARD15* binds muramyl dipeptide (MDP), which is the active moiety of peptidoglycan. This binding of bacterial peptidoglycan to MDP activates NFκB and mitogen-activated protein kinase signaling pathways, causing the production of various cytokines including TNF and IL-1β[10,16,17]. In normal circumstances, the pro-inflammatory cytokine secretion by intestinal APCs is minimal, yet bacterial killing occurs. This indicates that the intestinal immune

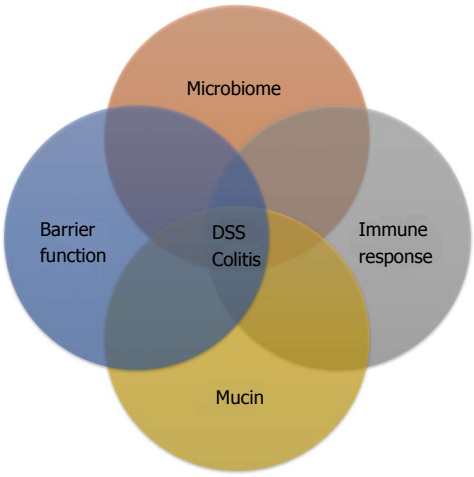


Figure 1 Factors that play important roles in the pathogenesis of inflammatory bowel diseases. DSS: Dextran sodium sulfate.

system can defend against luminal microbiota without appreciable tissue injury[10]. The importance of NFκB activation in the clearance of invasive bacteria is underscored by the observations that *CARD15* gene mutation fails to clear Salmonella from epithelial cells leading to increased bacterial interaction with the host's luminal defense mechanisms. Indeed, heterozygosity for a *NOD2* polymorphism confers a 1.75 to 4-fold increased risk whereas homozygosity confers even a much higher risk (11 to 27-fold) for development of IBD[10,18].

Microbiome and dysbiosis

A complex network of interactions exists between the host intestinal epithelial cells (IEC), host immune cells and the abundant intestinal microbiota. An altered balance of commensal pathogenic microbiota could lead to a pro-inflammatory milieu that exacerbates intestinal inflammation[11]. The dysbiosis theory suggests that intestinal microbiota of IBD patients change in diversity, composition and localization compared to that of healthy controls[19-24]. Fecal microbiota studies on IBD patients have revealed a decreased frequency in Bacteroidetes and Firmicutes and an increase of Proteobacteria and Actinobacteria phyla[21,23-26]. Advances in metagenomics sequencing of microbial RNA have confirmed decreases in bacterial composition and diversity in IBD patients compared to normal population[15]. Likewise, defects in the intestinal epithelial barrier integrity suggests increased uptake of luminal antigens that further leads to persistent immune activation[11]. Evidence suggests that reduced mucin production, through goblet cell depletion and epithelial cell tight junction dysfunction, by facilitating the access of gut luminal antigens to the intestinal mucosa, are also involved in the pathophysiology of IBD[15]. Additionally, *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum and one of the most abundant species in the healthy human colon, is underrepresented in IBD patients and altered in terms of disease activity specifically in CD patients[19,20,24,27-30].

It is still uncertain as to whether the dysbiosis is the primary cause of inflammation or merely a secondary phenomenon of IBD. The uncertainty stems from the fact that intestinal microbiota analysis in IBD patients is performed after the development of the disease rather than prior to development[22]. However, the pathogenic role of the microbiome in IBD pathogenesis and therapy is indicated by studies showing an improvement in IBD by antibiotic treatment[31,32]. Indeed, treatment of UC patients with antibiotics improves mucosal inflammation[15]. Further, remission of inflammation and mucosal healing was observed in CD patient's that underwent diversion of feces with subsequent disease reactivation after infusion of feces[33]. Likewise, defects in the intestinal epithelial barrier integrity suggests increased uptake of luminal antigens that further leads to persistent immune activation[11]. Evidence suggests that reduced mucin production, through goblet cell depletion and epithelial cell tight junction dysfunction, by facilitating the access of gut luminal antigens to the intestinal mucosa, are also involved in the pathophysiology of IBD[15].Collectively, these observations attest to the role of microbial dysbiosis in the induction of IBD[34].

Immune response

The innate and adaptive immune system activation and dysfunction as well as a loss of tolerance to enteric commensal bacteria contribute to the abnormal inflammatory response in the intestinal tract in patients with IBD[11,35]. Characteristic histological findings in IBD is an influx of innate immune cells (neutrophils, macrophages, dendritic cells and NK cells) as well as adaptive immune cells (B cells and T cells) into the lamina propria. With activation of immune cells, there is an elevation in the TNF-α, IL-1β, IFN-γ and cytokines levels. Recent advances in genome-wide associated studies and immunological studies suggest aberration in the mucosal innate response, innate microbial sensing, autophagy and unfolded protein response are potential pathogenic pathways in IBD[35].

Microbial antigen sensing by the innate immune system is mediated by pattern recognition receptors (PRR), such as Toll-like receptors (TLR) that recognize pathogen associated molecular patterns. The stimulation of PRRs results in a signaling cascade in which NFκB activation produces pro-inflammatory mediators, thereby ensuring an effective and appropriate innate response to presented antigens. Specific TLR that have been implicated in CD include nucleotide-binding oligomerization domain (NOD)-like receptors, specifically *NOD2*, which encodes an intracellular sensor and is stimulated by specific components of bacterial peptidoglycan that results in NFκB activation as mentioned in the earlier section on genetics[35].

Adhesion molecules, such as intercellular cell adhesion molecule 1 (ICAM1) are necessary for the circulating cells to adhere to the activated endothelium, which leads to extravasation of mononuclear and polymorphonuclear cells into the inflammatory tissue[11]. In addition, adhesion molecules mediate migration of the extravasated immune cells through the stroma to the source of maximal chemokine production, as well as through the epithelium to the lumen, where they produce crypt abscesses[11]. Historically, the NFκB pathway was thought to elicit a pro-inflammatory response, but selective gene-deletion studies have shown that this pathway produces both beneficial and deleterious effects[11]. NFκB activation stimulates expression of numerous molecules that are involved in the inflammatory response such as IL-1β, TNF, IL-6, IL-8, ICAM1 and other adhesion molecules[11]. NFκB also stimulates the expression of protective molecules, *CARD15*, cyclo-oxygenase 2, B defensins, TNF-induced protein 3 that inhibit the inflammatory response[11].

IL-23 is a key cytokine in orchestrating the crosstalk between innate and adaptive immunity and has a central role in driving early responses to microbes[35]. Interestingly, IL23R polymorphisms have been associated with both CD and UC, suggesting that the IL-23 axis might represent a shared inflammatory pathway in chronic intestinal inflammation[35]. Recent studies have shown that, besides its activity on Th17 cells, IL-23 can also act on cells of the innate immune system. Unconventional, innate-like T cell populations, which are particularly represented at mucosal sites, have been found to respond to IL-23 stimulation and secrete Th17-related cytokines[35].

The adaptive immune system is highly specific and confers long lasting immunity and is adaptable with its antigen specificity and maturation[35]. The inhibitory cytokines IL-10 and TGF-β in Peyer's patches, mesenteric lymph nodes and lamina propria are involved in T-cell tolerance in the intestine[10]. Immunologically Th0 cells are activated and differentiate into Th1, Th2 or Th17 cells based upon the clearance of specific pathogens, but a dysregulated T cell response with abnormal development of activated T cells subsets may lead to the onset of inflammation by an excessive release of cytokines and chemokines with have multiple pathogenic effects[35]. Moreover, there is a genetic association between the inhibitory cytokine IL-10 and UC, in which IL-10 participates in the down-regulation of intestinal inflammation[10]. CD had been thought to be a Th1 mediated disease, secreting copious amounts of IFNγ, TNF-α, and IL-12; whereas UC was thought to be a Th2 mediated response, with signature cytokine secretion from IL-4, IL-5 and IL-13[15]. However, recent data has suggested this paradigm is not quite so straightforward[15]. Furthermore, data suggests that Th17 cell production of IL-17 and IL-23 play important roles in the pathogenesis of IBD, with DCs isolated from CD patients producing more IL-23 than UC patients[15].

Th17 cells are a subset of helper T-cells that are induced by IL-6 and TGF-β and expanded by IL-23[15]. Signaling is mediated through the engagement of heterodimeric IL-23 with its heterodimeric receptor that activates the JAK-STAT signaling pathway, which then regulates the transcription of several genes[10]. IL-23 secreted by macrophages and dendritic cells may contribute to Th17 proliferation, survival or both[10].

PATHOPHYSIOLOGY OF DSS-INDUCED COLITIS: AN INVALUABLE ANIMAL MODEL

Numerous animal experiment in the last 25 years have employed the DSS colitis model as a chemical induction of intestinal inflammation model that morphologically and symptomatically resembles epithelial damage seen in human ulcerative colitis[1,36,37]. The DSS model, initially reported by Okayasu *et al*[38], and has been used to investigate the role of leukocytes in the development of colitis in animal models and is now one of the most extensively employed experimental models abetted by its simplicity and reproducibility[38,39].

DSS is a water soluble, negatively charged sulfated polysaccharide with a highly variable molecular weight ranging from 5 to 1400 kDa. Murine colitis results from administration of 40-50 kDa DSS added to drinking water. In the DSS model, the sulfated polysaccharide does not directly induce intestinal inflammation, but rather acts as a direct chemical toxin to colonic epithelium resulting in epithelial cell injury. The proposed and most accepted mechanism by which DSS induces intestinal inflammation results in the disruption of the intestinal epithelial monolayer lining, leading to the entry of luminal bacteria and associated antigens into the mucosa and allowing the dissemination of proinflammatory intestinal contents into underlying tissue[2,4,6,38,40-42].

The effectiveness of the DSS-induced colitis depends on several factors, including DSS concentration (usually 1%-5%), duration and frequency of administration (acute or chronic), molecular weight of the manufactured DSS, strain of animals (C3H/HeJ, C57BL/6 and BALB/c mice strains are more susceptible), and microbial environment of animals (*i.e.* germ-free versus specific pathogen-free). Furthermore, depending upon these various factors, the animals may develop acute colitis, chronic colitis or even colitis induced dysplastic lesions (Table 1).

In various protocols, 40-50 kDa DSS is added to sterilized drinking water at various concentrations to produce the desired inflammatory effect. At this molecular weight the tissue distribution reveals that DSS penetrates the mucosal membrane of the intestine[4]. Acute colitis is induced by administering DSS to the chosen strain of mice, often C57BL/6 or BALB/c male mice, for a period of 6-10 d (average 7 d). Alternatively, chronic colitis is induced by

Table 1 Factors that influence effectiveness of dextran sodium sulfate to induce colitis

Factors	Variables	Description
DSS	Molecular weight	40-50 kDa for tissue penetration (larger molecule does not penetrate colonic tissue well and smaller molecule has poor distribution)
	Dosage concentration	Ranges from 1.5%-3% used most frequently (1% with mild symptoms and delayed onset)
	Duration of therapy	Acute: 5-10 d administration
	Manufacturer/batch	Chronic: 4-5 repeating cycles of DSS and sterile water
		Various manufacturers with differing potency
Host	Genetically susceptible strain of animal	Certain strains are more susceptible to DSS colitis than other strains. Susceptible strains: C3H/HeJ, C57BL/6, BALB/C
Environment	Housing Conditions	Group *vs* individual unit, frequency of cage changes alters coprophagy by host
	Microbial State	Germ-free *vs* specific pathogen free *vs* wild type

DSS: Dextran sodium sulfate.

administering 4-5 repeated cycles of DSS, each cycle involves administration of various DSS concentrations for 1 wk followed by sterile water for 7-14 d[1,38,43,44]. The severity of colitis can be augmented based upon the duration of DSS administration as well as the concentration of DSS[1]. During DSS administration, mice can exhibit pronounced weight loss (about 5%-10% reduction by day 5), altered stool consistency leading to diarrhea and hematochezia[41]. A significant physiological indicator of animal stress and imminent demise occurs if weight loss is greater than 20% of initial weight[41]. Following the desired protocol, the mice are then sacrificed and tissue samples are collected for histological analysis and other assays[8,41].

Observations of the chronological changes induced by DSS administration have revealed that the signs of disease appear as early as 1 d of treatment primarily identified by changes in the expression of tight junction proteins such as occludin, Zonula occludens-1 (ZO-1) and various claudins[4,8,40]. The altered expression of tight junction complexes and increased epithelial apoptosis has been identified in human IBD and is thought to contribute to leaks in the epithelial barrier[4]. These modest initial effects are followed by increasingly worsening symptoms, including increased intestinal permeability, severe bleeding and mortality[8]. The typical histological changes induced by acute DSS include mucin and goblet cell depletion, epithelial erosion, ulceration and infiltration of granulocytes into the lamina propria and submucosa resulting in immune responses[2,8]. In chronic DSS protocols, additional histological changes such as crypt architecture disarray and widening of the gap between the base of the crypt and muscularis with deep mucosal lymphocytosis tend to appear a few weeks after DSS induction[8]. Furthermore, transepithelial migration of neutrophils resulting in cryptitis and crypt abscess, a common histologic finding in human IBD, is reproduced in mice subjected to the chronic DSS administration[8]. With prolonged administration of DSS in rodents, squamous metaplasia of rectal mucosa, adenomatous changes and adenocarcinoma can also be seen histologically[45].

It has been identified that the molecular weight of DSS is critically important to the induction, severity and of DSS induced colitis[4]. Carcinogenic activity in the colon is achieved by DSS of 50 kDa while larger and smaller molecular weights (520 kDa and 10 kDa, respectively) failed to induce activity due to inadequate tissue penetration[4]. Additionally, the repeated pulses of DSS administration utilized in chronic phases of DSS colitis results in dysplasia that frequently resembles the clinical course in human UC[31].

The knowledge of the unique or exact mechanisms that result in carcinogenesis underlying colitis-associated cancer (CAC) in humans is lacking. The transition from inflammation, to dysplasia and hence cancer is not fully elucidated, but it has been proposed that a host of multiple factors are integral in the role of CAC including; immune response, activation of oncogenes, inhibition of tumor suppressors, as well as alterations in intestinal microbiota[46]. A variety of murine models of CAC have been developed. The most widely used and best studied is a chemically induced colitis-associated model that incorporates a combination of a potent carcinogen, azoxymethane (AOM) and DSS[47]. Long term administration or repeated cycles of DSS has been shown to induced chronic colitis and subsequent dysplasia in rodents[48]. In this murine model, as in human CAC, the degree of inflammation correlates with dysplasia and is associated with nuclear translocation and mutational activation of β-catenin which results in increased activity of the Wnt signaling pathway[46,48]. The change in Wnt signaling results in enhanced inflammatory immune response with release of pro-inflammatory cytokines (IL-6 and TNF-α), which results in elevated levels of c-myc, a known oncogene and activator of cell cycle progression[46]. Additionally, many important inflammatory components are increased and activated during CAC (NFκB, Janus Kinase, cyclooxygenase-2 and inducible nitric oxide synthase) leading to further infiltration of lymphocytes, plasma cells and macrophage to sites of chronic inflammation[46]. The limitation of the models is that Kras or p53 mutations, that are typical in human CAC, are not present in murine AOM/DSS-induced CAC models[46].

DSS colitis is associated with increased production of various cytokines and chemokines. Following

the induction of DSS colitis various tissue derived cytokines have been shown to be upregulated as early as the first day of DSS-induction[4]. The different inflammatory mediators assessed include TNF-α, the hallmark of DSS induced colitis, IL-6, IL-10, IL-17, IL-1β, TGF-β, mucin, TLR2/4 gene expression, MPO activity[1,4]. Differences in inflammatory profiles was expressed between acute and chronic DSS phases[4]. It was shown that in acute inflammation in DSS converts to a predominant Th-2 mediated response in the chronic state with noted decreased levels of TNF-α, IL-17 and elevated levels of IL-4,-6,-10 and IFNγ[4,49]. IL-6, IL-1β tissue levels correlate with IBD activity and IFNγ secretion has been linked to IL-17 section which tends to be expressed during chronic inflammation[31]. It has been shown that the cytokine profiles in DSS colitis phases correlates with barrier function, histological and clinical parameters lending the model as an integral tool in the study of cytokine role in induction and recovery from inflammation[4]. The bulk of research on IBD pathophysiology till date has focused on the immunological response and less attention has been paid to defects in barrier function that can potentially increase bacterial contact with the epithelium[50]. In animal studies assessing epithelial damage, DSS has been considered a toxicity model as induction of intestinal inflammation is not direct. Rather the chemical exerts an epithelial injury resulting in intestinal epithelial barrier disruption causing an influx of luminal bacteria and associated antigens into the mucosa and submucosa and thus triggering an inflammatory reaction[51]. An alternative mechanistic action for the induction of inflammation in the DSS model is through dysbiosis of murine gut microbiota leading to immunoregulation defects, mucin and goblet cell depletion and barrier dysfunction. Although the exact mechanism through which DSS induces colonic mucosal inflammation is not completely understood, recent results indicate that sulfate groups of the DSS molecules destabilize the mucus layers and make it more permeable to bacteria[52]. Hence, the DSS model is not simply a toxicity model, but also a barrier dysfunction model that encompasses mucus loss and the eventual bacterial penetration frequently found during intestinal trauma[53].

DSS DISRUPTS INTESTINAL BARRIER FUNCTION

The function of the intestinal epithelium is to simultaneously provide a barrier between the host and external environment while facilitating selective permeability that limits migration of harmful molecules but allows appropriate absorption of nutrients, ions and water[19]. This dynamic relationship of selective permeability is dependent upon specialized structures composed of tight junction complexes[40]. Tight junctions (TJ) are protein complexes consisting of Occludin, ZO, Claudins and junctional adhesion molecules (JAM) that are located at the apical ends of the lateral membranes of IEC and form a physiologically active barrier that can alter permeability based on the cellular environment[40,54-56] .

Dysfunctions of intestinal barrier lead to increased intestinal permeability that have been associated with the pathogenesis of IBD[2]. Barrier dysfunction in IBD has been identified as shifts in tight junction protein expression, and function with poorly adherent mucosa in the inflamed intestinal mucosa[2,40,43,54,57,58]. This change in composition and function corresponds to an increase in intestinal permeability with entry of commensal bacteria and decrease in transepithelial resistance[2,40,57]. The question remains whether the alteration in the intestinal barrier function is a primary necessity for the inflammatory response or a secondary development from the inflammatory response[40].

Several studies have shown that the appearance of intestinal inflammation is not the initial, inciting event, but rather the TJ complex changes and the subsequent increase in colonic permeability precede the development of intestinal inflammation[38,40,42,43,57,59]. In mice DSS colitis leads to a decrease in TJs expression that is followed by an increase in permeability and clinical manifestations of colonic inflammation[19]. Specifically, the protein pattern of TJ undergo rapid changes, as evident in the increased expression of claudin-2 and depletion of various claudins and zona occludens-1[19,40,54]. Therefore, the breach in the mucosa barrier is seen as a secondary event to the increase in colonic mucosal permeability resulting in the influx of inflammatory cells into the intestinal mucosa[40,57].

As noted earlier, the TJ are comprised of protein complexes that include occludin, ZO, claudins and junctional adhesion molecules. Importantly, claudin proteins are intrinsically involved in the formation of the IEC barrier function[60]. Claudins comprise a multigene family of 27 members and are expressed in a pattern that is both organ and segment-specific. A majority of these claudins (claudins 1, 3, 4, 5, 7, 8) confer barrier properties and are often found in tight epithelia of distal intestine. Claudins interact in a tissue-specific manner to form a charge-selective and size-selective barrier and predominantly contribute to epithelial barrier function and regulate paracellular permeability in intact epithelium[61]. Several mouse studies have assessed the expression of various claudins in conjunction to DSS administration. So far, the results have been varied. One study found enhanced expression of the claudin-1 protein, whereas other studies found decreased expression of claudin-1[54,60]. These contradictory findings could be related to species differences as claudin-1 was increased in rats while decreased in mice when exposed to DSS. Further studies conducted by independent laboratories reported similar findings demonstrating an up-regulation of the pore-forming claudin-2 and decreased expression

in claudin-3,-5,-7,-8[60]. Furthermore, alterations in claudin expression resulted in similar outcomes with pronounced barrier dysfunction with aggravated mucosal damage and increased colonic permeability in DSS-induced colitis[60] .

Claudin-2 has been shown to provide a critical role in regulating colonic epithelial homeostasis and barrier function; therefore, regulating mucosal immune response and mucosal inflammation. A frequent regulatory step in inflammation is the increased expression of claudin-2 and it insertion into TJ strands. Upregulation of claudin-2 expression is found to start in the lower crypt and progress toward the surface epithelium[56]. The functional role of this modified expression and the consequential increase in intestinal permeability is still uncertain[56]. Consequently, recent human studies have examined potential alterations in claudin family functions n IBD patients and demonstrated a robust increase in claudin-2 expression[62]. Similarly, claudin-2 was found to assist the uptake of mucosal antigens[62]. A corollary finding in DSS-treated Cl-2TG mice revealed significant suppression of pro-inflammatory molecules with overexpression of claudin-2 suggesting its pivotal role in immune adaptation[62]. The paradoxical finding of increased regulatory CD4+ cells of unchallenged mice and decreases in immune cell infiltration of DSS-challenged mice suggests that claudin-2 induced epithelial permeability facilitates the interaction of host immune molecules and luminal antigens to promote adaptive tolerance and protection from colitis rather than increased sensitization[62]. Additionally, the DSS-challenged mice showed decreased apoptosis and increased epithelial proliferation further supporting the role of claudin-2 in intestinal epithelial cell regulation[62]. Conversely, claudin-2 knockout mice subjected to DSS exhibited severe colitis[62].

In addition to changes seen in claudin expression, other studies assessing barrier dysfunction assessed changes in ZO-1. Merely one day of DSS administration caused a statistically significant reduction of ZO-1[40]. Thus, the loss of TJ integrity led to an increase in permeability which occurred before any significant clinical or histological evidence of colitis[40]. Another study supporting this premise reported a redistribution of occludin and ZO-1, from the junctions in colonic epithelium after 4 d of administration of DSS[42]. As seen in the claudin studies, the ZO-1 data presented suggests that TJ complex changes are a prerequisite for the development of intestinal inflammation[40,42].

Another adhesion molecule with essential roles in the development and homeostasis of several tissues is the large group of proteins E-cadherin, that represent the major component of adherens junctions[63]. Studies suggest that E-cadherin may factor into the pathogenesis of UC as mice with E-cadherin deficiency had more severe colitis in the DSS model[63]. Further, similar to redistribution of occludin, ZO-1, E-cadherin and B-catenin also translocate from the junctions in colonic epithelium after 4 d of DSS treatment[2,42].

DSS EFFECTS ON MUCIN

Observations in several human studies found patients with colonic inflammation had alterations in colonic mucus and decreased effectiveness in its barrier function[64]. The gastrointestinal tract is quite remarkable with respect to the protective mucus barrier organization. The secretory, gel-forming mucins form the outer loose layer of mucus and the inner dense membrane-bound mucins covers and protects the surface epithelial cells[64,65]. Biochemically, mucins are usually very large, filamentous molecules with a large region within their polypeptides, which comprise relatively short tandemly repeated peptide domains which are highly O-glycosylated[66]. In humans, fifteen different mucins have been described and are assembled in the MUC gene family, with only a few encoded for activity in the colon[64,66]. Of the mucins, only Muc2 has been shown to be the principle secretory gel-forming mucin in both large and small intestines providing the functional barrier between epithelium and microbiota[64,65]. Muc2 is the predominant mucin produced by intestinal goblet cells, and is thickest in the healthy colon[67]. Whereas, the other membrane bound mucins with transmembrane regions are involved in cell signaling, adhesion, growth and modulation of the immune system[64,66].

As mucus is in direct contact with many microorganisms within the intestinal tract, defects in gene coding or protein folding of the mucins could lead to poor membrane integrity and ultimately a breach in the epithelial barrier or alterations in the mucosal-bacterial interactions. Prior *in-vitro* studies on intestinal cell lines revealed that the mucin expression and structure is influenced by cytokines, bacteria and their associated components[66,67]. Likewise, in UC the alteration of immunological or bacterial factors can influence mucin production[67]. Goblet cell depletion is a frequent histopathology finding in UC patients implying that the synthesis of Muc2 is decreased in association with smaller goblet cells thecae and histologic appearance of goblet cell depletion[67]. This, in turn, leads to further adverse effects resulting in chronic inflammation that is characteristic of IBD[64,66]. With these considerations in mind, it is still uncertain whether mucin decrease and goblet cell depletion are the primary contribution to IBD or the consequences of inflammation[67].

Mice deficient in Muc2 are characterized by a loss of mucus layer with bacteria not only in direct contact with epithelial cells but found deeper in the crypts (Table 2). These observations are absent in healthy animals. Furthermore, mice deficient in Muc2 ultimately are more susceptible to spontaneous severe colitis and eventually cause an increased risk of colon cancer development[52,68-70]. Beyond DSS mouse models, other models with defective mucus

Table 2 Role of the different classes of mucins in dextran sodium sulfate induced colitis

Mucin gene	Mucin class	Expression site (GI tract)	Chromosome	Pathological findings with DSS treatment	Ref.
Muc1	Membrane bound	All epithelia	3	Muc1$^{-/-}$ protective when challenged with DSS Increased thickness with adherent mucus	[64,66]
Muc3	Membrane bound	Intestine, enterocytes	5	Increased up-regulation of *Muc3* gene	[65,66]
Muc4	Membrane bound	All epithelia	n.d.	Large type I transmembrane glycoprotein Muc4$^{-/-}$ more resistant to colitis due to up-regulation of Muc2 due to increase proliferation of cytokines	[65,66,73]
Muc2	Secretory	Intestine, goblet cells	7	Muc2$^{-/-}$ more susceptible to spontaneous colitis, increased risk of CAC DSS produces fulminant colitis *vs* wild type	[66-68]
Muc5ac	Secretory	Stomach	7		[66]
Muc5b	Secretory	Tongue, sublingual glands	7		[66]

CAC: Colitis-associated cancer; DSS: Dextran sodium sulfate.

function all develop colitis[50,71,72]. In order to fully comprehend the role of mucins, specifically Muc2, in epithelial barrier protection and colitis, studies were conducted on Muc2 deficient mice. DSS administration to these mice produced fulminant colitis within days compared to the treated wild-type mice[68]. Further, a single missense mutation in Muc2 lead to spontaneous colitis with increased intestinal permeability and increased cytokine production in the distal colon[67]. Additionally, during DSS induction the thickness of the mucus gel decreased at the same time as the mice developed increasing colitis symptoms[64]. Analysis of IL-10$^{-/-}$ mice revealed thicker mucus layers than wild type, but in further analysis specific mucin properties were altered and the usually impenetrable inner layer was found to be penetrable to intestinal bacteria[52]. Therefore, mucus thickness alone was not shown to be a meaningful predictor or indicator of mucus barrier function[52].

Post-translations modifications on mucin are key to their functionality. Along these lines, an increased levels of Muc2 seen following DSS administration fails to control inflammation because of decreased sulphation in Muc2[66,67]. Further studies have shown that alteration in glycosylation of mucins resulted in decrease Muc2 synthesis and a diminished mucus barrier, thereby increasing the susceptibility to DSS-induced colitis[67]. The IL-10$^{-/-}$ germ free mice given normal enteric bacteria, presenting with a loss of sulphation of newly synthesized Muc2 molecules, were more prone to develop colitis and produced severe and chronic colitis[66]. Collectively, these observations underscore the importance of post-translational modification on mucin in its functional role in regulating intestinal barrier integrity[67].

In conjunction with the secretory mucins, the various cell surface mucins are considered to serve a critical function in mucosal protection[65,67]. Muc4 is a large type-I transmembrane glycoprotein component normally expressed on the surface of colonic epithelial cells. Muc4$^{-/-}$ mice were more resistant to colitis and CAC induced by AOM/DSS because of the compensatory upregulation of Muc2 expression in these mice[65]. Although not statistically significant, an upregulation of Muc3 (orthologue of human Muc17) was also observed[65]. Additionally, Muc4$^{-/-}$ mice induced with DSS had increased expression of pro-inflammatory cytokines (TNF-α and IL-1β) which could also be responsible for the observed upregulation of Muc2 and Muc3[65]. Although both Muc4 and Muc13 belong to the transmembrane mucin subtype, deletion of either of the two mucin genes results in opposing phenotypic response to DSS treatment. Muc13$^{-/-}$ mice had increased macrophage expression in the inflamed mucosa accompanied by increased expression of intestinal IL-1β and TNF-α mRNA and significantly increased loss of body weight, diarrhea score, fecal blood score and severe histologic damage including intestinal epithelial cell apoptosis following DSS exposure. This suggests Muc13 confers protection to colonic epithelial cells from apoptotic stimuli, preventing damage-induced cell death[67]. In contrast, Muc4$^{-/-}$ mice exhibit a protective phenotype (decreased loss of body weight, diarrhea score, fecal blood score and less severe histologic damage) which is related to a compensatory upregulation of Muc2 and Muc3[65,73].

Muc1, a non-gel forming mucin transmembrane mucin, when deleted resulted in protection of colonic epithelial cells similar to findings in Muc4$^{-/-}$ mice when challenged with DSS[64]. Muc1$^{-/-}$ deficient mice had increased thickness of adherent mucus resulting in mild colitis[64]. Additionally, germ-free mice had a thin colonic mucus barrier, but luminal exposure to the bacterial products lipopolysaccharides (LPS) and peptidoglycan quickly restores the firmly adherent mucus layer thickness to levels observed in conventionally housed mice[64]. Muc1$^{-/-}$ mice developed a more severe inflammatory response after exposure to *H pylori* compared with wild-type mice, demonstrating that cell surface mucins can modulate the inflammatory response to chronic infection[67]. As opposed to observations in Muc2 deficient mice resulting in severe colitis, the Muc1$^{-/-}$ mice developed a very mild colitis which is thought to be due to an increased colonic mucus barrier and a decreased ability to recruit T cells to the affected region[64].

DSS ALTERS THE MICROBIAL BALANCE

The intestinal microbiome plays an integral role in host immune development, tolerance and intestinal physiological processes. This has spurred large collaborative efforts aimed at identifying and characterizing the microorganisms which are associated with the health and disease in humans[25,32,74,75]. However, the sheer presence of microbiota in the intestinal tract alone is not enough to exert these physiological effects, rather variant microbe compositions, temporal changes in populations and relative abundance of specific microbes are important in homeostasis and specific disease states, namely IBD[66,74-76].

Effects on mucosal inflammation

Studies involving DSS-induced colitis have revealed the critical role that gut microflora play in the pathogenesis of mucosal inflammation and the related role of barrier function as a bulwark against extensive stimulation of the mucosal immune system. However, the exact mechanism through which bacteria induce inflammation has been elusive[22,76]. Indeed, a number of recent studies have identified compositional changes at the bacterial phyla and species levels that can influence phenotypic expression of both pro- and anti-inflammatory responses in humans and in murine models relevant to IBD[22,25,74,76].

As indicated earlier, microbiome composition alterations can result in normally underrepresented members of the microbiome to become dominant, leading to perturbations in structure and function of the microbiome. Various DSS-induced colitis studies have noted that murine microbiota alterations occurred early (within 3 d) and are characterized by diversity reduction and changes occurred prior to the clinical or biochemical evidence of inflammation[31,75,77]. Composition changes in DSS-induced murine colitis indicated a significant proliferation in Bacteroidaceae and Clostridiaceae families[38]. Further studies have corroborated these initial findings to suggest dramatic reductions in the genera of Bacteroidetes, Prevotella, Clostridium and Lactobacillus with corresponding increases in pro-inflammatory gut microbiotic components Bacillaceae, Enterococcales and Enterobacteriaceae[31,78,79].

Effects on barrier functions

Beyond the simple changes in microbiome composition that may herald alterations in physiologic homeostasis, other recent studies have examined the role that bacteria influence barrier function alteration. Evidence suggests that epithelial integrity is compromised in DSS-induced colitis leading to penetrance of microbes and associated antigens into the mucosa and[69,75,80,81]. One such study examining the anti-inflammatory effect of *Faecalibacterium prausnitzii* suggested that its immunomodulatory effects are mediated *via* decreasing the paracellular permeability to effectively reduce the severity of colitis and prevent colitis progression. *Faecalibacterium prausnitzii* decrease NF-κB activation and IL-8 secretion *in vitro* and impairs the colonic synthesis of pro-inflammatory cytokines while inducing the secretion of anti-inflammatory cytokine *in vivo*[19]. Likewise, various strains of bifidobacteria have shown promising anti-inflammatory effects[82]. Specifically, strains of *Bifidobacterium bifidum* have been reported to inhibit LPS-dependent NF-κB activation in IECs and induce anti-inflammatory macrophage, dendritic, and T cell populations[82].

Effects on mucin

Metagenomics studies following the administration of DSS revealed an elevation in *Akkermansia muciniphila,* a Verrucomicrobia member*,* which have been found to metabolize sulfur and lead to mucin degradation and correlate with disease activity in mice administered DSS[31,75,78]. Additionally, DSS induced colitis obliterated the difference in abundance and structure of bacterial communities between the two mucus layers[52,69]. There were fewer bacterial in the firmly adherent mucus but the abundance of bacteria in the mucus layer of the DSS treated animals was 10-100 times greater compared to the control group, suggesting DSS treatment increased the total count of infectious bacteria[22]. Antibiotic administration improved DSS-induced colitis akin to observations showing reduced inflammation in germ-free murine colitis model[31,32].

Changes during the recovery phase

Interestingly, there is a rapid shift of the gut microbial community toward a healthy profile in the recovery phase following the DSS treatment regimen. Within two days of stopping DSS administration, the mice gut microbiota showed relative abundance in Bacteroidetes/Prevotella, Bacillaceae, to levels comparable to those observed in healthy controls[31]. These data demonstrated the high degree of resilience of the gut microbiota and a rapid recovery of its healthy mutualistic profile after DSS-induced dysbiosis[31].

Probiotic treatment against dextran sodium sulfate-induced colitis

Probiotic therapies with Lactobacilli and Bifidobacteria produced favorable outcomes in murine colitis models[82,83]. Probiotic bacteria have been shown to decrease intestinal permeability and restore gut barrier integrity by modulation of tight junction proteins[83]. Pretreatment with *Lactobacillus reuteri* strains prevented the onset of DSS-induced colitis by reducing bacterial translocation and suppressing adherence of lactobacilli on colonic mucosa and[22]. Further, probiotic treatment suppressed upregulation of P-selectin in the colonic endothelium, which decreased leukocyte-

endothelial cell interactions and concomitant leukocytes recruitment to tissue[22]. However, no significant changes in microbial composition were observed indicating that the protective effects are linked to strengthening of the epithelial barrier integrity to reduce bacterial translocation[22,69]. Other studies have also examined the effects of different bacterial species on epithelial TJ in the DSS-induced colitis model. Various strains of bifidobacteria have shown promising anti-inflammatory effects[83]. *Bifidobacterium bifidum* S17 has been found to adhere to cultured IEC and displays potent anti-inflammatory activity both *in vitro* and in two murine models of colitis[83]. *Bacillus subtilis* supplementation resulted in improved barrier function compared to the DSS group, as evident by upregulated expression of TJ proteins (claudin-1, occludin, JAM-A, and ZO-1) and downregulated cytokine expression (IL-6, IL-17, IL-23, and TNF-α)[84]. Additionally, only certain, limited strains of *Bifidobacterium longum* have indicated an increased expression of ZO-1 and occludin resulting in reduced severity of DSS-induced colitis[83].

DEXTRAN SODIUM SULFATE-INDUCED CHANGES IN METABOLOME

DSS colitis has been shown to produce disturbances in the metabolism of phospholipids depicted by decreased levels of phosphocholine and glycerophosphocholine in the colon of mice[1]. Phosphocholine and glycerophosphocholine are the most important metabolites of choline and the major cellular constituents required for the assembly of biological membranes and disturbance in the metabolism suggests the possibility of distorted membrane integrity in the presence of DSS[1].

The role of dietary fat intake in exacerbating intestinal inflammation and modulation of immune function has been investigated extensively[85]. There is supporting evidence to show that a high fat intake is associated with an increased risk of ulcerative colitis[85]. Of note, two factors determine the role of lipid nutrition in health and disease: (1) the composition and (2) the total amount of fat in the diet. Bile acids are produced in the liver and excreted into the duodenum as conjugated bile salts to facilitate homeostatic functions in the gastrointestinal tract. Once in the large intestine, various microbial species enable the transformation of the bile salts into secondary bile acids, such as deoxycholic acid and urosodeoxycholic acid (UDCA), which vary in hydrophobicity[86]. Increased luminal bile acid hydrophobicity reportedly leads to gut barrier dysfunction through the disruption of cell membranes causing cytotoxicity, production of reactive oxygen species, epithelial growth factor receptor activation and tight-junction redistribution[86]. Conversely, UDCA which is more hydrophilic leads to stabilization of lipid membranes[86]. Thus, changes in bile acid composition favoring increased hydrophobicity leads to increased gut permeability allowing for enhanced translocation of bacteria and associated antigens across the tight-junction barrier of the gut epithelium leading to intestinal inflammation. One such study identified animals with a lower concentration of fecal cholic acid had increased histologic damage[86,87]. Additionally, various types of dietary fats promote colitis *via* alterations in gallbladder bile and gut microbiota through changes in taurocholic acid levels in bile which facilitated the growth of the *Bilophila wadsworthia,* an inflammatory Gram-negative anaerobe[86,88]. Overall, fecal bile acid hydrophobicity found to be mediated through a higher proportion of deoxycholic acid positively correlates with the severity of DSS colitis[86].

A potential causal link between dietary fat, especially Omega-3 and Omega-6 fatty acids, and susceptibility to induced colitis has also been reported. A dose dependent effect of the changes in composition of omega fatty acids in exacerbating colitis in various models including the DSS colitis model has been reported[85,86]. Fat in the diet alone does not alter the susceptibility to DSS, rather the modifications to the fecal bile acid composition has been proven to be deleterious[85]. Omega-3 fatty causes decreased adiponectin mRNA expression acid leading to increased inflammation in colonic mucosa[85]. However, another study reported decreases adiponectin RNA expression with no changes in serum adiponectin levels by high fat diet[85]. There are also reports that suggest that the pro-inflammatory effect of adiponectin is mediated by increased IL-6 production[85].

In addition to studies examining primary effect of bile acids on inflammation, the effects that bile acids on cell surfaces have also been investigated[89]. A member of the G protein coupled receptor superfamily, GP-BAR1 is a cell surface bile acid-activated receptor that has been found to be highly expressed in the ileum and colon and activated by secondary bile acids, specifically lithocolic acid and tauro-LCA[89]. Cipriani *et al*[89] asserted that this receptor regulates intestinal barrier integrity since mice lacking the GP-BAR1 receptor developed alterations in colonic histopathology and mucous cell distribution and function[89]. GP-BAR1$^{-/-}$ mice challenged with DSS exhibited an exacerbation of colitis that was not correlated with major immunological abnormalities, but rather an increase in intestinal permeability[89]. Furthermore, they have discovered that oleanolic acid, a natural GP-BAR1 ligand, attenuated colon inflammation and these anti-inflammatory effects of ciprofloxacin were lost in GP-BAR1$^{-/-}$ mice[89].

The central argument that metabolome changes play a part in colitis development is highlighted by the critical role microbiome composition plays in bacterial-host immune response. Certain enzymes, such as 7α-dehydroxylase that are endemic to gut microbiota, can convert primary bile acids into their secondary forms and therefore result in pro-inflammatory versus anti-inflammatory conditions in the host gut lumen.

Additionally, shifts in the community composition and structure can reduce potentially immunomodulatory mucosal-associated species, such as *Faecalibacterium prausnitzii*, *Clostridium leptum* and *Clostridium coccoides*, that act to maintain epithelial health through the production of short-chain fatty acids and stimulation of mucin production, thereby effecting the host's inflammatory response[25,27,75,90].

CONCLUSION

The last several years have provided indispensable insights into the histopathological and morphological changes in intestinal barrier function that likely contribute to the development and progression of murine colitis and aids in the understanding to the pathogenesis of human IBD. As stated prior, no isolated model has proven to sufficiently represent the complex clinical and histopathological characteristics of human disease. But, the DSS-induced colitis model is the most commonly used model that provides an inexpensive, simple and reproducible model to study various aspects of the role of mucin in barrier integrity, alterations in microbial balance and changes in the metabolome that relate to the pathogenesis of IBD. It is our hope that our further understanding of the role of intestinal barrier function in activating the innate and adaptive immune system in the intestinal tract that may lead to new therapeutic targets for human IBD.

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REVIEW

Autoimmune hepatitis: Standard treatment and systematic review of alternative treatments

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Abstract

Autoimmune hepatitis is a rare chronic inflammatory liver disease, affecting all ages, characterised by elevated transaminase and immunoglobulin G levels, positive autoantibodies, interface hepatitis at liver histology and good response to immunosuppressive treatment. If untreated, it has a poor prognosis. The aim of this review is to summarize the evidence for standard treatment and to provide a systematic review on alternative treatments for adults and children. Standard treatment is based on steroids and azathioprine, and leads to disease remission in 80%-90% of patients. Alternative first line treatment has been attempted with budesonide or cyclosporine, but their superiority compared to standard treatment remains to be demonstrated. Second-line treatments are needed for patients not responding or intolerant to standard treatment. No randomized controlled trials have been performed for second-line options. Mycophenolate mofetil is the most widely used second-line drug, and has good efficacy particularly for patients intolerant to azathioprine, but has the major disadvantage of being teratogenic. Only few and heterogeneous data on cyclosporine, tacrolimus, everolimus and sirolimus are available. More recently, experience with the anti-tumour necrosis factor-alpha infliximab and the anti-CD20 rituximab has been published, with ambivalent results; these agents may have severe side-effects and their use should be restricted to specialized centres. Clinical trials with new therapeutic options are ongoing.

Key words: Autoimmune hepatitis; Standard treatment; Second-line treatment; Adults; Children

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Core tip: The first part of this review summarizes the standard therapeutic approach for autoimmune hepatitis (steroids and azathioprine) and the evidence on which it is based. The second part reviews systematically published data on first and second line alternative treatments. This information is summarized in two comprehensive tables, one for adult and one for paediatric patients.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a rare inflammatory liver disease of unknown origin characterised by high transaminase and immunoglobulin G (IgG) levels, positive autoantibodies, and, histologically, by interface hepatitis[1-4]. The condition affects all ages, and has a female preponderance[5]. There is no single diagnostic test[1,2]. The International Autoimmune Hepatitis Group (IAIHG) established comprehensive diagnostic criteria in 1993[6], based on expert opinion, intended to be used for research purposes. After their evaluation in a number of studies, the criteria were updated in 1999[7]. A simplified, clinical practice-friendly version was published in 2008[8]. These criteria are intended to help in guiding diagnosis and decision on therapy initiation in patients presenting with a clinical picture suggesting AIH, and have received extensive external validation since publication[9-11].

AIH is divided in type 1 and type 2, the latter being rare in adults and representing 30% of juvenile AIH. The distinction is made serologically: type 1 AIH is positive for anti-nuclear antibodies (ANA), and/or anti-smooth muscle antibodies (SMA), while type 2 AIH is positive for anti-liver kidney microsomal antibodies type 1 (anti-LKM1) and/or anti-liver cytosol type 1 (anti-LC1)[12].

AIH is the first liver disease for which pharmacologic treatment has been shown to improve survival. Indeed, it has an excellent response to steroid-based immunosuppressive therapy, with a reported response rate of 75%-90%[2]. Steroid-response is a crucial feature of AIH, and it is part of the IAIHG revised diagnostic criteria[7]. Lack of response to steroids should prompt a review of the diagnosis.

Treatment indications

If untreated, AIH has a severe prognosis. This knowledge derives from early clinical trials, when "HBsAg-negative hepatitis" (as AIH was called then) patients were treated with corticosteroids *vs* placebo. One placebo controlled study reported a 5-year survival rate of 32% in untreated patients *vs* 82% in patients treated with steroids[13]. According to the guidelines on the management of AIH by the American Association for the Study of Liver Diseases (AASLD)[2], the 6-mo survival rate in untreated patients is about 60%. Therefore, once diagnosed, AIH should be treated promptly. Elderly patients with mild pauci- or a-symptomatic disease, who have a high risk of developing steroid side effects, may be an exception, and in this clinical context treatment *vs* watchful waiting should be carefully evaluated case by case[14-16]. Untreated patients need a close follow-up. Treatment must be always initiated in the presence of clinical symptoms, severe biochemical and/or histological disease activity. Younger subjects, particularly children and adolescents, who have a more aggressive disease, should be treated without delay[17].

Treatment aims

The aim of treatment is disease remission, which is reached if the following criteria are met: (1) absence of clinical symptoms; (2) normal transaminase levels; and (3) normal IgG levels. In children/adolescents, negative or very low-titre autoantibodies (< 1:20 for ANA/SMA; < 1:10 for anti-LKM1) are an additional criterion of remission[3], which remains to be evaluated in adults by longitudinal studies.

In the past, transaminase levels below twice the upper limit of normal (ULN) have been considered proof of remission, but it is now clear that patients with abnormal transaminase levels have progressive disease[2,18]. Once remission is achieved, the lowest possible dose of immunosuppressive drugs should be used to maintain long-term remission with no or minimal side effects.

Disease relapse is defined as transaminase levels rising above the ULN after remission[12]. Relapse occurs mostly if the dose of the immunosuppressive drugs is reduced, or in case of non-adherence. Non-adherence is a frequent clinical problem, particularly in adolescents[19] and young adults, and is often due to real or perceived treatment side effects. It should always be suspected in case of relapse while on a stable dose of immunosuppressive drugs.

AIM AND METHODOLOGY OF THE SYSTEMATIC REVIEW

The aim of this review is, in its first part, to critically summarize the evidence on which standard AIH treatment (prednisone and azathioprine) is based, and, in its second part, to provide a systematic review of the published data on alternative treatments. For the purpose of the systematic review of the literature on alternative AIH treatment, publications cited in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) were

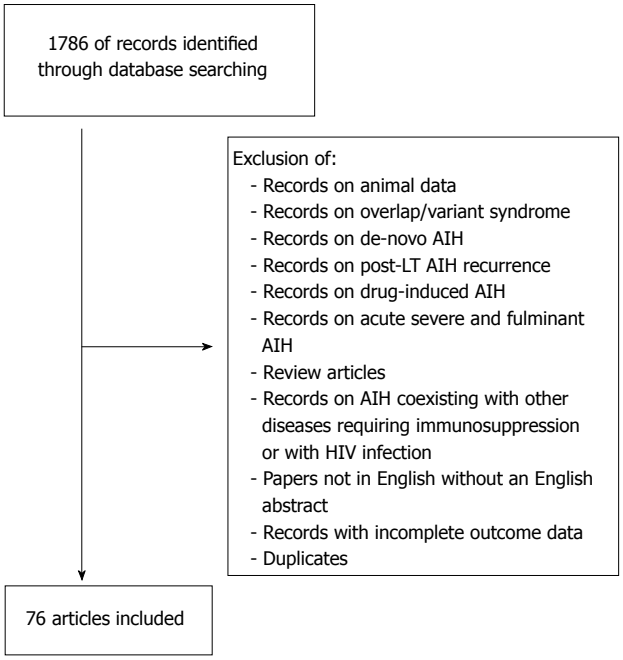


Figure 1 Selection of relevant articles for the systematic literature review on alternative AIH treatments. AIH: Autoimmune hepatitis; LT: Liver transplantation.

Table 1 Proposed schedule of prednisone tapering during remission-induction therapy in adults[25]

	Prednisone mg/d	Azathioprine
Week 1	60.0	Check transaminase levels every week before reducing the prednisone dose: if transaminase levels stop decreasing, add azathioprine 1-2 mg/kg per day, if jaundice is subsiding
Week 2	50.0	
Week 3	40.0	
Week 4	30.0	
Week 5	25.0	
Week 6	20.0	
Week 7	15.0	
Week 8-9	12.5	
Week 10-11	10.0	
If severe steroid side effects: consider reducing to 2.5 mg/d for 2 wk and then stopping prednisone		

Table 2 Proposed schedule of prednisone tapering during remission-induction therapy in children[2,12]

	Prednisone mg/kg/d	Azathioprine
Week 1	2.0	Check transaminase levels every week before reducing the prednisone dose: if transaminase levels stop decreasing, add azathioprine starting with 0.5 mg/kg per day, if jaundice is subsiding, at increasing doses up to 2-2.5 mg/kg/d until biochemical control
Week 2	1.75	
Week 3	1.50	
Week 4	1.25	
Week 5	1.00	
Week 6	0.75	
Week 7	0.50	
Week 8-9	0.25	
Week 10-11	0.10-0.20	
If severe steroid side effects: consider reducing to 2.5 mg/d for 2 wk and then stopping prednisone		

selected using the search words "autoimmune hepatitis" and "treatment". Citations were chosen on the basis of their relevance to the aim of this article (Figure 1). Fundamental characteristics of the abstracts judged pertinent to the review were noted, and full-length original articles were selected from the abstracts. Seventy-six articles were identified, 22 of them are not discussed in this review because of anedoctal reporting, the remaining 54 are included in Table 1 (adults) and Table 2 (children). Children/adolescents have a more aggressive disease, with a more frequent acute presentation[20] and therefore need a different management[17]. For this reason, the present review article discusses adult and pediatric treatment separately.

STANDARD TREATMENT

Why do we treat autoimmune hepatitis with steroids and azathioprine?

Standard treatment is based on steroids and azathioprine (Table 1). A systematic review of randomized controlled trials focused on these two drugs up to 2009 was published in 2010[21]. The exact azathioprine mechanism of action is unclear, but it is most probably linked to suppression of nucleic acid synthesis. The first evidence for steroid benefit in inducing remission and improving survival in treatment-naïve AIH stems from three trials performed in the 1970s, which demonstrated a significant better survival in patients with so called "HBsAg-negative chronic active liver disease" treated with steroids[22-24] in comparison to untreated patients. It should be noted that at that time the hepatitis C virus (HCV) had not been discovered and it is likely that some patients with HCV were included in the trials, although "HBsAg-negative chronic active hepatitis" was characterised by high globulin levels, female preponderance, and presence of autoantibodies, all features of AIH[13]. The benefit of steroid treatment would have probably been even greater if HCV-patients had been excluded[25]. In the Royal Free Hospital trial[22], 49 well characterised patients, including children, were randomised in a steroid-treated group (prednisolone 15 mg/d) and a placebo group. Mortality rate was 14% in the treated group, and 56% in the placebo group, with a follow-up ranging from 30 to 72 mo. The trial from the Mayo Clinic published one year later[23] included 63 patients, divided into four groups. Two groups were treated with protocols similar to current guidelines: the first group was treated with prednisone alone starting with 60 mg/d, tapered to a maintenance dose of 20 mg/d over 4 wk, the second group received prednisone 30 mg/d tapered to a maintenance dose of 10 mg/d combined with azathioprine at a fixed dose of 50 mg/d. The remaining groups were treated with azathioprine alone 100mg/d and placebo, respectively. The mortality rate in the first and second group was very low (6% and 7%), compared to a mortality rate of 36% and 41% in the groups treated with azathioprine

alone or placebo. The follow-up period ranged from 3 mo to 3.5 years. The side effect rate was lower in the azathioprine-prednisone group than in the prednisone alone group (10% *vs* 44%). A trial from King's College Hospital published in 1973[24] included 47 patients, divided into two groups, one treated with prednisone 15 mg/d, and the other with azathioprine alone, 75 mg/d, with a follow-up of two years. The mortality rate in the prednisone group was 5%, as compared to a mortality rate of 24% in the azathioprine group. From these early trials it is clear that prednisone is very effective in treating AIH, and that azathioprine alone is not able to obtain disease remission. Following these reports, strategies were sought to optimize the treatment schedule, *i.e.* to find the minimal doses of prednisone or prednisone/azathioprine able to control the disease with minimal side effects. A trial published in 1975[26] included 120 patients and compared four different schedules: (1) prednisone starting at 60 mg/d tapered to a maintenance dose of 20 mg/d; (2) prednisone starting at 30 mg/d tapered to 10 mg/d together with a 50 mg/d fixed dose of azathioprine; (3) prednisone at 60 mg/d tapered to a maintenance dose of 10 mg/d given on alternate days; and (4) placebo or azathioprine on a fixed dose of 100 mg/d without steroids, as control. Biochemical remission was achieved in 80% of patients in the first two groups, in 74% in the third group and in 34% in the control group. Histological remission was achieved in 57% and 60% of patients in the first two groups, but in only 19% and 24% in the third and in the control group. Side effects were less frequent in patients treated with prednisone/azathioprine from disease presentation, for which a lower dose of prednisone was used, leading to the conclusion that combined treatment is preferable. Of note, this trial enrolled "post-pubertal subjects", including patients from the age of 12 years. An additional trial published in 1982[27] compared a fixed low-dose prednisone alone (10 mg/d for body weight < 70 kg, 15 mg/d for body weight ⩾ 70 kg) in 37 patients with a fixed low-dose azathioprine alone (5 mg/kg per week for the first 2 wk, subsequently 10 mg/kg per week) in 47 patients. Mortality was very high in both groups at 1 year (27% and 28% respectively), indicating that a low prednisone dose and azathioprine alone are inadequate.

Despite the limitations of these early trials, prednisone ± azathioprine remains the mainstay of treatment for AIH, several reports showing high remission rates and favourable outcomes in both adult and juvenile AIH[20,28-38].

Of note, azathioprine monotherapy, though unsuccessful in the induction of remission, is effective in adults as maintenance therapy, at a dose of 2 mg/kg per day[39]. A 5-patient report suggests that it may be effective also in children[40]. In a recent retrospective series, 87% of 66 children with AIH were reported to maintain sustained biochemical remission (normal transaminase levels) in association with low 6-thioguanine nucleotides (TGN) levels (50-250 pmol 8 x 10 red blood cell cont) on an azathioprine dose of 1.2-1.6 mg/kg per day with or without associated steroids[41].

How to use prednisone and azathioprine

There is no treatment schedule applicable to all AIH patients. The suggested algorithms and treatment schedules must be tailored to the single patient, taking into account the severity of the disease, age and co-morbidities[1].

The AASLD guidelines published in 2010[2] recommend two alternative schedules: either prednisone alone at a dose of 60 mg/d or a combination of prednisone 30 mg/d and azathioprine 50 mg/d as initial treatment, favouring the latter because of fewer steroid side-effects[26]. However, as azathioprine can be hepatotoxic, particularly in cirrhotic and jaundiced patients[25], the more recent guidelines by the European Association for the Study of the Liver (EASL) recommend that it is added after two weeks of steroid monotherapy [predniso(lo)ne 1 mg/kg per day in adults], when partial disease control has been achieved[1]. In addition, this approach avoids the problem of distinguishing between azathioprine-induced hepatotoxicity and non-response, this distinction being an important issue in clinical practice. A retrospective series of 133 adult patients reports better results with a combination of steroids and another immunosuppressant (azathioprine in 96%, other unspecified drugs in 4%) from disease presentation compared to steroids alone or steroids followed by the addition of azathioprine/other immunosuppressants. Of note, only 2% of the patients included in this study were jaundiced at presentation[42], possibly explaining the high remission rate on azathioprine, without hepatotoxicity.

Prednisone should be rapidly tapered (Table 1) to minimise steroid side effects. This rapid decrease of the prednisone dose requires weekly checks of the transaminase levels to monitor response. Azathioprine should be added if the transaminase levels stop decreasing on steroid treatment alone (Table 1). Ultimately 85% of the patients will need azathioprine in addition to low-dose prednisone[12]. This protocol was originally used for children[25], but it is suitable to treat adult patients as well, because it allows to avoid azathioprine in a small proportion of patients and especially because it limits steroid side effects, which are often the reason for non-adherence. The initial recommended dose of azathioprine in adults is 50 mg/d or 1 mg/kg per day[2]. If steroid side effects are severe and require steroid discontinuation, the azathioprine dose is increased to 2 mg/kg per day.[39,43]

In children, the recommended treatment schedule is similar to that of adults, but a higher steroid dose is required due to the more aggressive disease course in this age group (Table 2). Children were included in early clinical trials[22,26], but a sub-analysis of paediatric patients was not performed, and the numbers were small. Current recommendations are based on series

from large centres, which report a remission rate of about 90% using predniso(lo)ne ± azathioprine[20,35,36]. Conventional treatment of juvenile AIH consists of prednisolone (or prednisone) 2 mg/kg per day (maximum 60 mg/d), decreased over a period of 4 to 8 wk in parallel to the decline of transaminase levels, to a maintenance dose of 2.5-5 mg/d (Table 2). Long-term low daily doses are not associated with impaired adult height[44]. The timing for the addition of azathioprine as a steroid-sparing agent varies according to the protocols used in different centres. In some, azathioprine is added only in the presence of steroid adverse effects, or if the transaminase levels stop decreasing on steroid treatment alone. In other centres azathioprine is added after a few weeks of steroid treatment in all patients, when the serum aminotransferase levels begin to decrease. Some centres use a combination of steroids and azathioprine from the beginning, but caution is recommended because of the azathioprine hepatotoxicity mentioned above[1,2,34]. The initial recommended azathioprine dose is 0.5 mg/kg per day[12], which can be increased to 1-2 mg/kg per day until normalization of the transaminase levels is reached. As in adults, azathioprine alone has been shown to be able to control the disease as long-term maintenance therapy, although only in retrospective series[40,41,45].

As AIH is very sensitive to prednisone, a maintenance dose of 5 mg/d is effective in controlling the disease, usually with, but sometimes without, azathioprine. Steroid reduction below 5 mg/d (or 2.5 mg/d in children) requires careful monitoring of transaminase levels, even if implemented after long-term disease remission. The dose should be reduced very slowly, *e.g.,* by 1 mg per month if 1 mg prednisone tablets are available, or, if not available, by reducing to 5-2.5 mg on alternate days for 1-2 mo, and then to 2.5 mg/d.

Side effects of steroids and azathioprine

Steroid side effects are dose and time dependent, and arise if a dose exceeding 7.5-10 mg/d is administered over several months[25]. The most common side effect is the development of cushingoid features. In a retrospective monocentric study of 103 adult AIH patients[46], mostly treated according to a standard protocol with a steroid starting dose of 1 mg/kg per day and a mean follow-up period of 95 mo, 15.5% developed cushingoid features. Although not severe, these changes are often a great concern for the patients, and may lead to non-adherence, with the dangerous consequence of poor disease control. Almost half of AIH patients discontinue steroids because of cosmetic changes (including acne) or obesity[47]. Severe, but less frequent steroid side effects include osteoporosis, brittle diabetes, cataract, psychosis and hypertension[2]. They are mainly related to the initial high dose, and are reversible[43,46]. Monitoring of these complications is advisable, including ophthalmologic controls and bone density scans on a regular basis.

Azathioprine side effects affect 10%-20% of patients and include hepatotoxicity, acute cholestatic hepatitis, pancreatitis, nausea and vomiting, rash, bone marrow suppression, veno-occlusive disease, opportunistic infections, and malignancy[2]. The most common side effect is bone marrow suppression, which is unpredictable, and can be aggravated by concomitant cytopaenia due to liver disease and hypersplenism. Haematological monitoring is necessary, particularly at the beginning of treatment. Measurement of erythrocyte concentrations of thiopurine methyltransferase (TPMT) activity may be advisable before institution of azathioprine therapy, but does not invariably predict response to the drug or toxicity[48,49]. TPMT genotyping predicts azathioprine haematological toxicity in those rare individuals with variant homozygosity, while heteroxygotes do not experience more toxicity than wild-type patients[50]. Five percent of patients develop early intolerance, most frequently with nausea and vomiting.

A possible complication of long-term treatment with azathioprine is the development of malignancies. In one study aiming at investigating disease control by azathioprine monotherapy at a dose of 2 mg/kg per day, 5 of 72 patients (7%) developed malignancies over a median follow up of 12 years[39]. Recently, two cases of T-cell lymphoma in adolescents treated with azathioprine for AIH were reported[51]. Thus, a lower azathioprine dose in association with low-dose steroids may be preferable for long-term maintenance therapy. Azathioprine is considered to be safe in pregnancy[52-54].

Measurement of the azathioprine metabolites 6-TGN and 6-methylmercaptopurine can be helpful in identifying drug toxicity and non-adherence, and in distinguishing azathioprine hepatotoxicity from disease non-response, as shown by a retrospective study in adults[55], and a small prospective study in children[56], but an ideal therapeutic level of the 6-thioguanine metabolites has not been established for AIH, unlike for inflammatory bowel diseases (IBD).

Treatment withdrawal

The AASLD[2] and the EASL guidelines[1] recommend a treatment duration of at least 2 and 3 years respectively, and both advise against a trial of treatment withdrawal before 2 years of complete biochemical remission. They recommend performing a liver biopsy before attempting treatment discontinuation, because histological inflammatory activity can still be present despite biochemical remission, predicting relapse. A recent report on 28 patients in whom treatment was withdrawn without histological evaluation, shows that the 54% of patients who did not relapse had transaminase levels less than half the ULN and IgG levels below 12 g/L on low-dose monotherapy (azathioprine/mercaptopurine or steroids) for at

least 2 years, suggesting that patients meeting these parameters may avoid pre-withdrawal liver biopsy[57]. This suggestion, however, requires confirmation by other centres.

Relapse after treatment withdrawal is frequent, having been reported in some 80% of patients[1,2,58]. Repeated relapses are associated with a poorer prognosis and a higher rate of drug side effects[2]. For this reason, patients experiencing a first relapse episode after appropriate evaluation of disease remission, should undergo life-long low-dose immunosuppressive therapy[1].

For AIH type 2, relapse is almost universal if treatment is completely withdrawn[20], and long-term low-dose maintenance therapy should be planned from the diagnosis. Since this condition mostly affects children, adolescents and young adults, life-long duration of the therapy should be discussed and carefully explained to the patients and their family.

ALTERNATIVE TREATMENTS

For patients who experience azathioprine side effects, ranging from the relatively frequent early gastrointestinal intolerance to the rarer and more serious bone marrow suppression, and for poor responders to standard treatment, alternative regimens are needed, primarily to avoid high-dose steroid side-effects. A systematic review of the published clinical data on pharmacological treatments different from prednisone and azathioprine is provided in this section. Treatments for whom there are only anecdotal data are not discussed cyclophosphamide[59], methotrexate[60-62], ursodeoxycholic acid[63-69], etanercept[70], plasma exchange[71], intravenous immunoglobulin[72], leukapheresis[73], chloroquine[74], thymostimulin[75], deflazacort[76,77], saireito[78], sympathomimetic amines[79], glycyrrhizin[80], fenofibrate[81].

Budesonide

Budesonide is a glucocorticosteroid with a potent topical effect and a high first-pass uptake (> 90%) in the healthy liver, thus appearing ideal for treating AIH. The first reports on its use included small numbers of patients at different stages of disease and gave controversial results[79-83] (Table 3). Subsequently, a large randomized controlled trial in 203 AIH patients (including 46 children/adolescents) was carried out, involving several European centres[82] (Table 3). Cirrhotic patients were excluded, because the first pass hepatic extraction of budesonide may be reduced in cirrhosis due to portosystemic shunting. In fact, severe complications have been reported in cirrhotic patients on budesonide[83,84], including portal vein thrombosis and Budd-Chiari syndrome, indicating that AIH patients with cirrhosis at diagnosis (at least one third) should not be treated with budesonide. The trial primary end-point was biochemical remission (defined as normalization of transaminase levels) in absence of steroid side effects. The overall results of the trial showed better response to budesonide/azathioprine than to prednisone/azathioprine treatment, the primary end-point being achieved in 60% of patients given budesonide *vs* 38.8% of those given prednisone[82]. These response rates, however, are below the remission rates achieved with standard treatment, and this has raised concerns. In the control arm, the prednisone dose was reduced as per-protocol, irrespective of the course of the clinical and biochemical response, an approach not recommended in AIH treatment[1], which should be tailored to individual patient response. The initial prednisone dose (40 mg/d) was low at least for children/adolescents[1,12], who should be treated with 2 mg/kg per day (up to 60 mg/d). All patients were prescribed azathioprine from the beginning, irrespective of the presence of jaundice, raising the possibility that the low response rate might be partly due to azathioprine hepatotoxicity[85]. The trial included treatment naïve patients and patients experiencing disease relapse, who are likely to represent a subgroup of poor responders[85]. Moreover, only transaminase levels were used to define biochemical remission, while the combination of normal transaminase and IgG/gammaglobulin levels best predicts absence of histological activity[46,86,87].

Though budesonide is still not recommended as first line therapy for AIH[1], it may be a valid alternative for the maintenance of remission long-time, particularly for patients experiencing steroid side effects. In a retrospective study 60 patients with either prednisolone side effects or dependence on a relative high dose of prednisolone were switched to budesonide[88]: the biochemical remission rate at 6 mo was 55%, and 25% of the patients needed to be switched back to prednisone due to budesonide side-effects or insufficient response. However, all patients who were in remission at the time of switching remained in remission. These findings indicate that budesonide is effective in maintaining remission in patients who have achieved it with prednisone, but also that it is not free of side effects, and that, not surprisingly, it is not effective in patients resistant to prednisone, as prednisone and budesonide share the same receptor.

A sub-analysis of the paediatric population (46 patients aged 9 to 17) enrolled in the budesonide trial[89] reported no significant difference in biochemical remission rate at 6 and 12 mo between the budesonide and the prednisone groups (32% and 33% at 6 mo and 50% and 42% at 12 mo, respectively) (Table 4). The frequency of steroid side effects was also not different, being 47% in the budesonide group and 63% in the prednisone group, apart from a lower mean weight gain in the budesonide group. The remission rate was well below that achieved with standard treatment, therefore, budesonide cannot be recommended for the treatment of children/

Table 3 Published data on autoimmune hepatitis treatment different from steroids and azathioprine in adults (from age 16)

Reference, yr	Country	Number and type of patients	Design	Outcome	Follow-up	Dose	Safety
Budesonide							
Danielsson *et al*[79], 1994	Sweden	13 naïve	Prospective	Significant decrase of mean transaminase levels	9 mo	6-8 mg/d	Plasma cortisol reduction in cirrhotic patients
Czaja *et al*[80], 2000	United States	10 AZA-NR	Prospective	3/10 BR	2-12 mo	9 mg/d	All patients had side-effects
Wiegand *et al*[81], 2005	Germany	12 naïve	Prospective	10/12 BR	3 mo	9 mg/d	3 discontinued due to side effects
Csepregi *et al*[82], 2006	Germany	10 naïve 8 AZA-NR	Prospective	7/10 naïve BR 8/8 AZA-NR BR	24 wk	9 mg/d	Steroids side-effects in cirrhotic patients
Zandieh *et al*[83], 2008	Canada	6 AZA-INT 3 PDN-INT	Retrospective	4/6 AZA-INT CBR 3/3 PDN-INT CBR	24 wk-8 yr	1.5-9 mg/d	Not reported
Manns *et al*[84], 2010[1]	Europe	208 naïve or relapsing	Prospective, randomized,	60% BR in budesonide 39% BR in PDN	6 mo	9 mg/d	Steroids side effects: 28% in budesonide arm, 53% in PDN arm
Mycophenolate mofetil							
Richardson *et al*[177], 2000	United Kingdom	3 AZA-INT 4 AZA-NR	Retrospective	5/7 BR	46 mo	2 g/d	Leukopaenia in 1
Zolfino *et al*[93], 2002	United Kingdom	3 second line	Retrospective	1/3 BR	Not reported	2 g/d	Not reported
Devlin *et al*[94], 2004	Canada	5 second-line	Retrospective	5/5 BR	Not reported	Not reported	1 pyelonephritis
Chatur *et al*[95], 2005	Canada	11 second-line	Retrospective	7/11 BR	10-54 mo	0.5-2 g/d	Leukopaenia in 1, diarrhoea in 1
Czaja *et al*[96],2005	United States	8 first- and second line	Retrospective	0/8 CBR	12-26 mo	0.5-3 g/ d	None reported
Inductivo-Yu *et al*[97], 2007	United States	15 second-line	Retrospective	Significant decrease of mean transaminase levels and of histological fibrosis and inflammation	41 mo	2 g/d	None significant
Hlivko *et al*[98], 2008	United States	17 naïve 12 second-line	Retrospective	16/19 BR	Not reported	0.5-2 g/d	10 discontinued for side-effects
Hennes *et al*[99], 2008[2]	Germany	27 AZA-INT 9 AZA-NR	Retrospective	57% AZA-INT BR 25% AZA-NR BR	16 mo	1-2 g/d	11 GI side effects
Wolf *et al*[178], 2009	United States	16 second-line	Retrospective	5/16 BR	Not reported	1-2 g/d	1 discontinued due to paresthesias
Sharzehi *et al*[100], 2010	United States	9 AZA-INT 12 AZA-NR	Retrospective	21/21 BR	12 mo	0.5-2 g/d	1 discontinued for GI side-effects
Baven-Pronk *et al*[101], 2011	The Netherlands	23 AZA-INT 22 AZA-NR	Retrospective	67% AZA-INT BR 13% AZA-NR BR	3-133 mo	0.5-3 g/d	6 discontinued for side-effects
Jothinami *et al*[102], 2014	India- United Kingdom	18 AZA-INT 2 AZA-NR	Retrospective	14 BR	5-83 mo	1-2 g/d	3 discontinued due to side-effects
Zachou *et al*[103], 2016	Greece	109 naïve	Prospective	83/102 BR at 3 mo	72 mo	1.5-2 g/d	2 discontinued for septicaemia; 5 dose reduction for leukopaenia or infections
Gazzola *et al*[179], 2016	Australia	51 AZA-INT 45 AZA-NR	Retrospective	27/49 AZA-INT BR 17/40 AZA-NR BR	Median: 31.9 mo	1-2 g/d	1 death, 2 hospitalisations, 8 GI side effects, 5 infections, 3 cytopoenia, 3 neuropsychiatric, 2 skin cancer, 1 lymphoproliferative disorder
Park *et al*[180], 2016	South Korea	1 AZA-INT	Retrospective	1/1 CBR	1 yr	1 g/d	None
Cyclosporine A							
Mistilis *et al*[121], 1985	Australia	1 AZA-INT	Retrospective	1/1 BR	1 yr	Not reported	None
Paroli *et al*[116], 1992	Italy	3 naïve	Prospective	3/3 BR	1 yr	5 mg/kg/d	Not reported
Person *et al*[118], 1993	United States	1 second-line	Retrospective	BR	Not reported	Not reported	Not reported
Sherman *et al*[114], 1994	United States	6 AZA-NR (1 paediatric)	Retrospective	5/6 BR at 10 wk	Not reported	Not reported	1 increased serum creatinine
Senturk *et al*[119], 1995	India	1 second-line	Retrospective	BR	1 yr	Not reported	None

Fernandes *et al*[113], 1999	United States	5 AZA-NR	Retrospective	4/5 BR at 3 mo	27 mo	3-5 mg/kg/d	Minimal
Malekzadeh *et al*[117], 2001	Iran	9 naïve 10 second-line	Prospective	79% BR and HI	26 mo	2-5 mg/kg/d	4 discontinued due to side effects
Zolfino *et al*[93], 2002	United Kingdom	1 second-line	Retrospective	NR	Not reported	Serum level 100-200 μg/L	Not reported
Malekzadeh *et al*[181], 2012	Iran	22 steroid-intolerant or NR	Retrospective	9 BR	60 mo	Not reported	Hirsutism (frequency not reported)
Tacrolimus							
Van Thiel *et al*[134], 1995	United States	21 naïve	Prospective	Mean 80% ALT drop at 3 months	3 mo	6.6-8 mg/d; blood levels 0.6-1.0 ng/mL	Mild mean creatinine elevation after 1 yr
Heneghan *et al*[137], 1999	United Kingdom	7 naïve	Prospective	BR in 86%		Not reported	Not reported
Zolfino *et al*[93], 2002	United Kingdom	5 AZA-NR	Retrospective	2/5 BR	Not reported	2-4 mg/d	Not reported
Aqel *et al*[130], 2004	United States	11 second-line	Retrospective	Normalization of mean ALT value	16 mo	0.5-1 mg/d(blood level < 6 ng/mL)	Minimal
Chatur *et al*[95], 2005	Canada	3 second-line	Retrospective	3/3 NR	10-54 mo	2-4 mg/d	1 discontinued for abdominal pain
Larsen *et al*[131], 2007	Denmark	9 AZA- or MMF-NR (1 pediatric)	Retrospective	9/9 BR	12-37 mo	2 mg/d (target blood level < 6 ng/mL)	1 mild tremor
Tannous *et al*[133], 2011	United States	13 second-line	Retrospective	12/13 BR	1-65 mo	2-6 mg/d (mean blood level 6 ng/mL)	1 HUS; 1 oral carcinoma
Than *et al*[135], 2016	German, United Kingdom	16 AZA-NR 1 AZA-INT	Retrospective	BR in most	60 mo	0.5-5 mg/d	1 LT; 4 PSC overlap
Al Taii *et al*[136], 2017[3]	United States	23 second-line	Retrospective	27% CBR 41% BR		5 mg/d (mean) serum level: 6.7 ng/mL (mean)	Significant increase of serum creatinine; 1 discontinued for GI hemorrhage
Sirolimus							
Chatrath *et al*[139], 2014	United States	5 AZA-NR	Prospective	4/5 BR	4-72 mo	2 mg/d	2 hyperlipidemia
Rubin *et al*[141], 2016	United States	2 second-line	Retrospective	1/2 BR	Not reported	3-6 mg/d	1 discontinued due to leg ulcer
Everolimus							
Ytting *et al*[143], 2015	Denmark	7 second-line	Retrospective	3/7 CBR 4/7 BR	1-3 yr	0.75-1.5 mg/d (target blood levels: 3-6 ng/mL)	Minimal
Rituximab							
Burak *et al*[144], 2013	Canada	3 AZA-NR 3 AZA-INT	Prospective	6/6 BR at 24 wk	72 wk	1000 mg on day 0 and 15	1 mild infection
Al-Busafi *et al*[182], 2013	Oman	1 steroid-resisitant	Retrospective	BR	Not reported	Not reported	None reported
Rubin *et al*[141], 2016	United States	1 second-line	Retrospective	1/1 BR	14 mo	475 mg/m^2 per week	None reported
Infliximab							
Weiler-Normann *et al*[154], 2013	Germany	11 second-line	Retrospective	8/11 BR	6 to > 40 infusions	5 mg/kg on 0, 2, 6, then every 4-8 wk	7/11 infections, 3 discontinued for side effects
Vallejo *et al*[156], 2014	Spain	1 AZA-NR	Retrospective	1/1 BR	3 mo	5 mg/kg given 3 times	Mild respiratory infection
6-mercaptopurine							
Pratt *et al*[167], 1996	United States	2 AZA-INT	Retrospective	2/2 CBR, 1/2 HI	24 mo in one not reported in the other	100 mg/d	None reported
Hübener *et al*[168], 2016	Germany/ United Kingdom	20 AZA-INT 2 AZA-NR	Retrospective	8/20 CBR 7/20 BR	18.5 mo	25-100 mg/d	4 discontinued for GI side-effects, 1 for leucopaenia
Elnegouly *et al*[183], 2017	Germany/ Austria	17 AZA-INT	Retrospective	11/12 CBR	1 yr	25-50 mg/d	2 discontinued for side-effects
Allopurinol							
Al-Shamma *et al*[170], 2013	United Kingdom	1 AZA-NR	Retrospective	1/1 BR	12 mo	100 mg/d	None reported

De Boer *et al*[171], 2013	The Netherlands	3 AZA-INT 5 AZA-NR	Retrospective	7/8 BR	13 mo	100 mg/d	1 discontinued for neuropathy
Al-Shamma *et al*[172], 2013	United Kingdom	1 AZA-NR	Retrospective	1/1 CBR	Not reported	100 mg/d	None reported
6-thioguanine							
De Boer *et al*[174], 2005	The Netherlands	3 AZA-INT	Retrospective	3/3 BR	Not reported	0.3 mg/kg/d	None reported
Van den Brand *et al*[175], 2017	The Netherlands	6 AZA-NR 6 AZA-INT	Retrospective	Significant median ALT decrease	12-75 mo	0.3 mg/kg/d	1 nodular regenerative hyperplasia

[1]The series includes 46 children (Woynarowski *et al*[91] 2013); [2]The series includes 4 adolescents, but only overall results are reported, and youngest age at diagnosis was 13 yr; [3]The series includes 6 adolescents, but only overall results are reported, and youngest age at diagnosis was 15 yr. BR: Biochemical response; AZA-NR: Azathioprine non-responder; AZA-INT: Azathioprine intolerant; CBR: Complete biochemical response; PDN: Prednisone; GI: Gastrointestinal; HI: Histological improvement; LT: Liver transplant; NR: Non-responder; ALT: Alanine aminotransferase; HUS: Haemolytic-uremic syndrome; PSC: Primary sclerosing cholangitis.

Table 4 Published data on autoimmune hepatitis treatment different from steroids and azathioprine in children

Ref.	Country	Number and type of patients (*n*)	Design	Outcome	Follow-up	Dose	Side effects
Budesonide							
Woynarowski *et al*[91], 2013	Europe	46 including naïve and second-line	Prospective	16% BR AZA+BUD 15% BR AZA+PDN at 6 mo	1 yr	6-9 mg/d	More weight gain in PDN group
Mycophenolate mofetil							
Lee *et al*[107], 2007	Malaysia	2 second-line	Retrospective	0/2 BR at 6 mo	6-18 mo	20-40 mg/kg/d	Not reported
Aw *et al*[106], 2009	United Kingdom	20 AZA-NR 6 AZA-INT	Retrospective	18/26 CBR	0.75-12 mo	20-40 mg/kg/d	7 Leukopaenia
Jiménez-Rivera *et al*[108], 2012	Canada	12 second-line	Retrospective	Not reported	Not reported	1000-1500 mg/d	Not reported
Dehghani *et al*[109], 2013	Iran	5 second-line	Retrospective	5/5 BR	None reported	Not reported	Not reported
Cyclosporine A							
Jackson *et al*[120], 1995	South Africa	1 AZA-INT	Retrospective	1/1 BR at 2 wk	19 mo	5 mg/kg/d	None
Debray *et al*[111], 1999	France	8 naïve 7 second-line (all type 2 AIH)	Retrospective	8/8 naïve BR 7/7 second-line (including 3 with ALF)	1-6 yr	4.7-5.6 mg/kg/d	Minimal
Ben Halima *et al*[122], 2002	Tunisia	1 first-line	Retrospective	1/1 BR	Not reported	Not reported	None
Sciveres *et al*[184], 2004	Italy	4 naïve 4 steroid/AZA-intolerant	Retrospective	8/8 BR at 2-8 wk	1.5-15 yr	4-10 mg/kg per day	2 gingival hypertrophy, 1 creatinine elevation
Cuarterolo *et al*[124], 2006	Agentina	86 naïve, type 1 AIH	Prospective	BR 94%	2 yr	4 mg/kg per day	8/84 creatinine elevation 3/84 hypertension
Nastasio *et al*[115], 2011	Italy	19 naïve[1] 10 second-line1	Retrospective	19/19 naïve BR at 4-18 wk 9/10 second-line BR	6.5 yr	Not reported	11 hyperthricosis, 13 gingival hypertrophy
Dehghani *et al*[109], 2013	Iran	3 second-line	Retrospective	3/3 BR	Not reported	Not reported	Not reported
Lee *et al*[107], 2015	Malaysia	2 second-line	Retrospective	1 /2 BR	6-18 mo	5 mg/kg per day, serum level 250-350 ng/mL	
Zaya *et al*[112], 2012	Croatia	9 naïve (1 type 2 AIH)	Retrospective	7/9 BR after 1 yr	24 mo	3-5 mg/kg per day	Minor
Jiménez-Rivera *et al*[108], 2012	Canada	9 naïve 15 second-line	Retrospective	Not reported	4 ± 2 yr	4 ± 0.8 mg/kg per day intially 4.9 ± 1.8 mg/kg per day in follow-up	Not reported
Tacrolimus							
Zolfino *et al*[93], 2002	United Kingdom	1 second-line	Retrospective	NR	Not reported	2 mg/d	Not reported
Marlaka *et al*[138], 2012	Sweden	20 naïve	Prospective	3/20 BR in monotherapy	1 yr	Target blood levels: 2.5-5 ng/ml	1 discontinued for side-effects; 2 developed IBD
Dehghani *et al*[109], 2013	Iran	2 second-line	Retrospective	2/2 BR	Not reported	Not reported	Not reported

Jiménez-Rivera *et al*[108], 2015	Canada	6 second-line	Retrospective	Not reported	Not reported	Not reported	Not reported
Sirolimus							
Kurowski *et al*[140], 2014	United States	4 second-line	Retrospective	2/4 BR	Not reported	Not reported	2 mo ulcers
Rituximab							
D'Agostino *et al*[150], 2013	Canada/ Argentina	2 second-line	Retrospective	2/2 CBR at 3/8 mo	26-38 mo	375 mg/m^2 weekly for 4 wk	None reported
Infliximab							
Rajanayagam *et al*[158], 2013	Australia	1 second-line	Retrospective	1/1 BR	19 mo	5 mg/kg 4 infusions at 4 wk interval	LT was not prevented
6-mercaptopurine							
Pratt *et al*[167], 1996	United States	1 AZA-NR	Retrospective	1/1 CBR and HR	36 mo	1.5 mg/kg	None reported

[1]Twelve patients had additional concomintant immunosuppressive drugs. BR: Biochemical response; AZA: Azathioprine; BUD: Budesonide; PDN: Prednisone; INT: Intolerant; NR: Non-responder; AIH: Autoimmune hepatitis; ALF: Acute liver failure; IBD: Inflammatory bowel disease; LT: Liver transplant; CRB: Complete biochemical response.

adolescents with AIH until a trial including strict diagnostic criteria and drug schedules appropriate for the juvenile disease is performed[85].

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is the prodrug of mycophenolic acid. It is an inhibitor of inosine monophosphate dehydrogenase, the rate-limiting enzyme in de novo purine synthesis on which, in contrast to other cells, B and T lymphocyte proliferation relies. MMF is widely used as second line AIH treatment, mostly combined with prednisone, both for patients intolerant to azathioprine and for patients with unsatisfactory response to standard azathioprine/prednisone treatment. Its use in AIH is based on retrospective series[90-103] (Table 3) with a total number of 313 patients treated, suggesting that MMF is partially effective in patients intolerant to azathioprine, but may not be effective in case of azathioprine poor response. However, a recent paper from Australia including 96 patients[104] reported a similar remission rate both in patients intolerant and poor responders to azathioprine (Table 3). One single prospective uncontrolled trial from Greece tested the use of MMF as first-line treatment[102,105] (Table 3). MMF was reported to be safe and effective in inducing and maintaining remission in treatment-naïve patients (83/102 patients achieved biochemical remission at 3 mo) and to have a rapid steroid sparing effect. However, it is not clear whether it offers an advantage over azathioprine, as a head-to-head comparison with azathioprine was not performed. A trial comparing azathioprine to MMF is currently ongoing (NCT02900443). MMF has the major disadvantages of being about 15 times more expensive than azathioprine, and, most importantly, of being teratogenic, which is highly relevant, since AIH affects mainly young females. The most frequent side effects are gastro-intestinal symptoms.

In juvenile AIH patients in whom standard immunosuppression is unable to induce stable remission, or who are intolerant to azathioprine, MMF at a dose of 20 mg/kg twice daily, together with prednisolone, has been used successfully used[90,106–108] (Table 4). A recent meta-analysis, including data from several small studies of second line treatments in children refractory to standard therapy shows that MMF is efficacious with a low side effect profile (in contrast to calcineurin inhibitors), supporting the notion that MMF should be the primary choice for second-line therapy in juvenile AIH[109].

Calcineurin inhibitors

Cyclosporine A: Cyclosporine A is a calcineurin inhibitor extensively used in the setting of transplant medicine. Important side effects are renal toxicity and cosmetic changes, particularly in association to high doses. In small retrospective series[90,108,110–115], small prospective open and uncontrolled trials[115,116] and single case studies[91,107,117-121] cyclosporine A has been reported to be effective - using variable doses, duration of treatment and follow-up - either as first-line option or in patients not responding to azathioprine and prednisone, both in children and in adults (Tables 3 and 4). Though the results of these reports appear to be encouraging, the quality and quantity of the data are insufficient to recommend its use. In paediatrics, cyclosporine A has been used as first line treatment for type 1 AIH in an attempt to reduce steroid side effects in a prospective multicentre study in 84 treatment-naïve children[122,123] (Table 4). Cyclosporine alone was administered for 6 mo, and the patients were subsequently switched to azathioprine and prednisone. Transaminase levels normalization was obtained in 72% of the subjects after six months of cyclosporine monotherapy, but IgG levels were not included in the remission criteria. Cyclosporine side effects included hypertrichosis (55%), gingival hyperplasia (39%), elevation of creatinine (9%) and hypertension (3%). The main limitation of this study is lack of direct comparison with standard treatment.

Animal data suggest that cyclosporine A may promote autoimmunity[124-127], and the first reports of de novo autoimmune hepatitis arising after liver transplantation were in children treated with

cyclosporine[128]. These observations call for caution in the use of cyclosporine in AIH.

Tacrolimus: Tacrolimus is a more potent calcineurin inhibitor than cyclosporine, has less cosmetic side effects, but similar drug class toxicity. In AIH, it has been used both for refractory cases and for patients intolerant to other immunosuppressive regimens. A few retrospective small case series in adults have been published, with variable remission criteria, sometimes including only transaminase levels[91,93,129-135]. The reported efficacy was good in a total number of 80 patients (Table 3). Two prospective open-label trials from the '90s are available, both in naïve patients[133,136] (Table 3). The oldest one included 21 adult patients[133], with a follow up of 1 year, after which a liver biopsy was repeated, but histological results are not reported. Half of the patients were anti-LKM1 positive; tacrolimus was used as monotherapy. Of note, the serum target level of tacrolimus was low (0.6-1 ng/mL). The mean decrease of transaminase and bilirubin levels was satisfactory, but the remission rate is not reported. In terms of side effects, the mean creatinine value increased significantly after 1 year of treatment. The second prospective trial in naïve patients included seven adult subjects and used lower tacrolimus doses combined with 20 mg/d of prednisolone. Transaminase levels, albumin, bilirubin and prothrombin time significantly improved in 6/7 patients[136].

In children, one prospective, single centre, open label trial including 20 treatment naïve patients is available: none was anti-LKM1 positive, follow up was 1 year, after which a liver biopsy was repeated[137] (Table 4). Target tacrolimus blood levels were 2.5-5 ng/mL. 14/20 patients needed azathioprine and prednisone in addition to tacrolimus to achieve remission. Histological improvement of inflammation was seen in 12/14 cases. No effect on the renal function was observed. This trial suggests that tacrolimus as monotherapy is not effective in juvenile AIH, but could be considered as steroid/azathioprine sparing agent.

More high-quality data are needed, both in adults and children, to assess tacrolimus efficacy in AIH.

m-TOR inhibitors

Sirolimus: Sirolimus is a macrolide molecule acting by inhibiting the mammalian target of rapamycin (mTOR), a protein that modulates the proliferation and survival of activated lymphocytes. It is produced by the bacterium Streptomyces hygroscopicus and was isolated in 1972 on Easter Island (Rapa Nui). Sirolimus is used to prevent rejection in solid organ transplantion.

There is very limited experience in the use of this drug for poor responders to standard AIH treatment. Retrospective data on 5 adult patients with AIH refractory to prednisone, azathioprine and mycophenolate are available[138] (Table 3). Only transaminase levels were used to define remission, median follow up was 24 mo, the target serum level was low, 10-20 ng/dL. Complete remission was achieved in 2/5 patients. Side effects were limited to hyperlipidaemia occurring in 2/5 patients. In paediatrics, a small retrospective series reports the use of rapamycin in 5 cases refractory to standard treatment (3/4 also to MMF)[139], including 1 case of non-adherence (Table 4). Two of the four patients showed an improvement in transaminase levels; tolerability was good, though 2/4 had mouth ulcerations not requiring drug discontinuation. The target sirolimus blood levels reported in the paper are 4-8 ng/mL. A report of two additional adult cases of difficult-to-treat AIH patients managed with sirolimus is even less encouraging: in one case sirolimus was discontinued due to legs ulcers, and in the other it was ineffective[140]. No drug serum levels were reported.

In conclusion, data on sirolimus in difficult-to-treat AIH patients are scanty and rather disappointing.

In the transplant setting, sirolimus has been reported to be effective in difficult-to-treat de novo AIH or AIH recurrence[141] in a small series of 6 paediatric patients. Three of them experienced infections while on sirolimus, including one case of colitis and fever leading to drug discontinuation.

Everolimus: Everolimus has a mechanism of action similar to sirolimus, and is used to prevent solid organ rejection, or at higher doses, as an anti-cancer drug. Only one report is available on the use of everolimus for the treatment of AIH. It is a retrospective series of 7 adult patients with insufficient response to standard or alternative treatments (budesonide, MMF, calcineurin inhibitors), or with severe treatment side-effects[142] (Table 3). Everolimus target blood concentration was 3-6 ng/mL. Complete biochemical response was obtained in 3/7 patients after 5 mo, but all patients, except one who was non-adherent, had significant decrease in serum transaminase levels, allowing reduction of the steroid dose. Histology did not show disease progression in four patients treated for 3-5 years. No severe side effects were reported, but one patient died from cholangiocarcinoma diagnosed 6 mo after starting everolimus, though cancer was not considered to be associated with the drug. In conclusion, in view of the very few data available, the role of everolimus in the treatment of AIH remains to be explored.

Biologicals

Rituximab: Rituximab is a monoclonal chimaeric (murine/human) antibody that specifically binds the CD-20 antigen, a phosphoprotein expressed on the surface of B-lymphocytes, leading to B-cell depletion. It is approved for the treatment of non-Hodgkin lymphoma, rheumatoid arthritis and ANCA-

associated vasculitis. It has also been used recently as rescue treatment in refractory AIH. In a single-centre open-label pilot study in Canada, 6 AIH adult patients who had failed treatment with prednisone and/or azathioprine[143] for intolerable side-effects (3/6) or refractory disease (3/6) were treated with two doses of 1000 mg rituximab administered two weeks apart (Table 3). Tolerance was good, only one patient developing minor infections. In all patients, transaminase and IgG levels decreased; a liver biopsy performed after 1 year in 4 of the 6 patients showed improvement of the inflammatory activity. Though a recent survey shows that rituximab is used for difficult-to-treat AIH patients in several centres[144], this experience has not been published. A few case reports of patients with AIH coexisting with other autoimmune diseases have been published[145-149], all demonstrating a positive effect of rituximab also on AIH.

In children, two cases of refractory AIH have been successfully treated with rituximab[149] (Table 4). In addition, the recently published preliminary results of a real-world expert management of paediatric AIH also reported the use of rituximab as rescue therapy[150].

In summary, rituximab has shown good efficacy in a small number of difficult-to-treat AIH patients, but its safety profile needs to be evaluated carefully, as the drug may have severe long term side-effects, including B-cell depletion[151].

Infliximab: Infliximab is a recombinant humanized chimaeric antibody used for the treatment of ulcerative colitis, Crohn disease, rheumatoid arthritis, psoriatic arthritis/plaque psoriasis, and ankylosing spondylitis. It acts mainly by direct neutralization of soluble tumour necrosis factor-alpha, but it has also pro-apoptotic and anti-proliferative effects on lymphocytes[152].

One small retrospective series from Germany on the use of infliximab as salvage therapy in 11 adult AIH patients reports[153] (Table 3) normalisation of transaminase levels in 8 and of IgG levels in 6. However, 7 patients developed infectious complications, and treatment had to be stopped because of side effects in three cases. Recently, preliminary results of an extension of this cohort of difficult-to-treat AIH patients was published: the cohort now includes 18 cases, 15 reaching biochemical remission[154]. Two case reports have also been published: one describing a difficult-to-treat AIH patient who achieved normalization of transaminases levels after 3 mo of infliximab treatment[155], and one reporting good disease control on infliximab in a young patient with AIH and adult onset Still disease[156].

In children, a 10-year old girl with aggressive disease, unresponsive to standard treatment, MMF and tacrolimus, has been reported to have a good response to infliximab, though liver transplantation was deferred but not avoided[157] (Table 4).

As for rituximab, specialized centres have unreported experience[144,150]. It is important to note that anti-tumour necrosis factor-alpha can induce hepatotoxicity resembling AIH[158-162], as well as other immune-mediated disorders, such as lupus erythematosus[163]. This should raise caution in using this agent, which should be reserved for treatement–resistant AIH cases in specialised centres.

Thiopurines

6-mercaptopurine: Azathioprine is the prodrug of 6-mercaptopurine (6-MP), and is non-enzymatically converted into 6-MP, which represents the biologically active form of the drug. 6-MP is used for the treatment of IBD, where it has been shown that 6-MP is better tolerated than azathioprine[164,165], despite the close biochemical relationship and shared metabolic pathways (Figure 2). In AIH, 6-MP was used successfully in 3 patients intolerant or unresponsive to azathioprine, including one paediatric patient[166], representing the only published experience in children (Tables 3 and 4). The largest series of AIH patients intolerant or unresponsive to standard treatment switched to 6-MP is a retrospective study on 22 adult cases[167] (Table 3). The two patients with insufficient response to standard treatment did not respond to 6-MP, whereas 15/20 patients intolerant to azathioprine showed either partial (7/15) or complete (8/15) biochemical remission. Five patients discontinued 6-MP, four for gastrointestinal side effects, and one for leukopaenia. Recently, preliminary data from an additional multicentre retrospective series of 17 patients, all azathioprine-intolerant, reported complete biochemical response in 11 of the 12 patients followed-up for at least 12 mo[168]. These data suggest that 6-MP can be an alternative for patients intolerant to azathioprine, but the available data are insufficient to formulate recommendations.

Allopurinol: Azathioprine hepatotoxicity can be due to a skewed metabolism of the drug, leading to a preferential generation of the hepatotoxic metabolite 6-methylmercaptopurine (6-MMP) instead of the metabolic active 6-thioguanine nucleotides (6-TGN). Allopurinol co-administration redirects the thiopurine metabolism towards 6-TGN. This strategy is used in the treatment of IBD. A case report suggests that allopurinol can be helpful also in AIH[169] (Table 3). A retrospective case-series of 8 AIH adult patients intolerant or with insufficient response either to azathioprine/prednisone (4/8) or 6-MP/prednisone (4/8), one patient in each group being also on budesonide, reported complete biochemical remission in 3/3 intolerant patients and in 4/5 unresponsive patients[170] (Table 3). All patients had skewed thiopurine metabolism. In one further case report of a patient with insufficient response to prednisone/

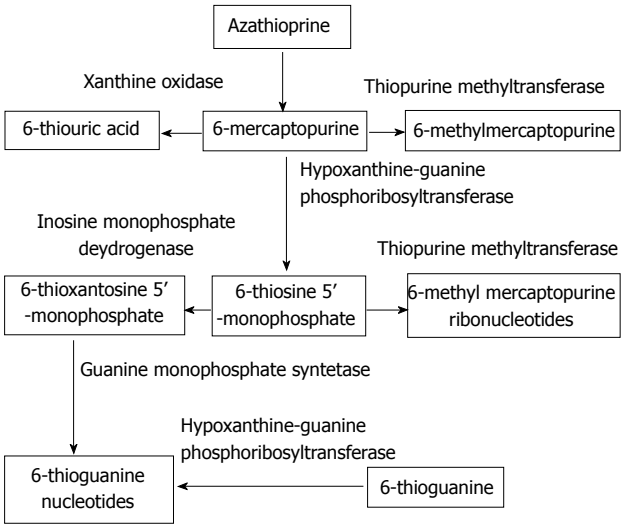


Figure 2 Simplified representation of the thiopurine metabolism. Azathioprine is non-enzymatically converted to 6-mercaptopurine, which is competitively converted into 6-methymercaptopurine, 6-thiouric acid and 6-thiosine 5'-monophosphate by different enzymes. The latter metabolite is further transformed into the metabolic active 6-thioguanine nucleotides.

azathioprine and shunted metabolism, allopurinol (100 mg/d) allowed rapid normalisation of transaminase levels and steroid reduction[171] (Table 3).

6-thioguanine: 6-thioguanine (6-TG) is enzymatically converted into 6-TGN, which are the active metabolites of azathioprine, bypassing the metabolic steps leading to the formation of the hepatotoxic metabolite 6-MMP (Figure 2). 6-TG is approved for the treatment of acute and chronic myeloid leukaemia, and chronic lymphatic leukaemia. It is used in IBD patients with insufficient response or intolerant to azathioprine or 6-MP[172]. Safety issues have been raised, particularly in respect to the development of nodular regenerative hyperplasia and sinusoidal obstruction syndrome[172]. In AIH, after an early preliminary report[173], a retrospective series of 12 adult patients switched from azathioprine or 6-MP to 6-TG for intolerance or insufficient response reported a median alanine aminotransferase levels drop from 81 IU/L to 30 IU/L (Table 3). Nodular regenerative hyperplasia developed in one case after 8 years of 6-TG treatment[174].

Due to the paucity of data and its potential hepatotoxicity, 6-TG cannot be recommended in AIH.

TREATMENTS UNDER INVESTIGATION

New compounds are currently under investigation in AIH. Preliminary results of a phase 1, first-in-human trial of preimplantation factor in AIH demonstrated good safety and tolerability, but a non-significant decrease in mean transaminase levels[175]. Other investigational drugs in AIH include VAY736, which leads to B-cell depletion and B-cell activating factor receptor blockade (NCT03217422), JKB-122, which is a toll-like receptor 4 antagonist (NCT02556372) and low dose interleukin 2 (NCT01988506).

CONCLUSION

The pharmacological treatment of AIH should be personalized, because of the heterogeneity of the disease. Treatment schedules in children differ, because of the more aggressive disease course in this age group. Standard treatment, based on steroids and azathioprine, is effective in the vast majority of patients, and side-effects can be minimised by rapid prednisone tapering. Budesonide was tried as first-line treatment in an attempt to reduce steroids side-effects, but the results of a randomized controlled trial do not allow to universally recommending it as first-line treatment instead of prednisone. A minority of patients prove difficult-to-treat, either because of severe side effects from standard treatment, or resistant disease. Mycophenolate mofetil is the most widely used second-line drug, and also the drug with the highest amount of available data. Calcineurin inhibitors are alternative options, but data on their efficacy are scanty. Infliximab and rituximab may represent an additional treatment option for selected difficult-to treat cases, but their use should be restricted to specialised centres because of potentially severe side effects. New pharmaceutical treatments are currently under investigation.

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MINIREVIEWS

Colorectal cancer, screening and primary care: A mini literature review

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Abstract

Colorectal cancer (CRC) is a common health problem, representing the third most commonly diagnosed cancer worldwide and causing a significant burden in terms of morbidity and mortality, with annual deaths estimated at 700000. The western way of life, that is being rapidly adopted in many regions of the world, is a well discussed risk factor for CRC and could be targeted in terms of primary prevention. Furthermore, the relatively slow development of this cancer permits drastic reduction of incidence and mortality through secondary prevention. These facts underlie primary care physicians (PCPs) being assigned a key role in health strategies that enhance prevention and prompt diagnosis. Herein, we review the main topics of CRC in the current literature, in order to better understand its pathogenesis, risk and protective factors, as well as screening techniques. Furthermore, we discuss preventive and screening policies to combat CRC and the crucial role served by PCPs in their successful implementation. Relevant articles were identified through electronic searches of MEDLINE and through manual searches of reference lists.

Key words: Colorectal cancer; Prevention; Diagnosis; Screening; Primary care

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Core tip: Colorectal cancer (CRC) is a common health problem, causing a significant burden in terms of morbidity and mortality. However, if detected early, the disease is highly curable. Primary care physicians are therefore in a unique position to enhance prevention and prompt diagnosis. The purpose of this paper was to

review the main topics of CRC in the current literature to provide a more comprehensive understanding of its pathogenesis, risk and protective factors, as well as screening techniques.

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INTRODUCTION

Colorectal cancer (CRC) is a global health burden, accounting for almost 700000 deaths per year worldwide[1]. CRC is the third most commonly diagnosed cancer worldwide and second in Europe[1]. According to the World Health Organization GLOBOCAN database, in 2012, almost 1.4 million new cases of CRC were diagnosed and almost 700000 deaths occurred worldwide[1].

CRC global incidence and mortality rates appear to be substantially higher for males than for females, with 21 new cases and 10.5 deaths per 100000 population compared to 17.6 new cases and 9.2 deaths respectively. In males, CRC ranks third in incidence, following lung and prostate cancers, and in females it ranks second, following breast cancer[1,2].

There is an over 10-fold geographical variation of CRC incidence throughout the world[3]. The highest incidence rates are observed in Australia and New Zealand, with the estimated age-standardized rates being 44.8 per 100000 population in men and 32.2 in women; Europe and North America follow close behind. The lowest incidence is observed in Africa, with the rates in Western Africa being only 3.5 per 100000 in men and 3.0 in women[1,3,4].

Incidence trends reported for the past few decades have revealed very interesting findings. In the United States, overall CRC incidence has been declining since the mid-80s, right about the time that CRC screening was introduced[5-7]. In Europe, incidence trend patterns show great diversity among countries, mainly due to differences in screening policies and prevalence lifestyle risk factors between countries. The largest increase in incidence was observed in Central-Eastern Europe over the past few decades[8-12].

CRC incidence increases with age, and cases are fairly uncommon before the 4th decade of life[1,3]. This is the reason why most screening programs are targeted to people over 50 years old. Nevertheless, recent studies have revealed an alarming increase in incidence between the ages 40 to 44, prompting consideration of lowering the recommended screening age[13,14].

Mortality rates have progressively declined in most economically developed countries, in contrast with poorer regions of the world, where mortality is either stable or increasing[1,11]. This reflects the diversity in screening services accessibility, specialized care and lifestyle risk factors[11]. The highest reported mortality rates are in Central-Eastern Europe, although the highest incidence to mortality ratio is observed in Middle-Western Africa[1,15].

In this paper, we aimed to perform a narrative literature review and compile all of the up-to-date knowledge on the current CRC medical literature. Our main objective was to summarize all the available information and provide gastroenterologists and primary care physicians (PCPs) with a comprehensive background for a better understanding of the current evidence.

SEARCH STRATEGY

We conducted a literature search in the PubMed database, with publication date limited to between January 1996 and August 2016, using the following Medical Subject Heading (commonly known as MeSH) terms: "colorectal neoplasms", "diagnosis", "early detection of cancer", "primary health care". The search was limited to English language. Editorials, Letters to the Editor and Case Reports were excluded. Inclusion criteria for papers were CRC topics in prevention, screening, detection and diagnostics, as well as follow-up in primary care. The titles and abstracts of all papers identified by the electronic search were manually assessed by two researchers working independently (AH, DA). Disagreements between the two reviewers were infrequent and resolved by consensus or arbitration of a third reviewer (MK).

Full texts of the articles that were considered eligible for inclusion were also scrutinized in order to offer a better approach on CRC issues related to pathogenesis, screening, diagnosis and management, as were articles related to early detection in the primary care setting.

A total of 159 studies were identified and assessed for eligibility. Among them, 7 overlapped and were excluded. Four articles were also excluded due to topical relevance to other types of cancer. Finally, 148 articles were assessed in detail for study inclusion. From these, 42 were excluded for not meeting the inclusion criteria. Figure 1 summarizes the process of identification and selection of studies.

PATHOGENESIS

The Adenoma-Carcinoma pathogenesis model is what gives endoscopic methods of screening the benefit of not only reducing mortality but also reducing incidence of CRC through early recognition and removal of adenomatous polyps from the colon[16]. The Adenoma-Carcinoma sequence applies to most CRCs and involves a sequential progression that takes, on average, a decade to occur. Many of the adenomas begin as small polyps that enlarge and become

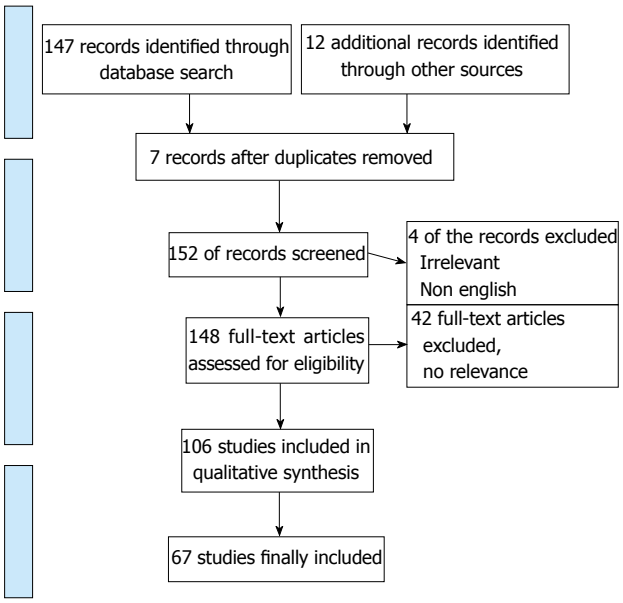


Figure 1 Study selection based on prisma diagram[112].

dysplastic and eventually cancerous[17-19]. Other CRCs may develop from non-polypoid adenomas, that are, by definition, more difficult to detect[20]. Nevertheless, there have been studies that suggest alternative developmental pathways to CRC, other than the traditional aforementioned model[21-23].

RISK FACTORS

The probability for developing CRC can be increased by both genetic and acquired/environmental factors. Although the impact of genetic susceptibility in an individual is much greater than the impact of acquired factors, the vast majority of CRC cases could be prevented through modifications in environmental factors[24-26]. A constructive way of classifying CRC risk factors is separating those that affect screening recommendations from those that do not. Hereditary syndromes, family history and inflammatory bowel diseases are the main risk factors that affect recommendations for screening[27]. In practice, risk factors that do not affect screening are the target of primary prevention strategies[24].

Hereditary colorectal syndromes comprise numerous specific genetic disorders that are associated with the development of CRC, altogether accounting for about 10% of CRC cases. The most common form is hereditary non-polyposis CRC, which reportedly accounts for only 2%-5% of CRC cases[28,29]. Family history, in addition to the genetic syndromes thus far known, constitutes a very significant risk factor for developing CRC, which appears to account for up to 25% of cases[28]. Although the mechanisms underlying this observation are not completely understood, studies have shown that individuals having first-degree family members diagnosed with CRC are at 2-3 times greater risk of developing CRC than the general population[28-31].

Although CRC, compared to other common cancers, includes a large percentage of hereditary cases, the vast majority of cases are sporadic, accounting for up to 70%[28]. The risk factors implicated in the mechanisms of sporadic disease are mainly environmental/acquired. Western lifestyle, cigarette smoking, alcohol intake, obesity and certain dietary habits are amongst the risk factors associated with increased risk for CRC[24,25,32-34].

PRIMARY PREVENTION

As expected, an important role in the etiology of CRC is attributed to lifestyle factors, since, as aforementioned, the majority of CRC cases are not associated with hereditary/familial factors[24,25,35]. Western lifestyle is a well-discussed risk factor for CRC, as it was readily observable by researchers that CRC incidence was consistently higher in industrialized countries[35]. This observation was further supported by the growing incidence in poorer regions as they adopted the western way of living[24,36].

Diet has been a popular subject of CRC research over the past few decades, both for its potential as a risk factor and as a protective factor. A number of researchers have argued the protective role of a diet high in fiber, with some studies showing a reduction in CRC incidence up to 50%[35,37-39]. Nevertheless, many recent reports have raised doubts about this argument, leaving the question of how protective dietary fibers really are, open for future prospective studies to answer[40,41]. Many authors have also asserted a protective role for calcium and vitamin D, as well as for other less verified dietary factors such as folate, vitamin B6, magnesium, garlic and omega 3 fatty acids[42-46]. On the other hand, frequent consumption of red meat and fat has been associated with increased risk for development of CRC[47-49].

Obesity has been consistently associated with increased risk for developing CRC, as well as with poorer outcomes following diagnosis[50-52]. In fact, a review of 29 studies reported that each 5 kg/m^2 incremental increase in body mass index is accompanied by CRC incidence increase of 24% in men and 9% in women[50]. In association with a healthy body weight, regular physical activity has been shown to reduce CRC incidence even more, with studies reporting up to 20%-30% lower risk[53,54].

Alcohol consumption as a risk factor for CRC has been a controversial subject, especially when referring to light and moderate consumption, but studies have consistently reported a higher risk for developing CRC among individuals with moderate to heavy consumption[55]. Tobacco smoking has been shown to double the risk of being diagnosed with a colon adenoma and to result to poorer outcomes following a cancer diagnosis, leading authors to recommend more intensive screening among smokers[24,56].

Although no accepted chemopreventive indications exist currently, many pharmaceutical agents have

shown preventive effects against CRC. Aspirin and COX-2 selective inhibitors are among the most investigated agents in regards to CRC prevention, and their regular use has shown ability to reduce incidence in individuals at both average and increased risk[57,58]. In the general population, the risks from their use seem to outweigh the benefits, but many advocate their use in certain individuals at increased risk for colorectal neoplasia[59].

SCREENING/SECONDARY PREVENTION

The fact that most CRCs take years to develop - following the Adenoma - Carcinoma sequence - permits the reduction of CRC mortality through screening, either by early detection and removal of the cancer or by detecting and removing the precancerous lesions[17-19].

There are roughly three categories of screening tests for CRC: stool-based, imaging, and endoscopic tests. Although stool-based tests can reduce mortality rates by early detection of asymptomatic cancerous lesions, imaging and endoscopic tests are capable of further reducing CRC incidence by detecting pre-cancerous lesions as well[16].

STOOL-BASED TESTS

Guaiac-based fecal occult blood test

Relying on the properties of alpha-guaiaconic acid, a phenolic compound extracted from Guaiacum trees, guaiac-based fecal occult blood test (gFOBT) can detect the presence of heme (of blood hemoglobin) in stool samples. Application of hydrogen peroxide onto guaiac paper causes alpha-guaiaconic acid to oxidize and turn blue. This reaction normally takes time, but heme (if present) catalyzes the reaction and within seconds a blue color change is visible[60-62]. This bioreactive method was proposed as a screening test for CRC almost half a century ago and has become the most frequently used screen for CRC worldwide[61].

While the gFOBT is cost affordable and non-invasive, it unfortunately bears many disadvantages. The interpretation of the result is subject to observer bias. Also, the reaction can be catalyzed by any peroxidase, such as heme found in meat, and false-positive results can lead to unnecessary colonoscopies; although, strict dietary restrictions that were proposed in the past seem to now be proven unnecessary[60,63]. False-negative results, on the other hand, can occur from ingestion of large doses of ascorbic acid (vitamin C)[64]. Aside from the dietary restrictions related to preparation for the gFOBT, the patient needs to provide three consecutive stool samples in order to achieve adequate sensitivity for occult blood[65]. The reported sensitivity and specificity vary between studies and different manufacturer brands, and efforts to introduce new, more sensitive guaiac-based tests resulted in lower specificity[66]. Finally, this test cannot detect polyps, since they do not bleed, and its sensitivity for advanced adenoma is relatively low[67].

Fecal immunochemical test

Fecal immunochemical test (FIT) detects blood in stool by using a specific antibody against human hemoglobin. As such, FIT is not affected by diet or observer bias, giving it a greater specificity than gFOBT. Besides specificity, however, its sensitivity for both cancer and adenomas has been shown to be superior to that of gFOBT[68,69]. According to a recent meta-analysis, the mean reported sensitivity and specificity for FIT detection of CRC is 79% and 94% respectively[70]. In addition, FIT requires fewer samples than gFOBT, making it more convenient for patients and thus increasing compliance[71]. Quantitative results can be provided with this method as well, facilitating the ability to determine positive cut-off points for different populations, patient characteristics, or system capabilities and resources[72].

In summary, stool-based tests are non-invasive and inexpensive methods capable of detecting occult bleeding. However, they are practically incapable of detecting polyps, since the latter do not usually bleed, and they have low sensitivity for detecting adenomas. Consequently, their role in reducing CRC incidence is close to none, but their implementation as a screening tool can reduce CRC mortality by providing early recognition of cancerous lesions. Comparing the two methods, FIT appears superior in terms of sensitivity and specificity (for both CRC and adenomas) and in terms of patient compliance. It is reasonable then to expect that, although more expensive, FIT could be more cost-effective than gFOBT since it could prompt less unnecessary colonoscopies.

IMAGING TESTS

Double-contrast barium enema

In double-contrast barium enema (DCBE), the colon is studied through X-rays obtained after coating the mucosa with barium and distending the colon with air, both of which are inserted transrectal. The DCBE is considered a safe method and has been used frequently in the past, but its use has been dramatically reduced as novel imaging methods become available. The reported sensitivity of DCBE for large polyps (> 10 mm) is only about 50%, and false positive results can occur due to inadequate bowel preparation[73,74].

Computed tomographic colonography

This method was first described more than 20 years ago, and provides 2- and 3-dimensional endoluminal images of the colon upon reconstructing of computed tomography or magnetic resonance images of the air-

distended colon[75,76]. The reported diagnostic value of computed tomographic colonography (CTC) has varied between studies, but as newer techniques of CTC are developed it is closing in on colonoscopy in terms of sensitivity and specificity for detecting CRC[77]. In a recent meta-analysis, the overall sensitivity and specificity of CTC was 66.8% and 80.3% respectively, both lower than the values for colonoscopy. For polyps > 10 mm though, the meta-analysis showed greater sensitivity and specificity (91.2% and 87.3% respectively)[78].

CTC appears to be more preferred by patients than colonoscopy; in addition, it has a very low risk of bowel perforation and requires no sedation[79,80]. On the other hand, CTC requires follow-up colonoscopy after positive results (to perform excision/biopsy), exposes the patient to radiation, and the lack of standardized methods leads to variable diagnostic performance[77,78]. The need for aggressive bowel preparation has been an issue, but newer techniques have been reported involving laxative-free CTC using "fecal tagging" with an ingested contrast agent[81]. Many authors include in CTC's advantages the potential of discovering extra-colonic pathology in asymptomatic patients, but this argument is controversial since these findings can sometimes lead to unnecessary patient anxiety, costly investigations and overdiagnosis[82-83].

Colon capsule endoscopy

The colon capsule endoscopy (CCE) method for CRC screening was initially introduced in 2006, and roughly consists of swallowing a pill-shaped device which is capable of photographing the gastrointestinal tract as it passes through it[84]. Initially, CCE did not gain significant acceptance as a screening tool for CRC, mainly because of its cost and relatively low diagnostic value compared to colonoscopy[85]. After introduction of the second-generation CCE (CCE-2) in 2009, the subject of CCE has become very popular in the medical literature[86]. The reported average sensitivity and specificity for the CCE-2 is 86% and 71% respectively, and since 2012 it has been prompted as an acceptable screening method for CRC by the European Society of Gastrointestinal Endoscopy[87]. Compared to colonoscopy, CCE might be a lot more preferable for the patient, but it is more expensive, lacks excision/biopsy ability and requires very aggressive bowel preparation[87,88].

ENDOSCOPIC TESTS

Flexible sigmoidoscopy

Flexible sigmoidoscopy (FS) enables the trained physician to visualize the distal gastrointestinal tract up to the splenic flexure, using a flexible, 60 cm long endoscope[89]. FS requires only minimal bowel preparation, no diet restrictions and no sedation, and can be performed by non-gastroenterologists (*e.g.,* PCPs) or even trained nurses[89-91]. Obviously, FS is unable to detect lesions in the proximal colon, which makes it lacking in sensitivity compared to colonoscopy[90]. In a meta-analysis, FS appeared to reduce CRC incidence and mortality among screened patients, by 32% and 50% respectively[92].

Colonoscopy

The traditional method of colonoscopy provides visualization of the entire large bowel and the distal part of the small bowel by using a flexible, 120-cm to 160-cm long endoscope[93]. It is considered by most the 'gold standard' in CRC screening, mainly because of its high sensitivity and specificity for detecting cancerous and precancerous lesions. It also provides the ability to excise or biopsy detected lesions during the same procedure[6,13,77,90]. Unfortunately, it is also an expensive method and not free of risk; it also requires sedation and extensive bowel preparation. The reported rate of major complications, such as bleeding or bowel perforation, is approximately 0.1%-0.2%, but could become significantly higher when excisions or biopsies are performed and in elderly or comorbid patients[93].

PREVENTION: SCREENING IMPLEMENTATION AND THE ROLE OF PCPS

It is beyond doubt that the burden of CRC can be significantly reduced through primary and secondary prevention. Scientific research over the past few decades has offered, as aforementioned, a variety of options for CRC screening and a better understanding of risk and protective factors for the development of CRC. Unfortunately, underutilization of screening and a lack in preventive policies are being reported[94-98].

Some European countries still have not implemented national mass screening programs, and others that did have reported low participation rates[97,98]. In the United States, there has been a significant decrease in incidence and mortality following widespread implementation of screening, but the overall use of screening is still below national standards[94]. Additionally, it has been reported that uninsured people in the United States and people of low socioeconomic or educational status show much lower participation rates[99].

Many researchers have attempted to identify the causes of CRC screening underutilization and ways to enhance it. While the barriers and sites of potential improvement have been identified at the levels of the health care system and the patient, most of the authors have advocated for the key role of PCPs.

Patients, in many studies, have shown low awareness concerning CRC screening and its importance. In one particularly insightful study, by Aubin-Auger *et al*[97], some patients showed low interest in CRC

screening, while others expressed the belief that CRC screening concerns only high-risk individuals or individuals that do not follow a healthy lifestyle. In a systematic review by Holden *et al*[100], the authors found that most patients not being screened state their reason as "not thinking about it". Strongly indicative of how important public awareness is for CRC screening participation was the "Presidential Effect", a term given to describe the increase in CRC screening participation of United States' citizens after the nation's President, Ronald Reagan, was diagnosed with colon cancer in 1985[101]. Similar reports of the impact of public figure announcements on cancer have been made, but data show that information given occasionally and in an unorganized manner often leads to short term results and mis- or overutilization of screening services[102,103].

At the level of health care systems, barriers and possibilities for enhancing CRC screening participation are more obvious. Several studies have revealed the efficacy of organized mass screening programs, especially when using patient reminders[100,104-107]. Participation rate has also appeared to be further enhanced when PCPs are involved in the invitation process[104,105]. Findings from studies have also led to authors advocating in favor of informational campaigns that increase public awareness of screening[108]. Another interesting finding in the literature is that patients with "usual source of care" are more likely to be screened for CRC[100]. This further highlights the importance of PCPs and family physicians in modern health care systems.

Research has also indicated barriers at the level of PCPs, with troubling findings in some cases. Screening recommendation rates by PCPs seem to remain low[100,109]. Several PCPs in surveys have reported a lack of knowledge and training, and some have even reported not finding screening to be effective[97,98,109,110]. The major role of PCPs in the effort to decrease CRC incidence and enhance screening participation has become more than obvious. PCPs constitute the first level of contact in a national health system for individual patients and their families. Their role in preventive medicine, through interventions in lifestyle habits, can effectively reduce CRC incidence, as well as that for other diseases. The unique patient-physician relationship in primary health care, in terms of trust and continuity of care, can effectively contribute to patient compliance, as clearly demonstrated throughout the literature.

Interventions through health care system organization, education of PCPs on CRC screening, prevention and counseling techniques, and in public awareness will, therefore, drastically decrease the burden of CRC[111].

CONCLUSION

Despite the significant improvements in screening techniques and our understanding of risk and protective factors, CRC remains a major global health burden. PCPs face a unique challenge in their capabilities and efforts to alter this phenomenon; their role in implementing screening and preventive policies is key to reducing the burden of CRC.

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MINIREVIEWS

Behavioral gastroenterology: An emerging system and new frontier of action

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Abstract

Behavioral gastroenterology is a new interdisciplinary science that explores the influence of unhealthy lifestyles and psychological factors on the digestive system and addresses the prevention, diagnosis, treatment, and rehabilitation of digestive diseases. Moreover, the concept of whole-course intervention with a focus on disease prevention and a new model of integrated therapy based on alterations of lifestyle and psychology are being gradually established. This paradigm may substantively impact the prevention and treatment of digestive diseases.

Key words: Behavioral gastroenterology; Lifestyle; Psychological factors

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Core tip: As a new interdisciplinary subject, behavioral gastroenterology shows a new concept of whole-course intervention with a focus on disease prevention and a new pattern of integrated therapy based on lifestyle and psychosocial adjustments. It will help both clinicians and patients to alter the old idea of focusing on traditional drugs or surgery, and this new paradigm is expected to become gastroenterology's new frontier of action.

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INTRODUCTION

Behavioral medicine, a phrase first coined at the Yale meeting in 1977, has been developing rapidly. In 2003, the new discipline of behavioral cardiology took a broad view, concluding that heart disease is not inevitable but rather may develop largely from psychosocial stress and unhealthy lifestyles such as smoking, overeating, and physical inactivity[1]. Likewise, the effects of psychological-behavioral factors and unhealthy lifestyles on digestive diseases are no less impactful than those of traditional risk factors. Thus, by combining behavioral medicine and gastroenterology, a new pattern of psychological-behavioral treatment for gastroenterological patients has gradually emerged.

Based on the research that has integrated both traditional Chinese medicine and modern medicine, we established a behavioral gastroenterological group in the behavioral-psychosomatic medicine branch of the Guangdong Medical Association in 2012. The new concept of behavioral gastroenterology was first proposed in 2013. As an emerging field of clinical practice and an important part of behavioral medicine, behavioral gastroenterology comprehensively explores unhealthy lifestyles and behavioral-psychological factors that can affect the digestive system. This new discipline also addresses the prevention, diagnosis, and treatment of digestive diseases and rehabilitation of patients. Furthermore, a new concept of whole-course intervention with a focus on disease prevention and a new pattern of integrated therapy based on lifestyle/psychosocial adjustments have gradually been established. It is believed that both lifestyle and psychosocial factors can be improved by behavior modification, during which the patient and the practitioner work together.

BEHAVIORAL FACTORS AND DIGESTIVE DISEASES

Traditional Chinese medicine attaches great importance to lifestyle such as diet and daily routine, which are closely related to health and longevity. Similarly, modern medical research has also demonstrated that the onset of many digestive diseases is closely related to deviations in diet, sleep habits, work shifts, and poor lifestyle choices such as excessive alcohol consumption and tobacco use.

Dietary behavior

Certain types of foods are closely associated with symptoms of gastrointestinal diseases. For example, coffee, black pepper, chocolate, and onions often cause epigastric burning and heartburn, milk and dairy products, beans, bananas, and carbonated drinks often produce gas, and fresh meat, fried foods, wheat, manufactured goods, cakes, sweets, chocolate, citrus fruits, beans, and onions often can cause early satiety. Wheat (93.33%), milk and manufactured goods containing milk products (83.33%), turnip (71.67%), bacon (55%), banana (53.33%), and onion (46.67%) often lead to intestinal smooth-muscle dysfunction, which constitutes a type of functional dyspepsia (FD)[2]. Additionally, food consumption plays a key role in irritable bowel syndrome (IBS): more than 60% of IBS patients report the onset or worsening of symptoms after meals - within 15 min for 28% of patients, and within 3 h for 93% of patients[3]. Most IBS patients (84%) report meal-related symptoms as a reaction to at least one specific food, such as milk and dairy products, beans, or apples[4,5].

Eating behavior is also associated with digestive diseases. Gastroesophageal reflux disease (GERD) correlates with eating greasy or spicy food or drinking carbonated beverages[6]. Patients with nonalcoholic fatty liver disease often consume high-energy foods and carbohydrates but ingest fewer foods rich in polyunsaturated fatty acids, vitamins, and minerals[7]. Consumption of certain foods is closely correlated with digestive diseases. Long-term intake of hot foods and beverages positively correlates with the incidence of esophageal cancer[8]. Fast intake (12 min or less) has been reported by about 40% of FD patients but in only 17% of healthy people. Compared with nonspecific FD, significant differences in the frequency of meal skipping and fast intake have been reported by patients with dismotility-like FD and ulcer-like FD[2]. A large-sample multicenter study in China found that refractory FD patients often have unhealthy eating habits such as skipping meals, eating extra meals, or a preference for sweet food and gas-producing food[9].

Exercise behavior

A sedentary lifestyle is a risk factor for constipation and nonalcoholic fatty liver disease[10-11]. Tuteja *et al*[12] reported that exercise does not affect symptoms such as constipation but can improve patients' quality of life. A meta-analysis revealed that physical exercise can reduce the risk of the occurrence of esophageal cancer, especially adenocarcinoma[13].

Sleep behavior

GERD patients often report poor sleep quality[14]. Fass *et al*[15] reported that 50.2% of 505 IBS patients suffer sleep disorders such as easy wakening during nighttime sleep or fatigue in the morning. Their study also found that poor sleep quality decreases the threshold of visceral pain and that sleep disorders can induce and aggravate the symptoms of IBS. Sleep disorders have also been reported to be associated with the severity of symptoms and the comorbidity of anxiety for FD patients[16].

Work behavior

A study found that working a rotating shift can

significantly increase the risk of gastrointestinal disorders and peptic ulcers[17]. Nojkov *et al*[18] reported that the incidence of IBS is 48% for nurses with rotating shifts and 31% for nurses with strictly daytime work, and the probability of functional abdominal pain was 81% for nurses with rotating shifts, 54% for those with day shifts, and 61% for those with night shifts. Sleep quality is closely associated with IBS and the incidence of abdominal pain, which might be related to disruption of the circadian rhythm caused by rotating shifts.

Smoking and drinking habits

A large population-based study from developing countries indicated that smoking is not associated with GERD or epidemiologic FD; however, smoking is significantly associated with clinical FD, postprandial fullness, and epigastric pain[19]. Lunney PC *et al*[20] found that the condition of patients with Crohn's disease can be worsened by smoking, and those investigators recommended that such patients quit smoking. In addition, long-term consumption of alcoholic beverages can induce lesions of the liver or pancreas, and alcohol directly damages the mucosa of the esophagus and stomach, modifies the sphincter pressure, impairs gastrointestinal mobility, and alters gastric acid output[21].

PSYCHOLOGICAL FACTORS AND DIGESTIVE DISEASES

Psychological factors have received attention with respect to the occurrence and development of digestive diseases. A variety of psychological anomalies can be found in FD or IBS patients, especially those involving symptoms of anxiety and depression[22]. A series of refractory functional gastrointestinal disease studies by our groups in recent years revealed that the incidence of anxiety and depression are 61.5% and 63.3%, respectively, for refractory FD patients, and the severity of anxiety and depression is greater than for non-refractory FD patients[23]. Moreover, of 1057 patients, the incidence of anxiety and depression for FD patients with weight loss was 56.04% and 59.90%, respectively, and a higher incidence of anxiety and depression was found for patients of group A (lost $\geqslant$ 5% of initial body weight) than for those of group B (lost < 5% of initial body weight)[24]. Cruz Ruíz *et al*[25] reported that 58% of 100 IBS patients suffered anxiety, whereas 62% of those patients suffered depression, and female patients accounted for more than half. The incidence of symptoms of depression and anxiety was 66.2% and 65.5%, respectively, for the refractory IBS group, which was greater than that for the non-refractory IBS and control groups[26]. Similarly, a significantly higher percentage of refractory globus patients have anxiety and depression compared with non-refractory globus patients. And the prevalence of moderate-to-severe anxiety and depression was higher for the refractory globus group than for the non-refractory globus group[27].

A study from Zhou *et al*[28] revealed that functional constipation patients have higher scores for depression and anxiety, and the anorectal squeezing pressure is negatively correlated with these scores; these results indicate that depression and anxiety may contribute to functional constipation. GERD patients are more likely to have emotions such as anxiety, depression, and pessimism than the general population, and such psychological disorders may make people vulnerable to GERD[29].

BEHAVIORAL AND PSYCHOLOGICAL INTERVENTION

Behavioral gastroenterology advocates a new pattern of integrated therapy involving the adjustment of unhealthy lifestyles and psychological problems. This strategy is a shift from the former paradigm, which ascribed greater importance to the treatment of severe digestive diseases and complications. Furthermore, a series of effective behavioral and psychological interventions have been gradually established.

Dietary behavior intervention

Intermittent fasting is an internationally popular diet method that is effective for the control of body weight and the improvement of metabolic syndrome. Intermittent fasting generally entails normal intake for 5 d a week, but intake of a quarter of the normal calories for the other 2 d (usually 500 calories for females, 600 calories for males). A multicenter and large sample research demonstrated that patient body weight was reduced by 4.3 kg on average after 6 wk of intermittent fasting[30]. Halberg *et al*[31] found that healthy people who underwent 2 wk of intermittent fasting exhibited no obvious body weight loss, but they did show improved insulin sensitivity - that is, the capacity for glucose storage and fat decomposition was increased. This result may be the key to improving metabolic syndrome.

A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) (Table 1) has been used recently for the management of functional gastrointestinal symptoms for IBS patients. Eswaran *et al*[32] showed that the low FODMAP diet led to a significant improvement in IBS symptoms, particularly pain and bloating. Staudacher *et al*[33] reported that more patients in the low FODMAP group expressed satisfaction with their symptom response (76%) compared with the standard-diet group (54%).

Intake control has received increasing attention, and intake reduction has been adopted by the majority of people. The strategy of "eating until you are 60% full", which was reported by the Healthy Aging Research Center of the University of London, was

Table 1 FODMAP food list

Food category	Low FODMAP food (things good to intake)	High FODMAP food (things to avoid/reduce)
Vegetables and Legumes	Alfalfa, Bean sprouts, Brussel sprouts, Butternut squash, Callaloo, Carrots, Celery, Chick peas, Chilli, Chives, Cho cho, Choy sum, Collard greens, Corn, Cucumber, Fennel, Green beans, Green pepper, Ginger, Leek leaves, Lentils	Garlic, Onions, Artichoke, Asparagus, Baked beans, Beetroot, Black beans, Cassava, Cauliflower, Falafel, Leek bulb, Mange Tout, Mixed vegetables, Mushrooms, Peas, sugar snap, Savoy Cabbage, Shallots, Taro
Fruit	Ackee, Bananas, Bilberries, Blueberries, Breadfruit, Cranberry, Clementine, Dragon fruit, Grapes, Kiwifruit, Lime, Mandarin, Orange, Passion fruit, Paw paw, Papaya, Pineapple, Plantain, peeled, Raspberry, Strawberry, Tamarind, Tangelo	Apples, Apricots, Avocado, Blackberries, Blackcurrants, Cherries, Currants, Dates, Goji berries, Grapefruit, Lychee, Mango, Peaches, Pears, Pineapple, Plums, Prunes, Sultanas, Tamarillo, Watermelon
Meats, Poultry, and Meat substitutes	Beef, Chicken, Kangaroo, Lamb, Pork, Prosciutto, Quorn, mince, Turkey, Cold cuts/deli meat/cold meats	Chorizo, Sausages, Processed meat - check ingredients
Fish and seafood	Canned tuna, Fresh fish (*e.g.*, Cod, Haddock, Plaice, Salmon, Trout, Tuna), Seafood (*e.g.*, Crab, Lobster, Mussels, Oysters, Prawns, Shrimp)	–
Cereals, Grains, Breads, Biscuits, Pasta, Nuts, and Cakes	Wheat free breads, Gluten free breads, Almonds, Biscuit, savoury, Biscuit, shortbread, Brazil nuts, Bulgur, Buckwheat, Brown rice, Chestnuts, Cornflour, Polenta, Popcorn, Potato flour, Pretzels, Quinoa, Rice, Sorghum, Walnuts	Wheat containing products, Almond meal, Amaranth flour, Barley including flour, Bran cereals, Bread, Cashews, Cous cous, Einkorn flour, Freekeh, Gnocchi, Muesli bar, Pistachios, Rye, Rye crispbread, Semolina, Spelt flour
Condiments, Dips, Sweets, Sweeteners, and Spreads	Aspartame, Acesulfame K, Barbecue sauce, Capers in vinegar, Capers, salted, Chocolate, Garlic infused oil, Golden syrup, Glucose, Marmalade, Marmite, Soy sauce, Stevia, Sweet and sour sauce, Sucralose, Sugar, Vegemite, Vinegars	Agave, Caviar dip, Fructose, Fruit bar, Hummus, Honey, Jam mixed berries/strawberry, Pesto sauce, Quince paste, Relish /vegetable pickle, Stock cubes, Sugar free sweets containing polyols, Sweeteners, Tahini paste, Tzatziki dip
Drinks and protein Powders	Drinking chocolate powder, Espresso, regular, black, Fruit juice (125 mL and safe fruits only), Malted chocolate powder, Tea (Chai tea weak, Green tea, Peppermint tea, White tea), Water	Beer, Coconut water, Cordial, Fruit juices made of apple, pear, mango and Orange, Rum, Sodas containing High Fructose Corn Syrup (HFCS), Soy milk, Sports drinks, Wine
Dairy foods	Butter, Dairy free chocolate pudding, Eggs, Margarine, Soy protein, Tempeh, Tofu, Whipped cream, Yoghurt with lactose free	Buttermilk, Cheese, Cream, Custard, Gelato, Ice cream, Kefir, Milk, Sour cream, Yoghurt

observed to extend the average lifespan of mice for five or six months - that is, it prolonged life by 30%. This would correspond to 20 years in humans.

Exercise behavior intervention

De Schryver *et al*[34] found that regular physical activity (including 30 min of brisk walking and 11 min a day family project) could significantly improve defecation problems (*e.g.,* overly frequent defecation, laborious defecation, and hard stools) caused by a lack of activity in middle-aged patients with chronic idiopathic constipation and significantly shorten the transit time in the sigmoid colon and rectum colon.

In addition, the results from Matsuzaki *et al*[35] revealed that the intensity of regular exercise is independently associated with gastric emptying in healthy individuals, and gastric emptying was significantly faster in the low-intensity exercise group than in the moderate-intensity exercise group. It is trusted to be an optimal exercise intervention for the treatment of FD.

Life behavior intervention

Nowak *et al*[36] implemented lifestyle adjustments for 23 patients with GERD during one month such as not leaning in during the 2 to 3 h after a meal, having more meals a day but less food at each meal, and a low-fat diet. The frequency and severity of GERD symptoms significantly decreased for 22 patients, and 11 patients reduced their use of GERD-specific drugs.

A report from Eherer *et al*[37] revealed that abdominal breathing training combined with proton pump inhibitors was more effective than proton pump inhibitors alone for the treatment of GERD. Thus, non-drug lifestyle intervention therapy can effectively improve symptoms and reduce the medication burden of patients.

Psychological intervention

Psychological factors are considered to be closely related to functional digestive diseases such as FD, IBS, GERD, and globus. Therefore, it is very important to quickly determine the causes of the psychological problems and help solve them. At the same time, appropriate drugs that can alleviate emotions should be used.

We performed a series of treatment research on the application of low-dose antidepressants in depressive patients with functional gastrointestinal diseases. Amitriptyline (25 mg/d) was used to treat globus patients. After 4 wk of treatment, the amitriptyline group showed significantly greater improvement in the Glasgow Edinburgh Throat Scale score and sleep quality than the group that received conventional treatment (pantoprazole, 40 mg/d)[38]. Paroxetine (20 mg/d) was also applied for globus treatment. After 6 wk of treatment, 71.7% of the subjects in the paroxetine group (33/46) exhibited a treatment response, which was significantly greater than those for the amitriptyline group (46.2%, 24/52) and the lansoprazole group (14.0%, 7/50). A more distinct improvement of emotional well-being, quality of life,

and quality of sleep was observed in the paroxetine group than in the lansoprazole or amitriptyline group[39]. Additionally, mirtazapine (30 mg/d) was utilized to treat the FD patients with weight loss. After 8 wk of treatment, mirtazapine not only alleviated symptoms associated with dyspepsia and depression linked to FD for patients with weight loss but also significantly increased body weight (mainly the visceral fat in body fat)[40].

The low-dose administration of antidepressants mentioned above may not only alleviate the symptoms of depression but also improve the patient's digestive symptoms. Because these satisfactory effects can be achieved at low medical cost, the treatments are worth promoting in the clinic.

CONCLUSION

Unhealthy lifestyle and psychological factors are closely related to the occurrence, development, curative effect, and prognosis of digestive diseases. Thus, the new concept of whole-course intervention with a focus on disease prevention, and the new model of integrated therapy for the adjustment of unhealthy lifestyles and psychological problems are being established. As a new interdisciplinary subject, behavioral gastroenterology will help both clinicians and patients to alter the old idea of focusing on traditional drugs or surgery while ignoring lifestyle adjustments, and this new paradigm is expected to become gastroenterology's new frontier of action.

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ORIGINAL ARTICLE

Basic Study

Pharmacological evaluation of NSAID-induced gastropathy as a "Translatable" model of referred visceral hypersensitivity

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Author contributions: Hummel M and Whiteside GT equally conceptualized study design, analysis, and interpretation of study results; Hummel M wrote the manuscript; Knappenberger T collected and analyzed the data; Reilly M collected rotarod data and prepared the figures depicting the data.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Purdue Institutional Animal Care and Use Committee (IACUC Protocol number: 2014-100) in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Ethical Guidelines of the International Association for the Study of Pain (www.iasp-pain.org) and are reported in accordance with the ARRIVE guidelines (www.nc3rs.org.uk). All efforts were made to minimize the number of animals used and to avoid any undue pain.

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Abstract

AIM

To evaluate whether non-steroidal anti-inflammatory drugs (NSAIDs)-induced gastropathy is a clinically predictive model of referred visceral hypersensitivity.

METHODS

Gastric ulcer pain was induced by the oral administration of indomethacin to male, CD1 mice (n = 10/group) and then assessed by measuring referred abdominal hypersensitivity to tactile application. A diverse range of pharmacological mechanisms contributing to the pain were subsequently investigated. These mechanisms included: transient receptor potential (TRP), sodium and acid-sensing ion channels (ASICs) as well as opioid receptors and guanylate cyclase C (GC-C).

RESULTS

Results showed that two opioids and a GC-C agonist, morphine, asimadoline and linaclotide, respectively, the TRP antagonists, AMG9810 and HC-030031 and the sodium channel blocker, carbamazepine, elicited a dose- and/or time-dependent attenuation of referred visceral hypersensitivity, while the ASIC blocker, amiloride, was ineffective at all doses tested.

CONCLUSION

Together, these findings implicate opioid receptors, GC-C, and sodium and TRP channel activation as

possible mechanisms associated with visceral hypersensitivity. More importantly, these findings also validate NSAID-induced gastropathy as a sensitive and clinically predictive mouse model suitable for assessing novel molecules with potential pain-attenuating properties.

Key words: Visceral hypersensitivity; Pain; Translation; Guanylate cyclase C; Non-steroidal anti-inflammatory drugs; Transient receptor potential channel

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Core tip: Recently, standard animal models of pain have been vehemently challenged for their inability to successfully predict human clinical outcomes. Further, few animal models have been represented with reasonable translational value for conditions presenting with visceral pain. Non-steroidal anti-inflammatory drug -induced gastropathy represents a translatable model of visceral hypersensitivity in which several pain targets have demonstrated reliable sensitivity when assayed. Further, this model is robust enough that proper pharmacological evaluation can be conducted. Overall, this model has the potential to efficiently triage molecules with pain-attenuating properties for their utility in gastrointestinal disorders that include pain as a hallmark symptom.

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INTRODUCTION

Stomach ulcers are common and afflict millions of individuals annually (www.mayoclinic.com)[1]. Like many gastrointestinal (GI) disorders, stomach ulcers cause significant pain, a hallmark feature of many ailments affecting visceral organs. Although stomach ulcers predicate a type of visceral hypersensitivity, surprisingly the mechanisms underlying the pain associated with gastric ulceration as with some other GI conditions [*i.e.*, irritable bowel syndrome (IBS), functional dyspepsia, pancreatitis] remain poorly understood[2-4]. Several preclinical models of gastric or duodenal ulceration have been established that highly resemble human ulcers in terms of pathological features including the indomethacin-induced gastropathy and acetic acid models[5-9]. While these and other models have been used to aid the development of novel disease ameliorating therapies (*i.e.*, proton pump inhibitors and histamine receptor antagonists) as well as elucidate the notable involvement of *Helicobacter pylori* infection in ulcer formation, a direct behavioral measure of the pain associated with ulceration in these models has not been reported[10]. Furthermore, despite the success of these drugs in healing ulcer lesions, sensory aberrations leading to such pain have not been clearly delineated and often remain a chief complaint for many patients[10,11]. This is especially true for those individuals who are actively treated for ulcers but who also require concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin, analgesics commonly used for other chronic conditions such as osteoarthritis (OA) and cardiovascular disease[12,13]. In conditions like OA, these agents are used to treat musculoskeletal pain, but paradoxically cause or exacerbate stomach pain on their own[13,14]. NSAIDs and salicylates are ulcerogenic and therefore, chronic use can exacerbate existing gastric injury or lead to new ulcer formation[15]. For these and other patients, it has been hypothesized that persistent or unresolved visceral pain, despite the etiology, may be due to aberrations in primary afferent function or hypersensitivity, peripheral sensitization, and/or psychological/genetic abnormalities[16-20].

With this in mind, we characterized the pain associated with gastric ulceration. By combining a clinically relevant stomach ulcer model with a predictive behavioral endpoint, we investigated some potential mechanisms producing visceral hypersensitivity. To this end, we used the indomethacin-induced gastropathy model to model the mucosal injury and concomitant pain associated with NSAID use. This model recapitulates the human condition in that indomethacin is orally administered to mice to produce mucosal damage, inflammation and referred visceral hyperalgesia[21-23]. Like in other GI disorders, ulcer pain is diffuse. Moreover, it can be referred to somatic structures and may present itself atypically given the dichotomization of sensory fibers that innervate visceral tissues[24-27]. Therefore, since ulcer pain is reportedly present upon palpation or mechanical stimulation of the abdomen both in dogs[28] and humans[29], we extrapolated this to mice and quantified the referred abdominal hypersensitivity by measuring the number of behavioral responses evoked by von Frey fiber stimulation[23,30]. We then investigated the pharmacological role of guanylate cyclase C (GC-C) and opioid receptors as well as TRPs, ASICS and sodium channels in this regard since all have been implicated in visceral hypersensitivity and/or functional bowel disorders to some degree[31-35].

MATERIALS AND METHODS

Animals

Male CD-1 mice (Harlan Laboratories, Indianapolis, IN, United States) weighing 20-25 g were housed on cob bedding (five/cage) in a climate-controlled room and maintained on a 12-h light/dark cycle with free access

to food and water. Animals were acclimated to the Purdue Pharma L.P. animal facility for one week prior to testing.

Animal care and use statement

All studies were approved by the Purdue Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Ethical Guidelines of the International Association for the Study of Pain (www.iasp-pain.org) and are reported in accordance with the ARRIVE guidelines (www.nc3rs.org.uk). All efforts were made to minimize the number of animals used and to avoid any undue pain.

Ulcer pain model

As previously described, mice were fasted overnight then dosed orally with 30 mg/kg indomethacin to develop the ulcer model[23,30]. Control animals received vehicle (10 mL/kg, p.o.). Morphine was administered 2 h post-indomethacin while the other compounds were administered 3 h post-indomethacin or 1 h before testing. Stomachs from a separate set of vehicle- and indomethacin-dosed mice were dissected and photographed to ensure ulcer model development.

Behavior

Referred abdominal hypersensitivity from indomethacin-induced gastric ulceration was quantified by measuring the threshold to withdrawal from the application of a tactile stimulus to the abdominal area[23]. Briefly, the abdominal area was shaved and the mice were subsequently placed inside Plexiglas boxes situated on elevated wire screen mesh flooring conducive to von Frey probing. Following a habituation period, baseline tactile sensitivity was assessed. Tactile hypersensitivity was then measured 4 and 24 h following indomethacin administration using von Frey filaments applied to the upper abdomen (bending force 0.005-3.58 g starting with the 0.407 g fiber). The upper abdominal region was stimulated with an incremental series of eight monofilaments of increasing logarithmic stiffness. The 50% withdrawal threshold was determined using the up-down method of Dixon, modified by Dixon[36] and Chaplan *et al*[37]. First, an intermediate von Frey monofilament (number 3.58) was applied to the abdomen causing a slight bending. If a positive response was noted (abdominal/whole body withdrawal or licking), a smaller filament was then tested. Conversely, if no response occurred, the next larger filament was tested. If an animal did not respond to monofilament application, a cut-off value of 3.58 was assigned to that mouse[23,30]. A separate set of naïve mice were also dosed with the highest doses of the compounds of interest. von Frey thresholds were determined 4 h and 24 h post-indomethacin administration, noting that at least four hours is needed before a pain response can be reliably measured.

Rotarod

To delineate efficacy and assess changes in motor performance following compound administration, naïve mice were tested on an automated accelerating rotarod (Ugo Basile, Italy). For training and testing, the rotarod speed was increased from 4 to 40 rpm over a 300 second period. The maximum time spent on the apparatus was set to 300 s. Mice received two training sessions separated by a minimum of 2 h on the first day then 24 h later they were administered compound or vehicle. The latency to fall was assessed 1 , 2 and 24 h post-compound administration. It is important to note that only the highest dose tested in the efficacy assessment was evaluated in the rotarod assay.

Blinding and randomization

All testing was conducted in a blinded manner with experimenters involved in the study being unaware of the group assignment of any animal they were testing. Dosing solutions were coded using A-E. Animals were dosed in a blinded fashion after pre-treatment baseline assessment such that animals were assigned to treatment groups based on baseline response thresholds so that group means were approximately equal (*i.e.*, randomization). Briefly, animals were ranked by response threshold from lowest to highest and treatments were assigned as follows (*e.g.*, A, B, C, D, E, B, C, D, E, A, C, D, E, A, B, D, E, A, B, C, *etc.*). The animals were then dosed in sequence based on animal number, so that the distribution of treatment across a given set of animals was not predictable.

Statistical analysis

All data are expressed as the mean ± SEM. Data were analyzed using a two-way repeated measures analysis of variance (ANOVA) using the factors of treatment and time. The Bonferonni multiple comparisons test was subsequently performed for post-hoc comparison using GraphPad Prism software (version 5.04; GraphPad Sofware, Inc., United States). Statistical significance was set to $P < 0.05$. The statistical methods of this study were reviewed by Salvatore Colucci, a biomedical statistician employed by Purdue Pharma L.P.

Compounds

Indomethacin, AMG 9810, HC-030031, carbamazepine, amiloride HCl and morphine were obtained from Sigma-Aldrich (St. Louis, MO, United States). Linaclotide, and asimadoline HCl were obtained from Toronto Research Chemicals (Toronto, ON, Canada). AMG 9810, HC-030031, asimadoline and linaclotide were administered p.o. as suspensions in 0.5% methyl-cellulose. Amiloride HCl was formulated in 25% hydroxypropyl-β-cyclodextran and administered intraperitoneally while morphine was dissolved in saline and given subcutaneously. Also, with the exception of morphine, all compounds were administered one hour prior to behavioral testing (three hours post-

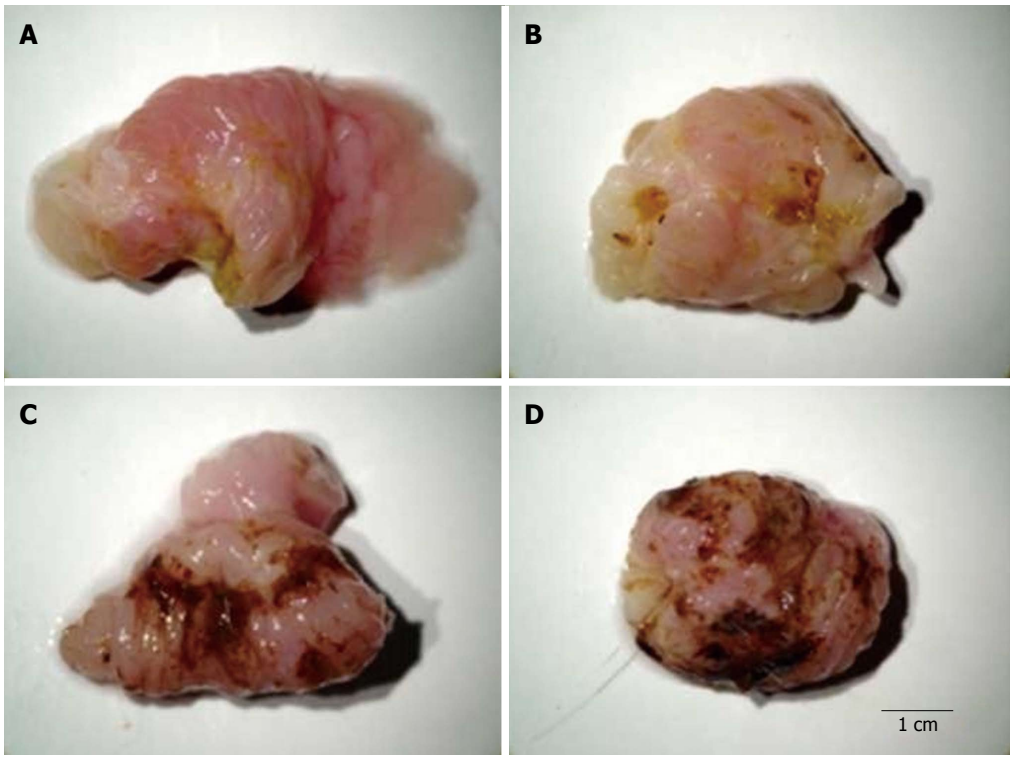


Figure 1 Representative digital photographs of stomachs dissected from mice orally dosed with vehicle (A) or 30 mg/kg indomethacin (B-D). Stomachs were scored as follows: A: score 0 = no lesion (normal mucosa = vehicle); B: score 1 = light petechial; C: score 2 = diffuse bleeding or aphtha; and: score 3 = multiple aphtha.

indomethacin dosing). Morphine was administered two hours before testing (or two hours after indomethacin). Route, vehicle and pre-treatment time for all compounds were chosen based on preliminary dosing experiments, pharmacokinetic (PK) data and/or previously published work. All doses are expressed as the free base and were administered in a dose volume of 10 mL/kg.

RESULTS

Ulcer model

Model development: We removed stomachs from vehicle- and indomethacin-dosed mice (4-h post-indomethacin administration). Mice were humanely euthanized. Their stomachs were grossly dissected, rinsed with saline and then photographed to demonstrate model development as shown in Figure 1. It is important to note that we found that visceral sensitivity is present in indomethacin-dosed animals irrespective of the degree of mucosal injury present. Behaviorally, severe mucosal damage does not always produce the most robust pain response.

Efficacy assessment

Opioid receptors: We examined the effect two distinct opioid receptor agonists had on visceral hypersensitivity. Firstly, we assessed the effect morphine (Figure 2A), the prototypical mu opioid receptor (MOR) agonist, had on the evoked visceral pain response. Results showed successful ulcer model development both 4 and 24-h post-indomethacin dosing ($P < 0.05$ *vs* vehicle), and that morphine attenuated the indomethacin-induced visceral hypersensitivity with a minimal effective dose (MED) equal to 10 mg/kg. Although there was a main effect of treatment [$F (4, 54) = 36$, $P < 0.05$ *vs* indomethacin] and a significant interaction with time [$F (8, 79) = 21$, $P < 0.05$ *vs* indomethacin], the effect morphine elicited on the pain behavior was only noted 2 h post-dosing (4 h post-indomethacin) for each of the higher doses tested, 10-30 mg/kg. Morphine was not efficacious in the assessment conducted 24-h post-indomethacin dosing. Nonetheless, the short-lived efficacy is consistent with the half-life of this drug. Subsequently, we next examined the effect asimadoline, a selective kappa opioid receptor (KOR) agonist, had in this pain model as well. Results showed consistent model development both 4 and 24-h post-indomethacin dosing ($P < 0.05$ *vs* vehicle). Asimadoline attenuated the indomethacin-induced visceral hypersensitivity with a MED= 10 mg/kg (Figure 2B). Like morphine, asimadoline was also efficacious in the model (10-30 mg/kg). There was a main effect of treatment [$F (4, 63) = 25$, $P < 0.05$ *vs* indomethacin] and a significant interaction with time [$F (2, 78) = 52$, $P < 0.05$]. However, unlike morphine, the kappa driven opioidergic effect was more prolonged such that the 30 mg/kg dose of asimadoline continued to attenuate the

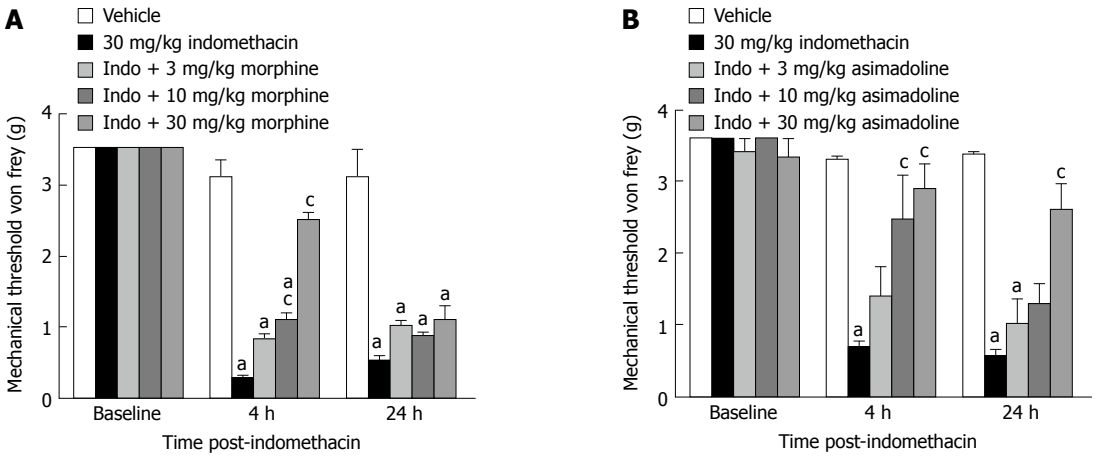


Figure 2 Effect of opioid receptor agonism on gastric ulcer pain. A: Dose-response and time course for the effect of a mu opioid receptor agonist on referred gastric ulcer pain. Morphine significantly attenuated referred gastric ulcer pain when dosed 10-30 mg/kg; B: Dose-response and time course for the effect of a selective kappa opioid receptor agonist on referred gastric ulcer pain. Asimadoline significantly attenuated referred gastric ulcer pain when dosed 10-30 mg/kg. All data are expressed as the mean ± SEM, where n = 8-10 mice/group. $^{a}P < 0.05$ *vs* vehicle, $^{c}P < 0.05$ *vs* indomethacin.

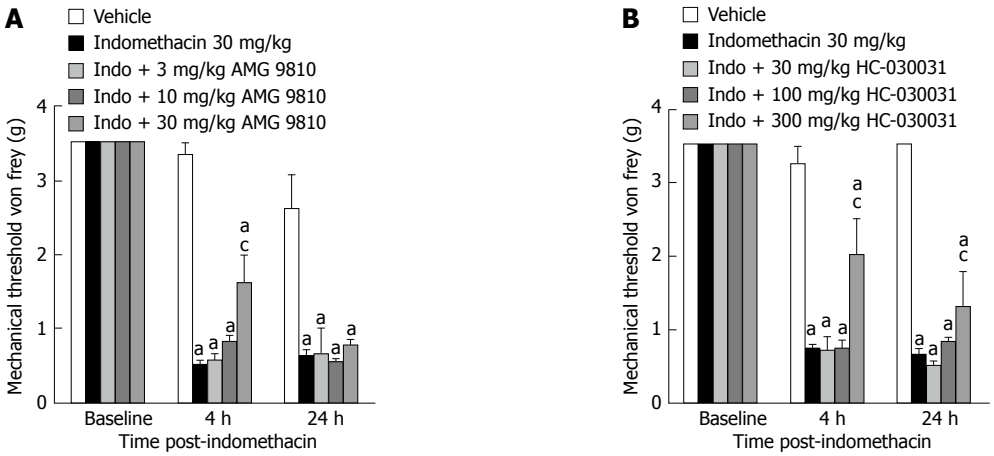


Figure 3 Effect of transient receptor potential channel blockade on gastric ulcer pain. A: Dose-response and time course efficacy for selective TRPV1 channel blockade on referred gastric ulcer pain. AMG 9810 significantly attenuated referred gastric ulcer pain when dosed at 30 mg/kg; B: Dose-response and time course for the effect of selective TRPA1 channel blockade on referred gastric ulcer pain. HC-030031 significantly attenuated referred gastric ulcer pain when dosed at 300 mg/kg. All data are expressed as the mean SEM where n = 10 mice/group. $^{a}P < 0.05$ *vs* vehicle, $^{c}P < 0.05$ *vs* indomethacin.

evoked pain response even at the 24-h assessment.

TRP channels

Next, we assessed the effect two distinct TRP channel receptor antagonists had on the visceral hypersensitivity. Firstly, we assessed the effect that AMG 9810, the most studied TRPV1 receptor compound, had on the evoked visceral pain response. In the ulcer pain model, indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 3A, $P < 0.05$ *vs* vehicle). Systemic treatment with the TRPV1 antagonist AMG 9810 attenuated the referred ulcer pain by increasing the mechanical threshold at which a behavioral response was elicited. Although there was a main effect of treatment [F (4, 44) = 43.2, $P < 0.05$ *vs* indomethacin alone] and a significant interaction with time [F (8, 75)= 15, $P < 0.05$], only the 30 mg/kg dose significantly attenuated the visceral hypersensitivity present 4 h post-dosing as revealed by post hoc comparison. Efficacy was not observed at the 24-h assessment.

We then examined the effect HC-030031, a selective transient receptor potential ankyrin 1 receptor (TRPA1) antagonist, had in this pain model as well. Indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 3B, $P < 0.05$ *vs* vehicle). Similar to TRPV1 blockade, the systemic treatment with the TRPA1 antagonist HC-030031 also significantly attenuated the referred ulcer pain in indomethacin dosed mice (Figure 3B). Although there was a main effect of treatment [F (4, 46) = 38, $P < 0.05$ *vs* indomethacin alone]and a significant interaction with time [F (8, 71)= 15.4, $P < 0.05$], only the 300 mg/kg dose attenuated the visceral hypersensitivity as noted

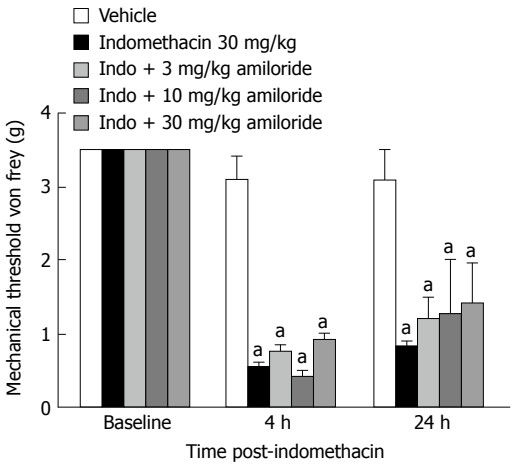


Figure 4 Effect of acid-sensing ion channels channel blockade on gastric ulcer pain. Dose-response and time course for the effect of non-selective ASIC channel blockade on referred gastric ulcer pain. Amiloride did not attenuate referred gastric ulcer pain at any of the doses tested. Data are expressed as the mean SEM where n = 10 mice/group. $^{a}P < 0.05$ *vs* vehicle.

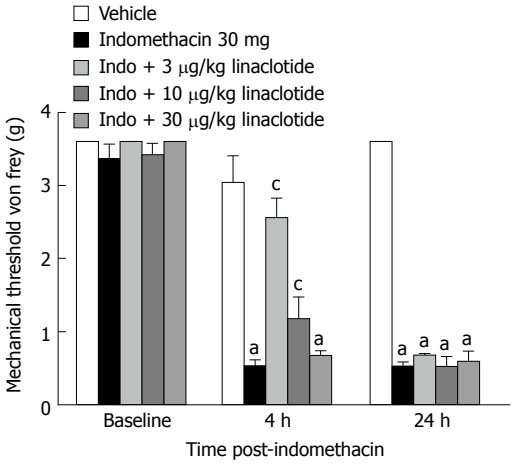


Figure 5 Effect of guanylate cyclase C agonism on gastric ulcer pain. A: Dose-response and time course for the effect of linaclotide on referred gastric ulcer pain. Linaclotide significantly attenuated referred gastric ulcer pain when dosed 3-10 μg/kg. Data are expressed as the mean SEM where n = 9-10 mice/group. $^{a}P < 0.05$ *vs* vehicle, $^{c}P < 0.05$ *vs* indomethacin.

by post-hoc comparison. Unlike the TRPV1 antagonist AMG9810, the effect of 300 mg/kg HC-030031 persisted at the 24-h assessment.

ASICs: We also investigated the sensory role of ASICs in ulcer pain. In the ulcer pain model, indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 4; $P < 0.05$). Surprisingly, however, the non-selective ASIC blocker amiloride did not significantly reduce the referred ulcer pain at any doses tested when evaluated by post-hoc comparison. Referred abdominal hypersensitivity was present in all dose groups 4 and 24-h post-dosing.

Guanylate cyclase-C: We determined if the newly developed GC-C agonist, linaclotide, was efficacious at attenuating ulcer pain. Indomethacin dosed mice demonstrated reduced mechanical thresholds compared to vehicle-dosed animals both 4 and 24-h post-dosing (Figure 5; $P < 0.05$). There was a main effect of indomethacin treatment [F (4, 62) = 486, $P < 0.05$] and a significant interaction with time, [F (2, 128) = 64; $P < 0.05$] 4 h post-dosing. 3-10 μg/kg linaclotide (MED = 3 μg/kg) significantly attenuated the indomethacin-induced mechanical hypersensitivity ($P < 0.05$). Surprisingly, the 3 μg/kg dose produced the most efficacious response while the 30 μg/kg dose was without effect. Visceral hypersensitivity was still notably present in mice that received 30 μg/kg linaclotide. That is, their tactile response was consistent with vehicle-dosed controls. The three doses of linaclotide tested were not efficacious in the ulcer pain model during the 24-h assessment.

Sodium channels: Lastly, we determined if the non-selective sodium channel blocker, carbamazepine, was efficacious in the ulcer model. Indomethacin dosed mice demonstrated reduced mechanical thresholds compared to vehicle-treated animals both 4 and 24-h post-dosing (Figure 6, $P < 0.05$). There was a main effect of indomethacin treatment (F (4, 58)= 14, $P < 0.05$) and a significant interaction with time (F (2, 71) = 65, $P < 0.05$). The 100 mg/kg dose of carbamazepine significantly reversed the referred tactile hypersensitivity noted during the 4-h assessment ($P < 0.05$). Those animals that received the 100 mg/kg dose of carbamazepine also demonstrated significantly less visceral hypersensitivity during the 24-h assessment as well ($P < 0.05$ *vs* indomethacin).

Side effect assessment

von Frey Threshold: To determine whether the compounds used in these experiments had any activity in the absence of visceral hypersensitivity, we assessed whether the highest doses tested in the efficacy model affected the von Frey thresholds of naïve animals. Post hoc analysis revealed that amiloride (Figure 7A; $P < 0.05$ *vs* vehicle), linaclotide, asimadoline and carbamazepine (Figure 7B; $P < 0.05$ *vs* vehicle) altered the von Frey thresholds of naïve mice when dosed at the highest dose examined in the ulcer model. Tactile sensitivity was enhanced 4-h after the dosing of linaclotide, asimadoline and carbamazepine, and 4 and 24-h after the administration of amiloride.

Rotarod performance: To place the efficacy of the compounds assessed into context with any inherent side effect liability, the highest dose of each compound examined in the ulcer model was subsequently evaluated in naïve mice using the rotarod assay. Morphine produced a slight but non-significant decrease

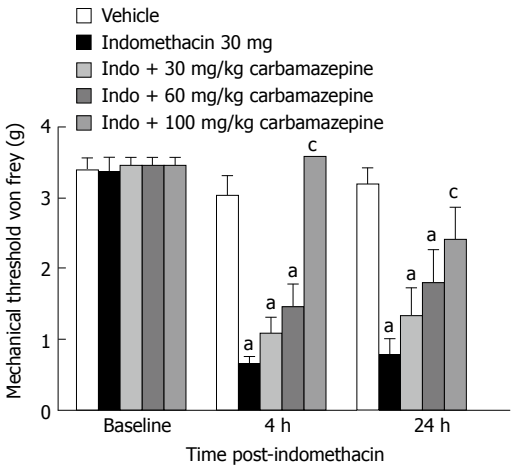


Figure 6 Effect of sodium channel blockade on gastric ulcer pain. Dose-response and time course for the effect of carbamazepine on referred gastric ulcer pain. Carbamazepine significantly attenuate referred gastric ulcer pain when dosed at 100 mg/kg. Data are expressed as the mean ± SEM with 9-10 mice/group. $^{a}P < 0.05$ *vs* vehicle, $^{c}P < 0.05$ *vs* indomethacin.

in rotorad performance one hour after administration (Figure 8A), while carbamazepine produced significant deficits in rotarod performance both 1 and 2 h following dosing (Figure 8B; $P < 0.05$ *vs* vehicle). Conversely, AMG 9810, HC-030031, linaclotide, asimadoline and amilioride did not produce any deficits in motor performance as show.

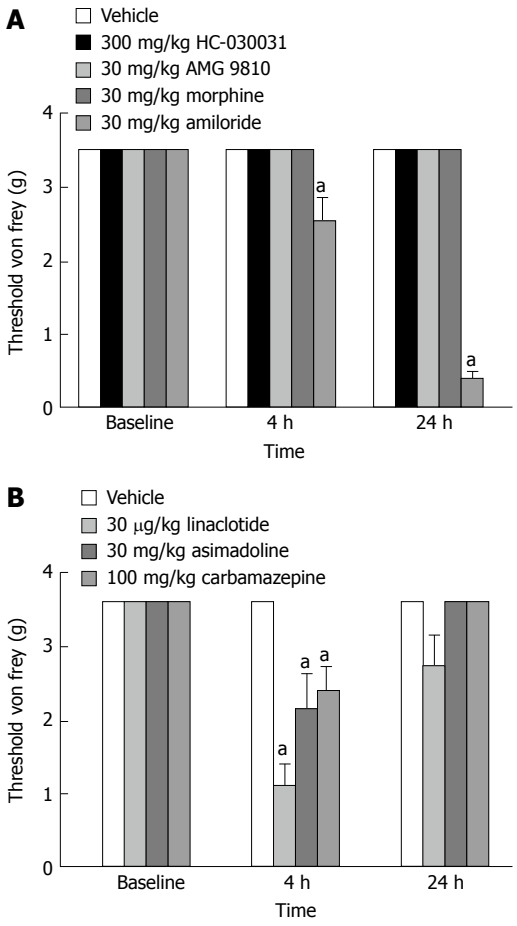


Figure 7 Effect of compound administration on von Frey threshold in naïve mice. A: The non-selective ASIC blocker amiloride significantly decreased abdominal threshold in mice 4 and 24-h post-administration when dosed at 30 mg/kg. B: Linaclotide, asimadoline and carbamazepine significantly decreased abdominal threshold in mice 4-h post-administration. Data are expressed as the mean ± SEM, where *n* = 9-10 mice/group. $^{a}P < 0.05$ *vs*

DISCUSSION

Pain is a characteristic feature of many chronic disorders affecting the GI tract. In this study we measured the pain associated with NSAID-induced gastropathy[13,14] and then investigated relevant pharmacological mechanisms underlying its development and maintenance.

Firstly, our findings show that the NSAID-induced gastropathy model in mice consistently produces stomach ulceration and referred abdominal hypersensitivity that can be reliably measured. Importantly, the model is robust enough that proper pharmacological evaluation can be conducted. Since gastric ulcers are a source of visceral pain which can be referred to somatic dermatomes upon palpation of the abdomen[28,29], this study was able to measure the threshold at which mice responded to abdominal application of von Frey filaments. Although this response was used as an index of the pain, it was no surprise that the mice demonstrated such robust behavior given the macroscopic changes depicted in Figure 1.

In characterizing the mechanisms associated with this pain model, we first assessed the effects of two distinct opioid analgesics. We determined that the MOR agonist, morphine, attenuated the ulcer pain in a dose- and time-dependent manner. This was not unexpected given the robust presence of MORs on enteric neurons of the GI tract, as well as this compound's well-published *in vitro* and *in vivo* pharmacological profile[38]. Interestingly and unknowing to us at the time of dosing was that the effect morphine had on ulcer pain may extend beyond its historical pain-ameliorating actions at the receptor level. That is, morphine may also provide gastric defense. An early study reported that morphine is protective to the stomach because it increases mucous production and decreases acid secretion[39]. However, contrary to this effect, it is also noteworthy to mention that morphine may be pro-ulcerogenic in certain circumstances as well. Esplugues and Whittle[40] had suggested that morphine can potentiate acid- and ethanol-induced gastric injury, although the experimental conditions employed herein did not support this result. Nonetheless, in this investigation, we demonstrated that morphine attenuated referred visceral pain at doses that did not alter von Frey thresholds or produce ataxia in naïve mice. However, it is important to note that the utility of MOR agonists for the treatment of visceral pain needs to be judiciously scrutinized given their effect on GI

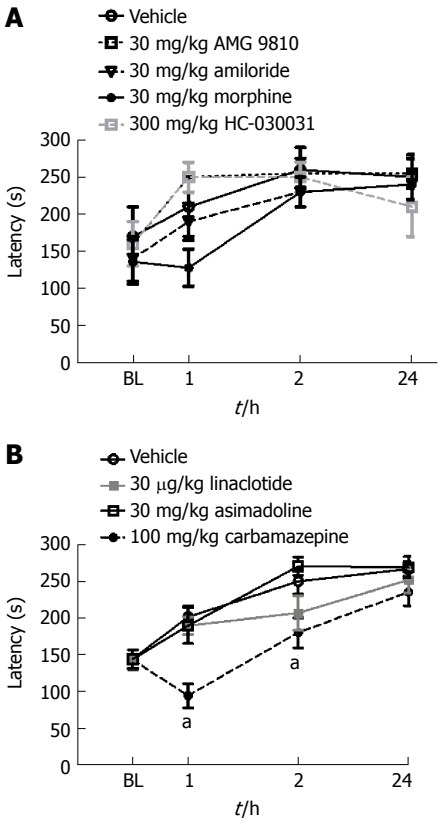


Figure 8 Effect of compound administration on rotarod performance. A: AMG 9810, amiloride, morphine and HC-030031 did not produce any significant deficits in rotarod performance 1-24 h post-administration; B: Linaclotide and asimadoline were without effect while carbamazepine significantly altered rotarod performance 1 and 2-h post-administration, $^{a}P < 0.05$. All data are expressed as the mean ± SEM, where $n = 10$ mice/group.

motility.

We next examined the efficacy of asimadoline, a potent KOR agonist. Asimadoline has 500-fold selectivity for kappa versus MORs and it is purported to produce its analgesic actions peripherally rather than centrally[41,42]. Previous work in animals has suggested that KOR agonists may represent a more viable approach to treating painful visceral conditions due to the nature of KOR expression and function[43,44]. In this regard, we hypothesized that asimadoline may be beneficial in attenuating visceral hypersensitivity. Interestingly, on a dose-per-dose basis, asimadoline was more efficacious than morphine in the model. Like morphine, it did not produce any deficits in rotarod performance at the highest dose tested; however, the 30 mg/kg dose did enhance tactile sensitivity in naïve animals. While Delgado-Aros *et al*[45] found that asimadoline produced hyperalgesia in patients dosed with high doses of the compound, there is a disconnect between human and rodent. In the human condition, these results may suggest that the KOR system becomes engaged during pathological conditions of persistent hypersensitivity[41,46] and that it does not significantly alter noxious sensation in healthy states[44]; however, these findings/assumptions are not consistent with ours since the mice tested were naïve and not subjected to ulcer induction. Although continued research is necessary to further our understanding on how KOR agonists modulate visceral pain, there is clinical evidence to support such an indication. Most notably, Cara Therapeutics have recently developed a peripherally restricted compound, CR665, which has demonstrated efficacy in human visceral pain[47-48].

TRP channels have received a significant amount of attention for their alleged role in pain including visceral hypersensitivity[35,49]. Since TRP channels transduce noxious stimuli at the peripheral terminals of nociceptors, these channels are well-poised anatomically to function as the first integrators of the pain response[50]. In support of this Akbar and colleagues[51] demonstrated a 3.5-fold increase in the density of TRPV1 immuno-reactive fibres in the colonic biopsies of patients with IBS compared to healthy controls. Transient receptor potential vanilloid-1 (TRPV1) is a ligand-gated nonselective polymodal cation channel that integrates a variety of pain stimuli[49]. Since noxious GI events are conveyed to the CNS by vagal and spinal afferents, and TRPV1 is expressed within both dorsal root and nodose ganglia innervating the GI tract[52,53], we examined whether selective blockade of the TRPV1 receptor would attenuate ulcer pain. Our results showing the ability of AMG 9810 to attenuate pain in the ulcer model suggests that the irritating effects of indomethacin on the gastric mucosa may engage the TRPV1 receptor either directly or indirectly such that these afferent neurons become hypersensitive to the hydrochloric acid that is normally innocuous to the stomach. Overall, the net effect may be analogous to proton activation of the channel. Alternately, it also becomes noteworthy to consider early reports supporting the protective effects of capsaicin-sensitive afferent neurons on the gastric mucosa[16,54]. While activation is believed to be protective, ablation of capsaicin-sensitive neurons impairs mucosal defense. With regard to our findings, one may have expected antagonism of TRPV1 to be detrimental. However, unlike nerve ablation which removes the entire nerve terminal and its functionality, an antagonist would leave the protective effects of these neurons intact.

Like TRPV1, TRPA1 is a non-selective cation channel that is a propagator of painful signaling that is activated by a variety of stimuli as well[55]. TRPA1 is mainly expressed in small diameter peptidergic nociceptors of the DRG, nodose and trigeminal ganglia along with TRPV1. TRPA1 has also been shown to be extensively expressed in enterochromaffin cells of colonic myenteric neurons as well as within non-neuronal tissue such as the small intestine and pancreas[55-57]. Therefore, together with its profile, co-expression pattern and connectivity to afferents that potentially impact the stomach, we hypothesized that TRPA1 would be a potential mediator of ulcer pain.

We found that the TRPA1 antagonist, HC-030031, attenuated abdominal hypersensitivity in the ulcer model. This result is in agreement with a study by Kondo *et al*[57] who demonstrated the efficacy of this compound in the context of a noxious gastric distention model. However, these findings are in direct contrast to a recent study by Kojima *et al*[58] who examined the effect of the TRPA1 agonist, ASP7663, in a similar model. Surprisingly, they maintained that TRPA1 agonism, not antagonism, represents a rational approach to treating visceral pain and that the analgesic effect of ASP7663 on colorectal distension-induced abdominal pain is mediated by direct desensitization of the TRPA1 channel.

Another potential transducer of noxious signals in the GI tract are ASICs (ASIC 1-3), a family of proton-gated sodium channels expressed within primary afferent neurons. Since a positive correlation between pain and local acidity has been reported and protons trigger inward currents through sodium channels in visceral sensory neurons causing hyper-excitability[21,59,60], we were surprised that amiloride did not attenuate the referred ulcer pain at any of the doses tested. Another study by Jones *et al*[61] examining the effect of amiloride on afferent fiber sensitivity to circumferential stretch of the colon also failed to see efficacy. The authors suggested that different ASIC subunits may exert opposing effects on mechanosensation. Likewise, different subunits may be involved in mechanical hypersensitivity of the colon as shown in a study using ASIC3 knockout mice[62] and this too may be reflective of the enhanced tactile sensitivity observed following dosing of the non-selective blocker amiloride to naïve animals and its lack of efficacy in the ulcer model.

Voltage-gated sodium channels have also received a significant amount of attention in recent years for their diverse role in pain. Given this, we investigated the role of the non-selective sodium channel blocker, carbamazepine, in the ulcer model. Unlike a prior efficacy report[63], we found carbamazepine produced a positive signal when administered at a high dose, 100 mg/kg. However, it is difficult to conclude if this was an efficacious response or a mixed result driven by the sedative properties of this molecule. As expected, carbamazepine also affected other measures of neurological function, such as rotarod performance and innate tactile threshold, thereby complicating interpretation of the reflexive endpoint used. Nonetheless, sodium channels appear to be important in pain as they have been causally linked to human conditions like IBS[64]. Notably, a number of selective sodium channel blockers targeting Nav 1.7 and Nav 1.8 in the periphery have entered clinical development[65]. Given the expression pattern of these targets as well as their selectivity profiles, one would expect that the not so distant future will provide novel sodium channel blockers that may alleviate conditions associated with visceral hypersensitivity although the discovery and developmental path for these compounds thus far has been very challenging.

Lastly, we examined whether linaclotide (Linzess™), a first-in-class, orally administered synthetic peptide of the guanylin peptide family, would produce an efficacious response in the ulcer model[33]. Linaclotide, a GC-C agonist, and its active metabolite are purported to bind to GC-C to act locally within the GI lumen to elevate cGMP. Mechanistically, Silos-Santiago *et al*[66] suggest that the cGMP significantly decreases the firing rates of sensitized afferent neurons in response to mechanical stimuli to lessen the pain response. In agreement with this study, we found that linaclotide was efficacious in attenuating ulcer pain when dosed 3-10 μg/kg. Surprisingly, however, the maximum effect was observed when linaclotide was administered at the lowest dose, 3 μg/kg. Although we would not have predicted this result, this finding did align with findings published previously by Eutamene *et al*[67] using the colorectal distension model. Similarly, this study showed that higher doses of linaclotide, 30 μg/kg, did not affect colorectal hypersensitivity, while lower doses did attenuate it. Interestingly, IBS clinical trial results examining the efficacy of linaclotide in patients confirmed these preclinical findings as well such that the FDA approved package insert for this compound states that continued abdominal pain (7% *vs* 5% placebo) is a possible side effect of therapy (www.linzesshcp.com). Although the mechanism for this paradoxical effect is unclear, it could be related to the physiochemical-pharmacokinetic properties of the molecule. Linaclotide has no oral bioavailability when given at efficacious doses; it is purported to act locally within the GI tract. However, with increasing dosage, the PK of linaclotide is documented to be non-linear. So, it is conceivable that such reduced anti-hyperalgesic effects at higher doses may be attributable to a corresponding loss of pharmacological specificity[67].

Visceral hypersensitivity underlies the pain experienced by patients presenting with various GI-related disorders. Although there is an unmet need to model visceral pain pre-clinically, this can be very challenging given the unique sensory innervation of visceral organs as well as our incomplete understanding of disease etiology[68]. Nonetheless, given the characteristics of visceral pain, both favorable (*i.e.*, not produced by noxious stimuli) and unfavorable (*i.e.*, pain is referred, diffuse, poorly localized)[27], we have demonstrated that the ulcer model is uniquely sensitive for assessing the effects of compounds directed against several well-known pain mechanisms. Furthermore, while we think this model is well-suited for drug discovery namely because of its ease and its reliability in performing stringent pharmacological evaluation (*i.e.*, dose-response data), it does have its limitations. For one, it is an acute model of visceral pain. While acute visceral pain is burdensome, the unmet need lies with the undulating hypersensitivity that persists from chronicity like in IBS. Also, most

discovery groups perform their pain studies in rats, so consistency with regard to species selection does differ as this model was validated in mice. Lastly, functional GI disorders represent a heterogeneous group of disorders that include genetic and environmental contributors. Notably, these factors are difficult to recapitulate in animals. While these limitations are well-recognized, together with its clinical relevance, we believe that the NSAID-induced gastropathy model may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts for this unmet need.

ACKNOWLEDGMENTS

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COMMENTS

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) and salicylates are ulcerogenic and therefore, chronic use can exacerbate existing gastric injury or lead to new ulcer formation. For these and other patients, it has been hypothesized that persistent or unresolved visceral pain, despite the etiology, may be due to aberrations in primary afferent function or hypersensitivity, peripheral sensitization, and/or psychological/genetic abnormalities.

Research frontiers

With this in mind, the authors characterized the pain associated with gastric ulceration. By combining a clinically relevant stomach ulcer model with a predictive behavioral endpoint, the authors investigated some potential mechanisms producing visceral hypersensitivity.

Innovations and breakthroughs

Pain is a characteristic feature of many chronic disorders affecting the gastrointestinal (GI) tract. In this study the authors measured the pain associated with NSAID-induced gastropathy and then investigated relevant pharmacological mechanisms underlying its development and maintenance.

Applications

The authors believe that the NSAID-induced gastropathy model may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts for this unmet need.

Peer-review

In this paper, the authors used the indomethacin to create NSAID- induced Gastropathy mouse model, and then investigated the pharmacological role of opioid and guanylate cyclase C receptors as well as TRPs, ASICS and sodium channels on this translatable model of visceral hypersensitivity. In summary, this study has demonstrated that the NSAID-gastropathy model has the potential to efficiently triage molecules with pain-attenuating properties for their utility in GI disorders that include pain as a hallmark symptom, and may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts. The overall structure of the manuscript is complete, and all figures are necessary and appropriate. The study has a certain significance and it may possibly help researchers to develop further studies.

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ORIGINAL ARTICLE

Basic Study

High yield reproducible rat model recapitulating human Barrett's carcinogenesis

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Abstract

AIM

To efficiently replicate the biology and pathogenesis of human esophageal adenocarcinoma (EAC) using the modified Levrat model of end-to-side esophagojejunostomy.

METHODS

End-to-side esophagojejunostomy was performed on rats to induce gastroduodenoesophageal reflux to develop EAC. Animals were randomly selected and serially euthanized at 10 (n = 6), 17 (n = 8), 24 (n = 9), 31 (n = 6), 38 (n = 6), and 40 (n = 6) wk postoperatively. The esophagi were harvested for downstream histopathology and gene expression. Histological evaluation was

completed to determine respective rates of carcinogenic development. Quantitative reverse transcription-polymerase chain reaction was performed to determine gene expression levels of *MUC2, CK19,* and *CK20,* and results were compared to determine significant differences throughout disease progression stages.

RESULTS

The overall study mortality was 15%. Causes of mortality included anastomotic leak, gastrointestinal hemorrhage, stomach ulcer perforation, respiratory infection secondary to aspiration, and obstruction due to tumor or late anastomotic stricture. 10 wk following surgery, 100% of animals presented with esophagitis. Barrett's esophagus (BE) was first observed at 10 wk, and was present in 100% of animals by 17 wk. Dysplasia was confirmed in 87.5% of animals at 17 wk, and increased to 100% by 31 wk. EAC was first observed in 44.4% of animals at 24 wk and increased to 100% by 40 wk. In addition, two animals at 38-40 wk post-surgery had confirmed macro-metastases in the lung/liver and small intestine, respectively. *MUC2* gene expression was progressively down-regulated from BE to dysplasia to EAC. Both *CK19* and *CK20* gene expression significantly increased in a stepwise manner from esophagitis to EAC.

CONCLUSION

Esophagojejunostomy was successfully replicated in rats with low mortality and a high tumor burden, which may facilitate broader adoption to study EAC development, progression, and therapeutics.

Key words: Esophageal adenocarcinoma; Gastroesophageal reflux disease; Levrat; Esophagojejunostomy; Experimental rat model; Mucin genes; Cytokeratins

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Core tip: The current study reportsrefined surgical techniques with improved tumor burdens for the modified Levrat model of end-to-side esophagojejunostomy in a rat for future *in vivo* studies of esophageal adenocarcinoma (EAC). For the first time, the model was established with significantly reduced mortality and morbidity and further validated through evaluation of conserved EAC disease progression markers, such as mucin and cytokeratins. The reported approach will allow for broader adoption of the model to allow for greater understanding of the complete disease progression spectrum from Barrett's esophagus to metastatic EAC and aid in the development of novel therapeutics.

Matsui D, Omstead AN, Kosovec JE, Komatsu Y, Lloyd EJ, Raphael H, Kelly RJ, Zaidi AH, Jobe BA. High yield reproducible rat model recapitulating human Barrett's carcinogenesis. *World J Gastroenterol* 2017; 23(33): 6077-6087 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6077.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6077

INTRODUCTION

Esophageal adenocarcinoma (EAC) incidence has increased dramatically in the western world in recent decades, with a 600% increase seen in the United States since the mid-1970s[1]. Despite recent advances in the development of multimodality patient-centered care plans, EAC still carries a poor prognosis with an overall 5-year survival rate of less than 15%[2]. Due to the increasing incidence and lethality of EAC, a better understanding of the underlying cancer biology and improved methods for prevention and treatment are urgently needed.

Barrett's esophagus (BE) is induced by chronic gastroesophageal reflux disease (GERD) and is a premalignant precursor to the progression of dysplasia and EAC[3,4]. GERD stimulates the replacement of squamous epithelium of the distal esophagus by intestinal-type metaplastic columnar epithelium, pathologically characterized by goblet cells and the expression of intestinal and differentiation markers, such as mucin genes (*MUC2*, *MUC5AC*), cytokeratins (CK7, CK20) and villin[5-9]. Furthermore, several studies have shown that transcription factors involved in the establishment of tissue differentiation, such as CDX2 and SOX2, play a key role in the development of BE and precede morphological changes[10,11]. Although there is great interest the underlying biology of BE carcinogenesis, the exact molecular mechanisms involved in the longitudinal progression to EAC have not been fully elucidated. Therefore, molecular biomarkers that are dysregulated across the progression spectrum would have significant clinical utility for early detection, risk identification, prognosis and therapeutic intervention.

Well-established small animal models efficiently recapitulate human de novo disease progression at the histological and molecular levels, providing clinical utility for development of effective treatment strategies. In 1962, Levrat *et al*[12] reported a surgical model of esophagoduodenostomy (ED) in rats to induce chronic gastroduodenal reflux. This model was further refined to demonstrate that ED with or without gastrectomy, esophagojejunostomy (EJ) with or without gastrectomy, and pancreatico-esophageal anastomosis could recreate disease progression to EAC without the administration of exogenous carcinogens[13-18]. Additionally, the study of gene expression profiles from BE to EAC confirmed significant homology between human and rat disease[19]. The modified Levrat model of end-to-side EJ with vagal nerve preservation to induce EAC through chronic gastroduodenoesophageal reflux (GDER) currently represents the gold standard of the model and has been extensively utilized to evaluate EAC disease progression and novel therapeutics. Therefore, this surgical approach provides a representative and translatable model for the study of reflux-induced carcinogenesis and downstream genetic alterations associated with EAC.

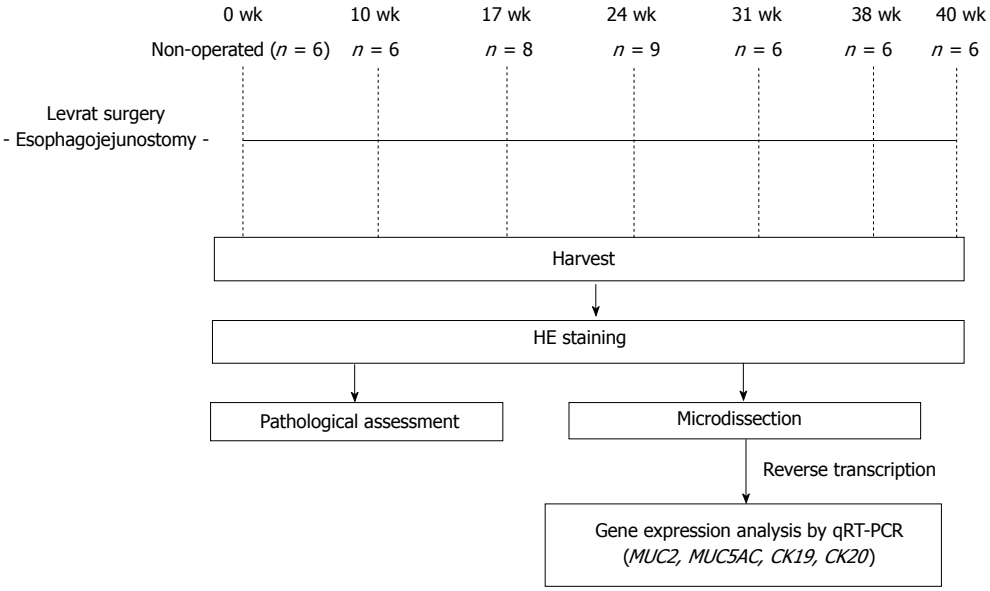


Figure 1 Study schema representing the major steps in the experimental design. qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

Through our extensive use of the modified Levrat model over recent years to study EAC, we have successfully optimized our protocols to maximize tumor burden and minimize mortality. The aim of the present study was to describe efficient use of the modified Levrat model through improved surgical techniques, perioperative care, management of complications, and analysis of molecular profiles. We feel the successful broader implementation of a reliable and replicable model that mimics human disease will enable other laboratories to reproduce it effortlessly and advance further research into the pathophysiology and biology of EAC.

MATERIALS AND METHODS

Ethics statement

This study was conducted with approval from the Institutional Animal Care and Use Committee of Allegheny General Hospital in Pittsburgh, Pennsylvania under Protocol #992. All animals received humane care in compliance with the standards set forth in "The Guide for the Care and Use of Laboratory Animals." All animals were weighed weekly and euthanized if acute decompensation occurred prior to study endpoint.

Experimental design

The cohort for this study was randomly preassigned from a larger protocol of 225 animals. Mortality and morbidity were calculated from the larger sample set to proportionally reflect the range and incidence rates of causes across sub-studies. The modified Levrat surgery of end-to-side EJ was performed on 200 to 250 g 6-wk to 8-wk-old male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) to induce chronic GDER and the subsequent spectrum of *de novo* premalignant lesions leading to EAC formation. Animals were randomly selected for serial euthanasia at 10 (n = 6), 17 (n = 8), 24 (n = 9), 31 (n = 6), 38 (n = 6), and 40 (n = 6) wk postoperatively. Additionally, six animals served as controls and were harvested with no surgical intervention. The higher effective n at 17 and 24 wk is reflective of animals that were prematurely sacrificed from the original designated time point due to health considerations. Pathological assessment was performed on all harvested esophageal specimens stained with hematoxylin and eosin (HE). Tissue samples were further evaluated for *MUC2*, *MUC5AC*, *CK19*, and *CK20* gene expression using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to compare each level of disease progression. These markers were selected based on known roles in human esophageal carcinogenesis. Study schema of experimental design is represented in Figure 1.

Modified levrat surgical model

Preoperative management: Prior to intervention, animals were acclimatized for at least one week and housed on a 12-h alternating light-dark cycle. Standard feed included a solid pellet diet and free access to tap water. One day prior to surgery, rats were provided with a gel-based diet (BioServ, Flemington, NJ; # S5769) to encourage stomach clearing to reduce the risk of anastomotic leak. Animals were nil per os (NPO) for 2-4 h before surgery. For sedation, animals were placed in an acrylic anesthetizing chamber for induction with 5% isoflurane and transferred to a nose cone mask for maintenance with 2% isoflurane in 1 mL/L of oxygen. The anesthetized rats were then placed on a circulating water heating bed to maintain adequate body temperature during surgery and were given prophylactic ketoprofen (3 mg/kg) and

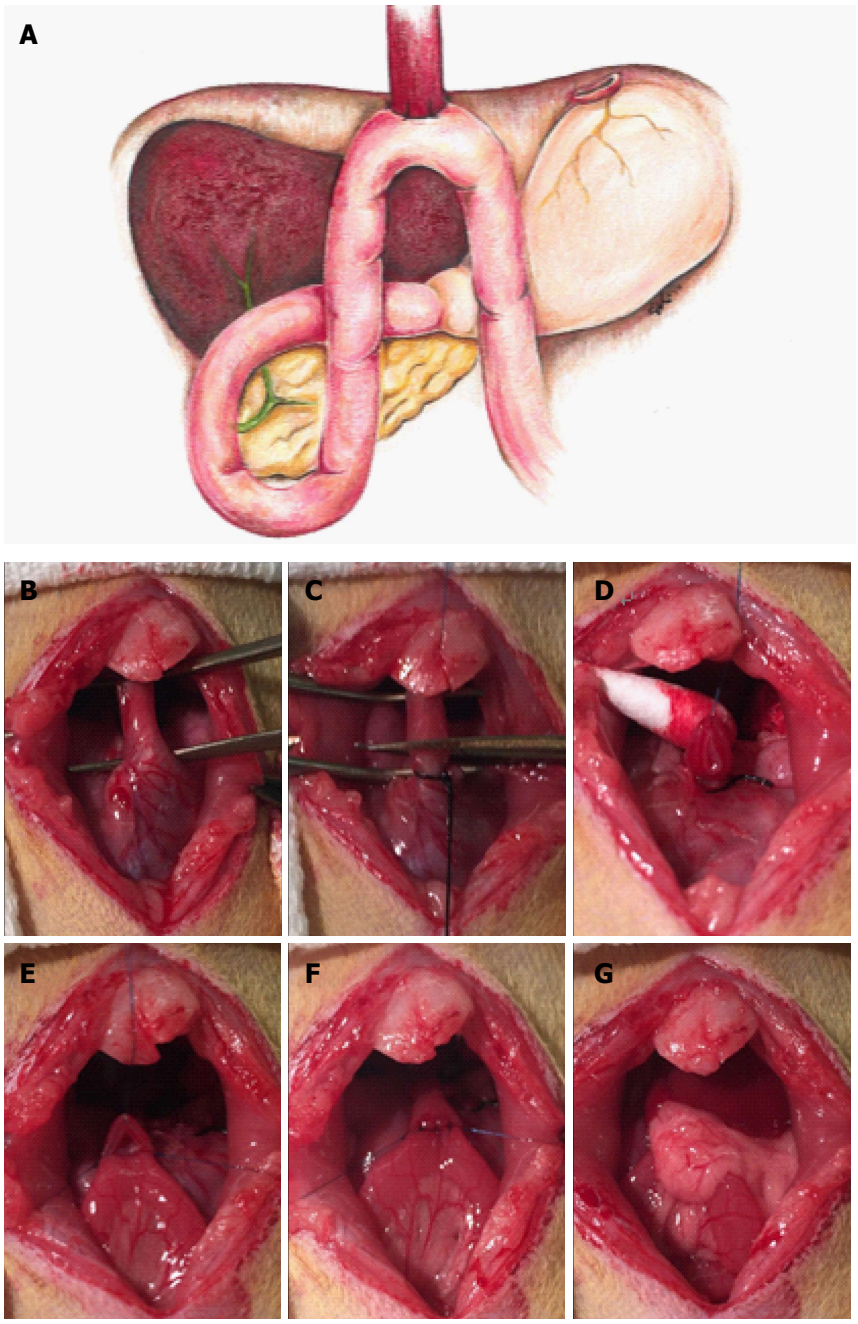


Figure 2 Diagram of modified Levrat model and images of the main steps of the surgical procedure. A: Illustration of end-to-side esophagojejunostomy with gastric preservation; B: Esophagus is mobilized, preserving the left and right vagus nerves; C: Esophagus divided from the stomach after ligation of gastroesophageal junction; D: Anterior esophageal suture placed through muscle and mucosa layers, allowing proper visualization of mucosa; E: Anterior and lateral esophageal sutures intact, creating visualization of the lumen; F: Completed anastomosis between the distal esophagus and jejunum with eight interrupted full-thickness sutures; G: Anastomosis returned to anatomical location wrapped in omentum.

enfloxacin (5 mg/kg) immediately prior to surgery.

Surgical techniques: All animals underwent an end-to-side EJ with gastric preservation through upper midline abdominal incision. The esophagus was mobilized, preserving the left and right vagus nerves (Figure 2B), and the gastroesophageal junction was ligated using 3-0 silk. A temporary 7-0 prolene suture (Ethicon, Somerville, NJ, United States) was delicately threaded through muscle layer only of the distal esophagus 1 cm above the silk knot to prevent retraction of the esophagus through the diaphragm. Care was taken to minimize tension on the esophagus to prevent recoiling of the mucosal layer into the lumen, and the esophagus was cut just above the gastroesophageal junction (Figure 2C). A loop of jejunum was identified 4 cm distal to the ligaments of Treitz and 3 mm jejunostomy was made using a #11 surgical blade. Residual intestinal fluid in the immediate area was drained using a sterile swab to

minimize risk of postoperative abscess formation and infection. An end-to-side anastomosis was constructed between the distal esophagus and jejunum in an antecolic manner with eight interrupted full-thickness 7-0 prolene sutures. Special care was taken to include the esophageal mucosa to ensure adequate mucosal-to-mucosal apposition (Figure 2D-F). The first 5 sutures were placed on the anterior wall, and the entire anastomosis was carefully flipped 180° to expose the posterior wall. Before placing the posterior sutures, patency of the proximal and distal lumen was confirmed. The final 3 sutures were placed, and the anastomosis was returned to anatomical position. This delicate flip allowed all suture knots to remain external to the lumen and minimized anastomotic failure due to postoperative obstruction. The omentum was wrapped around the completed anastomosis to prevent anastomotic leakage (Figure 2G), and the celiotomy was closed using 4-0 vicryl (Ethicon, Somerville, NJ, United States). All rats received a 25-30 mg/kg bolus of Lactated Ringer's Solution (LRS), were placed on 100% oxygen until awake, and placed in a cage with a raised wire mesh grate to eliminate direct exposure to obstructive bedding. Rats remained NPO for at least 1 h postoperatively to prevent aspiration.

Postoperative management: For the duration of the study, postoperative animals were housed in cages with raised grates to eliminate access to bedding. Any ingestion of foreign material post-surgery results in anastomotic failure and leak or obstruction. Durable plastic huts were provided as enrichment, and rubber stoppers on water bottles were continuously monitored for integrity. Animals continued to receive daily injections of LRS, ketoprofen (3 mg/kg), and enfloxacin (5 mg/kg) until postoperative day 3. Additionally, all rats were placed on a 10-d diet modification plan designed to gradually shift from liquid diet to full solid diet, in an effort to facilitate proper healing of the anastomosis, minimize aspiration-related complication, and encourage feeding after surgery. On postoperative days 0 to 3, rats received a nutritional diluted liquid supplement diet (Ensure; Abbot, Columbus, OH, United States) with acetaminophen (Tylenol®) for pain control. Animals were then transitioned to a gel diet (BioServ, Flemington, NJ; # S5769) (days 4-6), followed by a mushed pellet diet (days 7-9), and then regular solid pellet diet on postoperative day 10. The days on a specific modified diet were extended by 1-2 d if the animal seemed slow to recover from surgery. During the postoperative window, all rats were individually housed to closely monitor health, consumption, and stool production and to reduce chances of wound damage. On day 14, all rats were ear-tagged and pair-housed. At 12 wk, all rats were treated with iron dextran (50 mg/kg, i.m.) every two weeks prophylactically for anemia. All animals received a weight check at least once a week, and during periods of weight loss, rats were weighed more frequently, and modified diets were provided in a supplemental fashion. Animals having more than 45% weight loss or acute decompensation in the postoperative period were sacrificed, and all remaining rats were euthanized at their respective time points for histological evaluation.

Gross and histological evaluation

Tissue preparation and pathological assessment: Upon necropsy, the entire esophagus and jejunum, to a length approximately 1 cm distal to the anastomosis was harvested. After the specimen was cut open longitudinally, samples were rinsed in ice-cold phosphate buffered saline to remove debris, oriented to maximize exposure of suspicious areas, and flash frozen in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA; #4583). Next, frozen esophagi in OCT blocks were cut into 5 micron sections using a cryostat (Fisher Scientific, Waltham MA; Microm HM 550) and stained with HE for pathological assessment and gene expression analysis. Two experienced pathology experts independently performed the histological analysis to identify areas of disease. Histological changes were defined on the basis of the following established classification criteria: (1) esophagitis: intraepithelial inflammation, thick basal cell layer, elongated lamina propria papillae, and spongiosis; (2) proliferative hyperplasia: Increased thickness of the squamous epithelium (sometimes hyperkeratotic) with no cellular atypia; (3) BE: Replacement of normal esophageal squamous epithelium with columnar-lined epithelium containing goblet cells; (4) dysplasia: dysplastic squamous cell epithelium with enlarged, atypical nuclei and an increased number of mitotic figures, which may invade lamina propria of the epithelium but does not invade the submucosal layer; and (5) EAC: Mucinous, dysplastic glandular cell growth with both atypia and invasion through the basement membrane.

Gene expression analysis by PCR

In order to evaluate the expression of a subset of four biomarkers at each esophageal disease level, qRT-PCR was performed on macrodissected esophageal tissues, including 3 normal esophageal epithelium (controls), 5 esophagitis, 5 BE, 11 dysplasia, 19 EAC and 2 EAC (primary profiled) with metastasis. Briefly, two experienced pathology experts independently confirmed areas of the highest disease for each sample and marked HE slides for macrodissection. Based on the marked areas, 200 μmol/L of tissue was macrodissected using a cryostat, and special care was taken to ensure all collections were highly representative of the disease states. RNA was isolated from the tissue using a miRNeasy kit (Qiagen, Valencia, CA; #217004), and a reverse transcription reaction was performed using a RT^2 first strand kit (Qiagen,

Table 1 Breakdown of histological findings of each time point after esophagojejunostomy *n* (%)

Histology	Postoperative wk						
	0 wk	10 wk	17 wk	24 wk	31 wk	38 wk	40 wk
	n = 6	*n* = 6	*n* = 8	*n* = 9	*n* = 6	*n* = 6	*n* = 6
Esophagitis	0 (0)	6 (100)	8 (100)	9 (100)	5 (83.3)	5 (83.3)	6 (100)
Proliferative hyperplasia	0 (0)	6 (100)	8 (100)	9 (100)	6 (100)	6 (100)	6 (100)
Barrett's esophagus	0 (0)	1 (16.7)	8 (100)	8 (88.9)	6 (100)	6 (100)	5 (83.3)
Dysplasia	0 (0)	0 (0)	7 (87.5)	8 (88.9)	6 (100)	6 (100)	6 (100)
Adenocarcinoma	0 (0)	0 (0)	0 (0)	4 (44.4)	4 (66.7)	5 (83.3)	6 (100)
Metastasis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	1 (16.7)

Valencia, CA, United States; #330401), according to the manufacturer's protocol. qRT-PCR was performed with the RT^2 SYBR Green ROX qPCR MasterMix (Qiagen, Valencia, CA, United States; #330523) in a total volume of 25 μL using the following RT^2 Primer Assays: MUC2 (Qiagen, Valencia, CA, United States; #PPR69984A), MUC5AC (Qiagen, Valencia, CA, United States; #PPR59660B), CK19 (Qiagen, Valencia, CA, United States; #PPR44322A) and CK20 (Qiagen, Valencia, CA, United States; #PPR44539A). Real-time PCR reactions were conducted at 95 ℃ for 15 min, followed by 40 cycles of 94 ℃ for 15 s, 55 ℃ for 30 s, and 70 ℃ for 30 s, using a StepOnePlus real-time quantitative system (Applied Biosystems; Carlsbad, CA, United States). Raw data was exported from the real-time instrument software and relative gene expression was calculated using the ΔΔ-Ct method. B-Actin (Qiagen, Valencia, CA, United States; #PPR0650C-200) and RPLP1 (Qiagen, Valencia, CA, United States; #PPR42363C-200) were selected as endogenous controls. All samples were normalized against pathologically confirmed normal squamous esophagus and run in technical triplicates.

Statistical analysis

Statistical analysis was conducted by a biomedical statistician using SPSS software (IBM, Armonk, NY, United States; Version 23). The gene expression levels in normal squamous esophagus, esophagitis, BE, dysplasia, and EAC tissues were compared by an independent 2-tailed *t* test to identify significant differences in expressions. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Surgical model and outcomes

Fifteen percent of the operated animals died before the intended endpoint of the experiment, and necropsies were performed on all euthanized and found dead animals to identify the cause of death. Of these, 23.5% animals died within 2 wk post-surgery due to surgical and procedure-related complications, such as anastomotic leaks and continuous gastrointestinal hemorrhage. An additional 26.5% of the animal deaths were between 2-10 wk following surgery, with the major cause of death being stomach ulcer perforation. The remaining 50% of animal deaths were 10 wk after surgery, and the major causes of mortality were attributed to respiratory infection secondary to aspiration, stomach ulcer perforation, and obstruction due to tumor or late anastomotic stricture. Overall, the anastomotic leak rate for the study was 2.2%. A randomized cohort of 47 animals was preselected and utilized for histopathology and gene expression. The effective numbers of rats examined for study endpoints were as follows: non-operated (n = 6), 10 wk (n = 6), 17 wk (n = 8), 24 wk (n = 9), 31 wk (n = 6), 38 wk (n = 6), and 40 wk (n = 6) after surgery.

Histological findings

The results of histological findings for each disease level at each time point are shown in Table 1. All animals that underwent EJ showed histological features of esophagitis and proliferative hyperplasia from 10 wk following surgery (Figure 3B). BE was observed as early as 10 wk (16.7%; 1/6 animals) following surgery, and 100% of animals at 17, 31, and 38 wk showed the presence of BE (Figure 3C). Dysplasia was first recognized at 17 wk post-surgery (87.5%; 7/8 animals), and the incidence increased over time to 100% at 31-40 wk (Figure 3D). EAC was first observed at 24 wk (44.4%; 4/9 animals) post-surgery and sequentially increased to 100% at 40 wk. All of the neoplastic cases were localized to the esophagus just above the esophagojejunal anastomosis. Five of 6 (83.3%) EAC cases at 40 wk were well-differentiated mucinous carcinomas (Figure 3E). In addition, two animals at 38-40 wk post-surgery had confirmed macro-metastases in the lung/liver and small intestine, respectively (Figure 4).

Gene expression analysis

The relative *MUC2* gene expression was highest in BE, and it was progressively down-regulation across the BE-dysplasia-adenocarcinoma spectrum. When compared to normal squamous esophagus, BE, dysplasia, and EAC had significantly higher expression levels of MUC2 (P = 0.017, 0.005 and < 0.001, respectively). Additionally, there was significant difference in *MUC2* gene expression between esophagitis and BE (P = 0.028) (Figure 5A). The relative *MUC5AC* gene expression was highest in EAC and lowest in BE among esophageal disease types. Esophagitis, dysplasia,

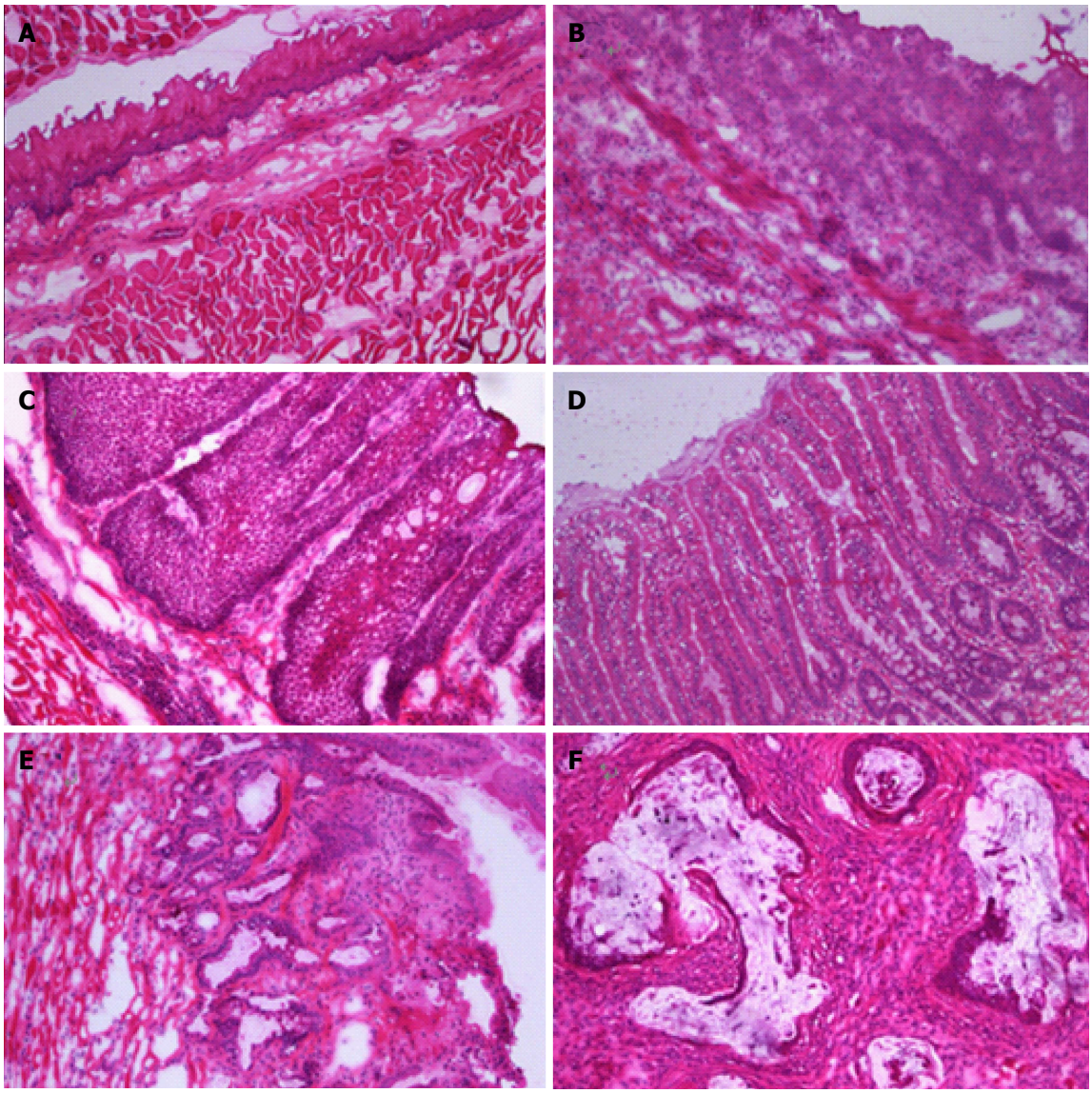


Figure 3 HE staining of esophageal disease progression (× 10). A: Normal rat esophagus; B: Esophagitis; C: BE with goblet cells; D: Dysplasia; E: EAC. EAC: Esophageal adenocarcinoma; BE: Barrett's esophagus.

and EAC had significantly higher expression levels of MUC5AC, compared to normal squamous esophagus (P = 0.03, 0.021, and 0.029, respectively) (Figure 5B). Both *CK19* and *CK20* gene expression increased in a stepwise manner from esophagitis to EAC. All four histological stages (esophagitis, BE, dysplasia, and EAC) had significantly higher *CK19* and *CK20* expression levels than normal squamous esophagus (*CK19*: P = 0.019, 0.015, < 0.001 and < 0.001, respectively; *CK20*: P = 0.047, 0.018, 0.001 and < 0.001, respectively) (Figure 5C and D).

DISCUSSION

The present study describes a detailed surgical technique and efficient perioperative management for the modified Levrat surgery of end-to-side esophagojejunostomy with gastric preservation. Implementation of the outlined standards provided maximal utilization of the model to produce a high tumor burden balanced with minimal mortality. Additionally, successful management of perioperative complications allowed us to extend the postoperative window to 40 wk to evaluate well-differentiated EAC and metastasis.

The overall mortality rate in this study was 15%, with 23.5% of the total mortality within the first 2 wk post-surgery, resulting in an overall procedure-related mortality of only 3.5%. This rate was lower than that reported by other investigators for the modified Levrat model[20,21]. Improved and aseptic surgical techniques and perioperative managements may have also contributed to the reduction of operative mortality. Of note, taking special care to include the esophageal mucosa when constructing the anastomosis was essential to obtain adequate mucosal-to-mucosal apposition. Additionally, covering the anastomotic site with omentum minimized the risk of anastomotic leakage post-surgery. A postoperative progressive

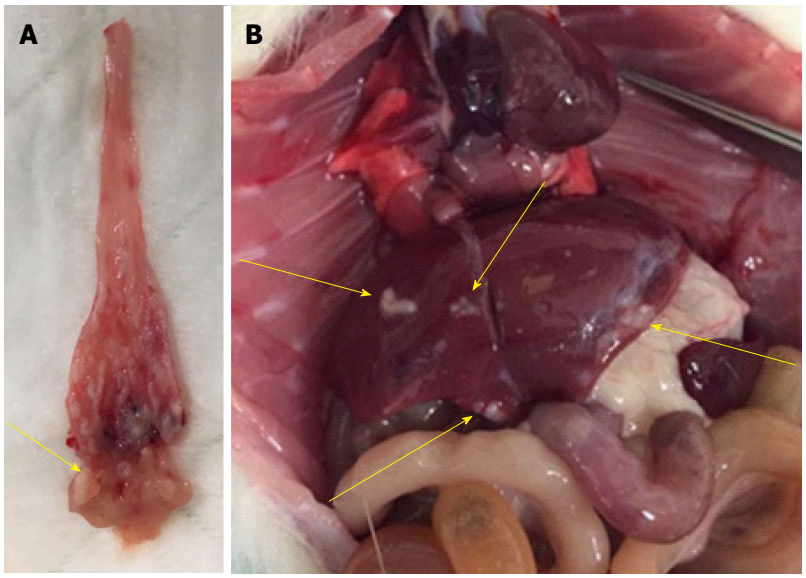


Figure 4 Primary esophageal adenocarcinoma tumor (A) and respective liver metastasis (B), as grossly observed in a 40-wk post-surgical rat.

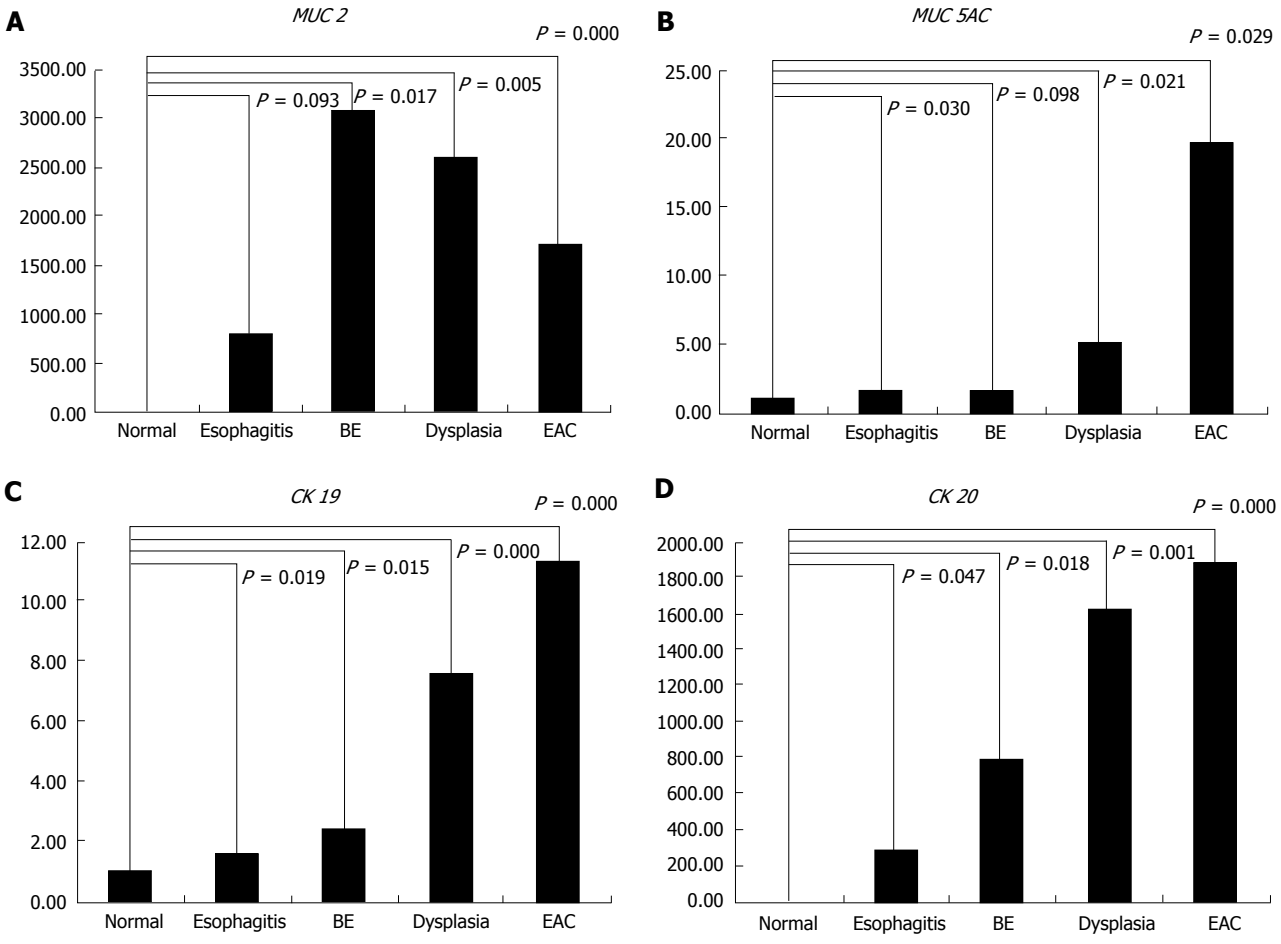


Figure 5 Panel A to D represents the relative gene expression level of *MUC2*, *MUC5AC*, *CK19*, and *CK20* for each esophageal disease level, respectively. BE: Barrett's esophagus; EAC: esophageal adenocarcinoma.

modified diet plan greatly contributed to the protection of the surgical site for the first 10 d after surgery to minimize leak rate. Additionally, supplemental diets provided during periods of weight loss or illness minimized acute morbidity, as anastomotic stricture or tumor growth presented a significant risk factor for obstruction.

As shown in the present study, the rat reflux model induced by EJ displayed sequential changes in the esophageal epithelium at the site of anastomosis and distal esophagus, similar to those seen in human esophagus. Dysplastic changes were first recognized

near the anastomosis in 87.5% rats at 17 wk post-surgery. Additionally, 83.3% (5/6 animals) and 100% (6/6 animals) of the animals evaluated at 38 wk and 40 wk post-surgery showed histological evidence of EAC at the site of anastomosis with an adjacent area of dysplastic BE, respectively. The overall incidence of EAC reported in this study was higher compared to other previously reported rates of only 17.4% at 30 wk and 74% at 40 wk for the rat surgical reflux model[22,23]. This difference is likely a direct result of improved surgical techniques and successful management of health complications that allowed for the extension of the perioperative window to 40 wk. Additionally, frequent iron injections may enhance esophageal carcinogenesis by increasing oxidative stress[24]. In other words, oxidase damage could be a contributing factor in the formation of EAC in the rat reflux model, and may similarly occur in human patients with GERD and iron overload. Moreover, the high incidence of EAC and the presence of distant metastasis at 38-40 wk post-surgery may indicate that the 38-40 wk time point is ideal for the study of EAC progression and metastasis in the modified Levrat model.

Since Levrat and colleagues first described the surgically induced reflux model of esophagitis by performing ED in rats, it has been extensively used to study esophageal carcinogenesis and to evaluate novel preventive and treatment strategies[25-27]. In our previous studies, we have utilized the modified Levrat' s surgical model for studying both chemoprevention and targeted therapy and have demonstrated potent efficacy for multiple cancer mechanistic inhibitors against EAC progression[20,28,29]. For the first time, we demonstrated macro-metastatic lesions originating from the primary tumors in the model[30]. Additionally, we further enhanced the utility of the model through combined magnetic resonance imaging (MRI) and small animal endoscopic biopsy to simultaneously track *in vivo* tumor volumes and molecular correlates longitudinally[31]. With these advancements, the animal model has been highly efficient, not only to develop preventative and treatment strategies, but also to provide a platform to better understand the molecular mechanisms that affect disease progression from inflammation to EAC.

The identification and validation of molecular biomarkers that modulate expression across the progression spectrum may serve as powerful tools for early detection, risk stratification, prognosis, and the development of treatment strategies for EAC. The present study assessed the expression levels of *MUC2, MUC5AC, CK19,* and *CK20* at each esophageal disease level harvested from surgically induced rat reflux model. Interestingly, compared to normal squamous esophagus and esophagitis, statistically significant *MUC2* expression was highest in BE and then progressively down-regulated through the neoplastic sequence.

Mucin genes are expressed in a site specific manner in the human gastrointestinal tract, and all MUC subtypes are aberrantly expressed in Barrett's metaplasia[32,33]. Although BE had a low *MUC5AC* expression level in this study, the expression pattern of *MUC2* was consistent with reported human esophageal literature[6,7,34]. Furthermore, this study demonstrated a relative stepwise increase in expression of *CK19* and *CK20* across the EAC progression. This result may support the possible role of these markers for early detection and risk stratification.

Limitations of the study included small sample size and precision of histological macrodissection. Although the sample set utilized for molecular analysis was small, a quantitative RT-PCR approach was utilized to determine gene expression. Additionally, an inherent limitation of macrodissection included possible inadvertent inclusion of heterogenous tissue that could conceal a specific cell-type signature. Therefore, further investigation with a larger sample size, or applying a microdissection technique to isolate a highly-enriched pure cell population may be beneficial.

In summary, the present study displays improved surgical technique and successful perioperative management of the modified Levrat model to enhance the value in context of a low mortality rate and consistently higher disease burden. This will enable broader adoption of the model to facilitate further research into the pathophysiology and biology of EAC. We further demonstrated a unique expression pattern of *MUC2*, *CK19*, and *CK20* that was relevant to human EAC progression, reinforcing the possible utility as conserved diagnostic molecular markers of BE and EAC.

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COMMENTS

Background

Esophageal adenocarcinoma (EAC) is an extremely lethal disease with an overall survival rate of less than 20%. Small animal models are required to efficiently replicate and study the biology and pathogenesis of human EAC.

Research frontiers

Currently, the treatment options for EAC are outdated, reflected by lack of improvement in patient outcomes over the last three decades, thereby establishing the need for the development of new and improved therapeutic paradigms. The modified Levrat surgery in a rat model has been previously utilized successfully to accelerate development of such agents for chemoprevention, but significant improvements are required to expand the applicability for treatment of established-disease.

Innovations and breakthroughs

These findings report a validated highly replicable protocol to study EAC carcinogenesis and subsequent utilization for treatment efficacy studies. For the first time, the authors demonstrate a significantly reduced mortality rate and increased tumor burden. Additionally, they further validate the translatable nature of the model through gene expression analysis of conserved markers of human Barrett's carcinogenesis, such as mucin and cytokeratin.

Applications

The reported methodology will allow for more efficient utilization of the model to study EAC disease progression and treatment options, which in turn will be translated to clinical settings to improve patient prognosis.

Peer-review

This is a well-designed basic study. Authors in this study generated modified End-to-side esophagojejunostomy (EJ) rat model. Using this model, authors determine respective rates of carcinogenic development and gene expression levels of *MUC2*, *CK19*, and *CK20*. In order to better understand the underlying biology and prevent and treat EAC, the modified EJ model generated in the present study and the data obtained are important for understand disease progression spectrum from Barrett's esophagus to metastatic EAC.

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ORIGINAL ARTICLE

Basic Study

Changes in expression of inhibitory substances in the intramural neurons of the stomach following streptozotocin-induced diabetes in the pig

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Abstract

AIM

Influence of chronic hyperglycemia on chemical coding of enteric neurons in stomach using pig as a model for human diabetic complications.

METHODS

Ten pigs were divided into two groups: diabetic (D group, n = 5) and control (C group, n = 5). Pigs constituting the experimental group were given streptozotocin (150 mg/kg). Animals were euthanized

six weeks after the induction of diabetes. The samples of stomach were collected from animals of both groups. The cryostat sections were processed for double immunofluorescence staining using primary antisera directed towards pan-neuronal marker (Hu C/D) proteins and/or neuronal isoform of nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP) and galanin (GAL).

RESULTS

In the control group in the myenteric ganglia (MG) of the corpus we have noted 22.28% ± 1.19% of nNOS positive neurons, while in diabetic group we have found 40.74% ± 2.22% of nNOS immunoreactive perikarya (increase by 82.85 %). In turn in the pylorus we have observed 15.91% ± 0.58% nNOS containing neurons in control animals and 35.38% ± 1.54% in the diabetes group (increase by 122.37%). In the MG of the antrum and submucosal ganglion (SG) in the corpus hyperglycemia did not cause statistically significant changes. With regard to VIP-positive cell bodies in the antrum MG in the control animals we have noted 18.38 ± 1.39% and 40.74% ± 1.77% in the experimental group (increase by 121.65%). While in the corpus we have observed 23.20% ± 0.23% in the control and 30.93% ± 0.86% in the diabetes group (increase by 33.31%). In turn in the pylorus VIP positive cells bodies constituted 23.64% ± 1.56% in the control group and 31.20% ± 1.10% in the experimental group (increase by 31.97%). In the submucosal ganglion in the corpus we have noted 43.61% ± 1.06% in the control animals and 37.00% ± 1.77% in the experimental group (decrease by 15.15%). Expression of GAL-positive perikarya showed statistically significant changes only in the MG of the antrum and pylorus. In the antrum GAL positive perykarya constituted 26.53% ± 1.52% in the control and 36.67% ± 1.02% in the experimental animals (increase by 38.22%). While in the pylorus GAL positive neurons in the control group constituted 16.32% ± 0.92% and 17.99% ± 0.38% in the experimental animals (increase by 10.23%).

CONCLUSION

Our results support the hypothesis that in the course of diabetes, long term episodes of high glucose serum level may influence the chemical phenotyping of enteric neurons.

Key words: Immunohistochemistry; Inhibitory neurons; Streptozotocin; Hyperglycemia; Pig

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Core tip: Our results revealed the neuronal plasticity of enteric neurons within porcine stomach in response to chronic hyperglycemia. We used the pig as a model for human gastrointestinal disorders occurring in people with diabetes. Our study highlights the important role of the enteric nervous system in response to high glucose serum level. We observed a substantial increase in the expression of nitric oxide, galanin and vasoactive intestinal peptide inside the enteric neurons. Since all of the investigated molecules have inhibitory properties, they may be involved in the impairment of the motor function of the stomach occurring in people with long-term diabetes.

Bulc M, Palus K, Zielonka L, Gajęcka M, Całka J. Changes in expression of inhibitory substances in the intramural neurons of the stomach following streptozotocin- induced diabetes in the pig. *World J Gastroenterol* 2017; 23(33): 6088-6099 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6088.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6088

INTRODUCTION

Diabetes is one of the most frequently diagnosed endocrinopathies worldwide[1,2]. Long-term episodes of hyperglycemia often occur during course of diabetes. As a consequence of poorly-controlled glucose serum level, numerous tissue and organ dysfunctions can occur[3]. The central, peripheral and autonomic neurons are particularly vulnerable to carboxylic stress, which is a consequence of hyperglycemia[4]. One of the many consequences of autonomic system damage are numerous disorders in the gastrointestinal tract[5-7]. They can involve any organ in the digestive system, such as the esophagus, stomach, gallbladder tract, pancreas, small and large intestines. Generally, they are referred to as diabetic gastrointestinal autonomic neuropathy[5,8]. These disorders are not directly life-threatening, but often lead to a significant impairment in the quality of life[9]. Up to 75% of patients with long-term diabetes experience gastric motor dysfunction, leading to the following symptoms: constipation, nausea, heartburn, post-prandial fullness, abdominal pain as well as diarrhea[10,11].

In physiological conditions, the enteric nervous system (ENS) regulates the function of the gastrointestinal tract. ENS (called the "enteric brain") is independent of the central nervous system control[12]. This unique arrangement of neurons is organized in the muscular and submucosal plexuses and can control secretion processes, absorption and gastrointestinal motility[13]. The functioning of enteric neurons is based on the secretion of a broad spectrum of neurotransmitters, which are structurally and chemically differentiated[14]. The most useful division of bioactive substances of enteric neurons include two classes of neurons: inhibitory and excitatory[12,13]. The nitric oxide (NO) produced by enteric neurons is a major non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter which mediates smooth muscle relaxation in the gastrointestinal tract and is, therefore, an important factor in the stomach and gut contractility[14]. Vasoactive intestinal peptide (VIP),

together with NO comprise the primary inhibitory NANC neurotransmitters[15]. The biological activity of VIP leads to hyperpolarization of the smooth muscle fibers. Moreover, the relaxation function of VIP involves increased production of NO in smooth muscle cells[14,15]. Consequently, VIP acts as an inhibition factor of gastric emptying and reduced gastric acid secretion. Simultaneously, VIP is involved in the regulation of blood flow in the submucosal layer[16]. It is also essential that this substance has an effective neuroprotective function. This has been confirmed by the increasing survival of neurons previously damaged by lipopolysaccharide, as well as in the case of axotomy[17,18]. Galanin (GAL) can play both inhibitory and excitatory functions, depending on the digestive tract region, as well as the animal species. For example, in the dog ileum galanin is an excitatory factor while in the guinea pig it exhibits an opposite action[19].

Notably, in the field of metabolic disorders, the pig has gained appreciable interest as its metabolic, biochemical and pathophysiological response to diabetes partly mimics that observed in humans[20-22]. It has been shown that the blood supply of the porcine pancreas is similar to the human pancreas and the number of insulin-producing cells is within a similar range as observed in humans, making it a valuable model for the study of diabetes[23]. The most suitable and generally accepted pig model of hyperglycemia is streptozotocine-induced type 1 diabetes, where carbohydrate metabolism and pancreatic insulin secretion remain at a very similar level to humans with long-term diabetes[20]. Thus, taking advantage of its close similarity to humans, we raised the question of how the porcine gastric enteric neurons adapt to hyperglycemia. Therefore, the aim of this study was to provide data on the adaptive changes in chemical phenotyping, including inhibitory substances [vasoactive intestinal polypeptide, galanin and neuronal isoform of nitric oxide synthase (nNOS)] in the gastric enteric neurons of streptozotocin-induced hyperglycemic pigs.

MATERIALS AND METHODS

Animals

Ten juvenile both sex pigs of the White Large Polish breed, weighing from 17.0 kg to 20 kg, were used in this study. The pigs were randomly divided into two groups: diabetic (D group, n = 5) and control (C group, n = 5) and were housed in cages suitable for pigs. Prior to the experiment initiation, the animals were given one week of acclimatization to observe their general health, to minimize physiological stress and to ensure the proper conduct of the study. All treatment of animals was conducted in compliance with the instructions of the Local Ethical Committee in Olsztyn (Poland) (decision number 13/2015/DTN) with special attention paid to minimizing any stress reaction.

Chemical induction of diabetes

After acclimatization, hyperglycemia in the diabetic group was induced by use of streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO, United States, S0130) 150 mg/kg of body weight, dissolved in a freshly-prepared disodium citrate buffer solution (pH = 4.23, 1 g streptozotocin/10 mL solution). For this purpose, pigs were anesthetized and the solution was administrated *via* intravenous needle inserted into an ear with continuous infusion for approximately 5 min. To avoid nausea and vomiting after streptozotocin injection, animals were fasted for 18 h before the experiment and the control pigs were injected with equal amounts of vehicle (citrate buffer).

General health condition evaluation

The pigs were continuously observed for 24 h after streptozotocin injection. In order to avoid temporary hypoglycemia, 250 mL of a 50% glucose solution per animal was administered intravenously. The pigs received a normal diet throughout the whole time of the experiment twice a day and tap water ad libitum. The blood glucose level was measured to confirm diabetes. The blood glucose concentration was estimated using an Aceent-200 (Cormay) biochemical analyzer, with the colorimetric measurement at a wavelength of 510 nm/670 nm. For this purpose, capillary blood from the ear was collected. The plasma glucose level was measured prior to the experiment initiation in both control and experimental groups. The next measurement was made 48 h after the induction of diabetes. Subsequent measurements of glucose levels were monitored weekly until the end of the experiment.

Tissue collection

Six weeks after streptozotocin injection, animals were deeply anesthetized *via* intravenous administration of pentobarbital (Vetbutal, Biowet, Poland) and perfused transcardially *via* the ascending aorta with 4% paraformaldehyde in a 0.1 mol/L phosphate buffer (PB, pH 7.4). The samples were post-fixed by immersion in the same fixative for 1 h, rinsed several times with phosphatase buffer (PB) and then transferred into 30% sucrose solution and stored at 4 ℃ until sectioning. The tissue blocks were cut in frontal or sagittal planes using a Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 μm and mounted on gelatinized glass slides.

Immunofluorescence procedure

The sections were processed for a double immunofluorescence staining. Briefly, after air-drying at room temperature for 45 min and rinsing in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4; 3 ×

Table 1 Serum glucose levels

Date	Control group (mmol/L)	SE	Experimental group (mmol/L)	SE
Before streptozotocin injection	5.01	0.10	5.030	0.10
1 wk after streptozotocin injection	5.08	0.10	17.36	0.38
2 wk after streptozotocin injection	4.91	0.18	20.72	0.24
3 wk after streptozotocin injection	5.19	0.06	21.58	0.27
4 wk after streptozotocin injection	5.31	0.12	20.08	0.09
5 wk after streptozotocin injection	4.84	0.32	22.26	1.21
6 wk after streptozotocin injection	5.20	0.10	21.45	1.11

Serum glucose levels in controls and after induction of diabetes by streptozotocin administration (up 1 to 6 wk).

10 min), the sections were incubated in a blocking buffer containing: 10% of normal goat serum (MP Biomedicals, United States), in 0.1 mol/L PBS, 0.1% donkey serum (Abcam, United Kingdom), 1% Triton X-100 (Sigma-Aldrich, United States), 0.05% Thimerosal (Sigma-Aldrich, United States) and 0.01% NaN_3 for 1 h at room temperature to reduce non-specific background staining. Subsequently, after another wash in PBS (3 × 10 min) the sections were incubated overnight at 4 ℃ with primary antibodies raised in different species and directed towards general neuronal marker Hu C/D proteins (mouse polyclonal: Invitrogene United States; code A-212711:1000 working diluted 1:1000), anti-nNOS antibodies (rabbit polyclonal: Chemicon, Billerica, MA, United States, cat. No. AB 5380; working dilution 1:4000), VIP (rabbit polyclonal; Biomol, Hamburg, Germany, cat. No. VA1285; working dilution 1:6000) and GAL (rabbit polyclonal: Millipore, Billerica, MA, United States, cat. No. AB 2233; working dilution 1:1000). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% BSA. On the following day, the sections were rinsed (PBS, 3 × 15 min) and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor 488, 1:1000 Invitrogen United States; code A21202, and donkey anti-rabbit Alexa Fluor 546 1:1000 Invitrogen, United States; code A10040) diluted in PBS containing 0.25% BSA and 0.1% Triton X-100) for 4 h. The sections were then rinsed three times (PBS, 3 × 5 min) and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, United States). The prepared specimens were viewed and photographed using an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, coupled with a digital monochromatic camera (Olympus XM 10) connected to a PC and analyzed with Cell Dimension software (Olympus, Tokyo, Japan). Standard controls, *i.e.*, pre-absorption of the neuropeptide antisera with appropriate antigen, omission, and replacement of the primary antisera by non-immune sera, were performed to test the antibodies and specificity of the method. The test was performed as follows: sections of the stomach were incubated with "working" dilution of the primary immunoserum, which had been previously pre-absorbed for 18 h at 37 ℃ with 20 μg of appropriate purified protein VIP (064-24, Phoenix Pharmaceutical), GAL (026-06, Phoenix Pharmaceutical) and nNOS (N3033, Sigma, St Louis, MO, United States). Additional negative controls, such as omission and replacement of all primary antisera with non-immune sera, were also performed. This procedure completely eliminated specific staining.

Counting of the nerve structures and statistical evaluation

The number of nNOS, VIP and GAL-like immuno-reactive (LI) enteric neurons was expressed as a percentage of the total number of Hu C/D positive perikarya. At least 700 Hu C/D labeled cell bodies of intramural ganglia and each part of the stomach were examined. Only neurons with well-visible nucleus were counted. To prevent the double-counting of Hu C/D immunoreactive neurons, the sections were located at least 100 μm apart. Data pooled from all animals groups were statistically analyzed using Statistica 10 software (StatSoft Inc., Tulsa, OK, United States) and were expressed as a mean ± SE. Significant differences were evaluated using Student's *t*-test for independent samples ($^aP < 0.05$, $^bP < 0.01$, and $^cP < 0.001$).

RESULTS

Plasma glucose level and physiological status

The baseline plasma glucose level measured in animals before STZ treatment and the development of hyperglycemia were both within standard reference values for the pig (5.01 mmol/L ± 0.10 mmol/L). During the weeks following STZ implementation, a consistent increase in plasma glucose concentration was observed. A significant 3.4 -fold (17.36 mmol/L ± 0.38 mmol/L) increase in glucose level was observed on the 7th day after STZ injection and 4-5 wk after the injection the highest increase (4.4 fold) level of glucose was observed (22.26 mmol/L ± 1.21 mmol/L). In the last week of experiment, the baseline serum glucose level decreased slightly, reaching 21.24 mmol/L ± 1.11 mmol/L. The mean glucose concentration during the experimental period is presented in Table 1.

Despite significant hyperglycemia, none of the diabetic animals showed any abnormalities throughout the entire period of the experiment. Moreover, none of the animals required exogenous insulin injection.

Immunofluorescence

All biologically active substances studied were presented in the investigated area of the stomach (Table 2). In the control group, the nNOS distribution varied and clearly depended on the analyzed area of the stomach (Figure 1A-S and Figure 2A-F). In the myenteric ganglia (MG) of the antrum, the total number of nNOS neurons was 37.12% ± 2.81% (Figure 1A-C), while in the corpus,

Table 2 The average percentage of neurons immunoreactive to bioactive substances used in this study

Stomach part	Control group			Experimental group		
	Antrum	Corpus	Pylorus	Antrum	Corpus	Pylorus
		nNOS[1]			nNOS[1]	
Myenteric ganglia	37.12 ± 2.81	22.28 ± 1.19	15.91 ± 0.58	41.93 ± 2.34	40.74[b] ± 2.22	35.38[c] ± 1.54
Submucosal ganglia	-	18.62 ± 1.66	-	-	19.79 ± 1.51	-
		VIP[1]			VIP[1]	
Myenteric ganglia	18.38 ± 1.39	23.2 ± 0.23	23.64 ± 1.56	40.74[c] ± 1.77	30.93[c] ± 0.86	31.20[a] ± 1.10
Submucosal ganglia	-	43.61 ± 1.06	-	-	37.00[a] ± 1.77	-
		GAL[1]			GAL[1]	
Myenteric ganglia	26.53 ± 1.52	17.73 ± 1.12	16.32 ± 0.43	36.67[b] ± 1.02	16.51 ± 0.92	17.99[a] ± 0.38
Submucosal ganglia	-	41.42 ± 0.88	-	-	40.49 ± 0.63	-

[1]Relative frequency of particular neuronal subclasses is presented as percent (mean ± SE) of all neurons counted within the ganglia stained for Hu C/D proteins. nNOS, VIP and GAL immunoreactive perikarya in various parts of the porcine stomach under physiological conditions (Control group) and during experimentally induced hyperglicemia (Experimental group). Statistically significant data ([a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$) in particular animal group are marked by superscripted alphabetical lettering. nNOS: Neuronal isoform of nitric oxide synthase; VIP: Vasoactive intestinal peptide; GAL: Galanin; Hu C/D: Pan-neuronal marker.

the quantity of nNOS positive cells bodies inside the MG was relatively lower 22.28% ± 1.19% (Figure 1G-I). Additionally, in submucosal ganglion (SG) in the corpus, nNOS-LI neurons were estimated at 18.62% ± 1.66% (Figure 2A-C). In the pylorus, in MG about 15.91% ± 0.58% nNOS-LI were observed (Figure 1M-O).

Although experimentally-induced hyperglycemia contributed to the expression of nNOS in enteric neurons, the changes were differentiated. Statistically significant changes were observed in the MG in the corpus from 22.28% ± 1.19% to 40.74% ± 2.22% (increase by 82.85%) (Figure 1J-L) increased of 18.46% and in the pylorus from 15.91%±0.58% to 35.38% ± 1.54% (increase by 122.37%) (Figure 1P-S) increased of 19.47%. In contrast, in the MG in the antrum and SG in the corpus, hyperglycemia did not cause statistically significant changes (Figures 1D-F and 2D-F).

The other investigated substance was VIP. As in the case of nNOS, VIP-positive cells were presented in all studied areas, but clear differences were noted between the various regions of the stomach (Figures 2G-L and 3A-S). In the control group the largest number of VIP-positive cells bodies were noted within the SG in the corpus 43.61% ± 1.06% (Figure 2G-I). Definitely fewer VIP-positive perikarya were observed within the MG in the antrum 18.38% ± 1.39% (Figure 3A-C), pylorus 23.64% ± 1.56% (Figure 3M-O) and MG of the corpus 23.20% ± 0.23% (Figure 3G-I).

In the diabetic group, statistically significant changes were observed in all investigated areas. The highest increase in VIP-immunopositive perikarya was noted in the MG inside the corpus from 18.38% ± 1.39% to 40.74% ± 1.77% (increase by 121.65%) (Figure 3J-L). With regard to MG in the antrum and pylorus, the changes in chemical coding were relativity smaller from 23.20% ± 0.23% to 30.93% ± 0.86% (increase by 33.31%) inside to antrum (Figure 3D-F) and from 23.64 ± 1.56% to 30.47% ± 1.10% inside to pylorus, respectively (increase by 31.97%) (Figure 3P-S). However, in the SG of the corpus, a decrease in the number of VIP-LI neurons was noted from 43.61% ± 1.06% to 37.00% ± 1.77% (decrease by 15.15%) (Figure 2J-L).

As in the case of previously studied substances, the distribution of GAL varied and clearly depended on the area of the stomach (Figure 2M-S and 4A-S). In the control group, the largest population of cells were observed in the MG of antrum 26.53% ± 1.52% (Figure 4A-C). In the MG in the area of corpus and pylorus, a comparable number of GAL-positive neurons were observed 17.73% ± 1.12% in the corpus (Figure 4G-I) and 16.32% ± 0.43% in the pylorus (Figure 4M-O). The highest population of GAL-positive perikarya was noted in the SG in the corpus 41.42% ± 0.88% (Figure 2M-O).

In the diabetic group, statistically significant changes were noted only in the MG in the antrum and pylorus, whereas increased expression of GAL-LI in enteric neurons was observed from 26.53% ± 1.52% to 36.67% ± 1.02% in the antrum (increase by 38.22%) (Figure 4D-F) and from 17.73% ± 1.12% to 17.99% ± 0.38% in the pylorus (increase by 10.23%) (Figure 4P-S). However, in the corpus in both the MG and SG, the population of GAL-LI neurons changed in a statistically insignificant manner compared to the control group (Figures 4J-L and 2P-S).

DISCUSSION

Autonomic neuropathy is a commonly occurring complication in poorly-controlled blood glucose level patients. Studies of the development mechanisms of this pathology have been conducted in many research center[4,5,8,23]. Moreover, different animal models have been used to determinate the exact mechanism of diabetes complications[24]. Thus, there are doubts as to whether the pig is a suitable model for the study of diabetic autonomic neuropathy occurring in patients with long-term diabetes. Unfortunately,

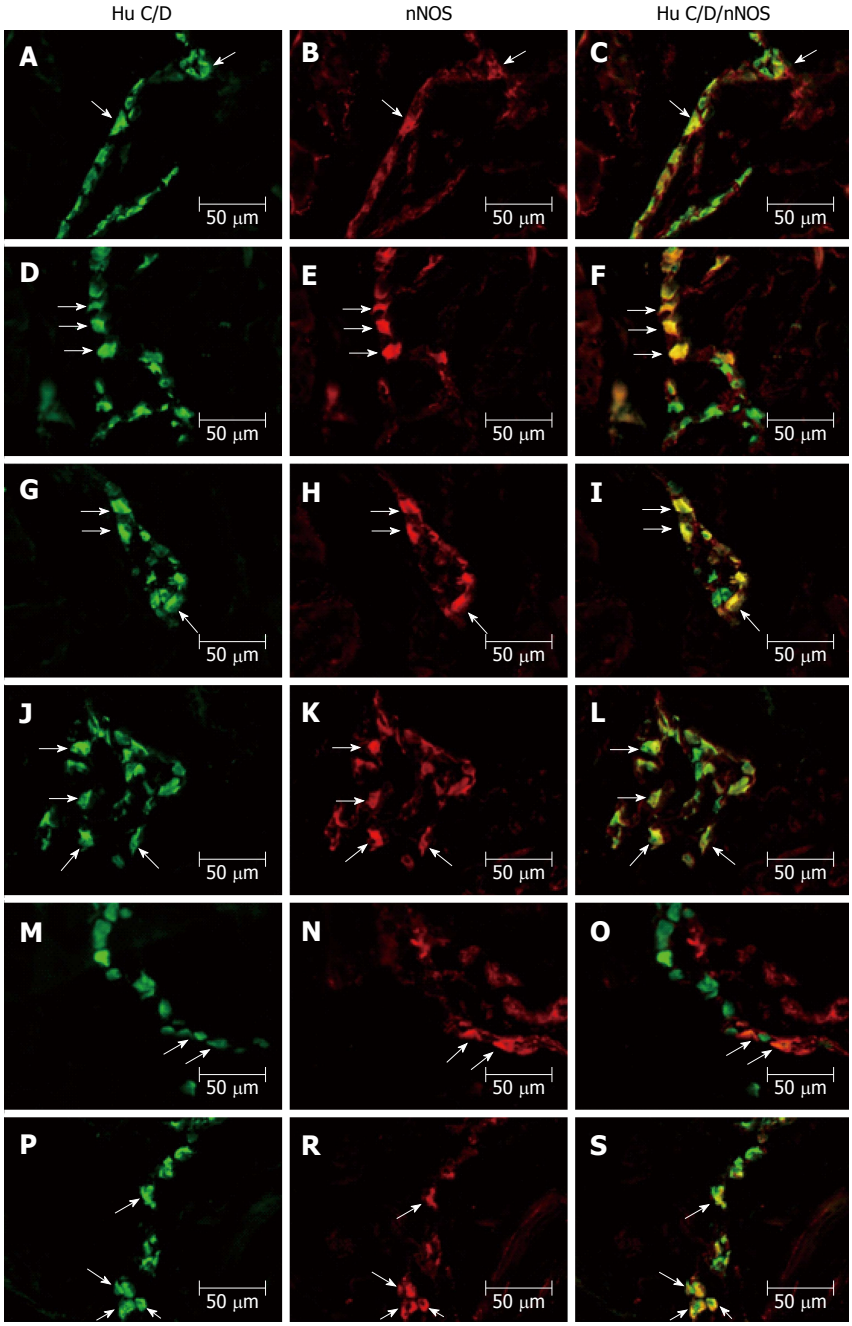


Figure 1 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to nNOS. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to nNOS; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and n NOS; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to nNOS; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and nNOS; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to nNOS; I: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and nNOS; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to nNOS; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to nNOS; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and nNOS; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to nNOS; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. nNOS: Neuronal isoform of nitric oxide synthase; Hu C/D: Pan-neuronal marker.

since spontaneous diabetes in large domestic animals such as the pig are extremely rare[25], it is important to select an appropriate method leading to the induction of diabetes and, thus, the development of hyperglycemia. Total pancreatectomy is a technique involving a considerable mortality rate of animals and is

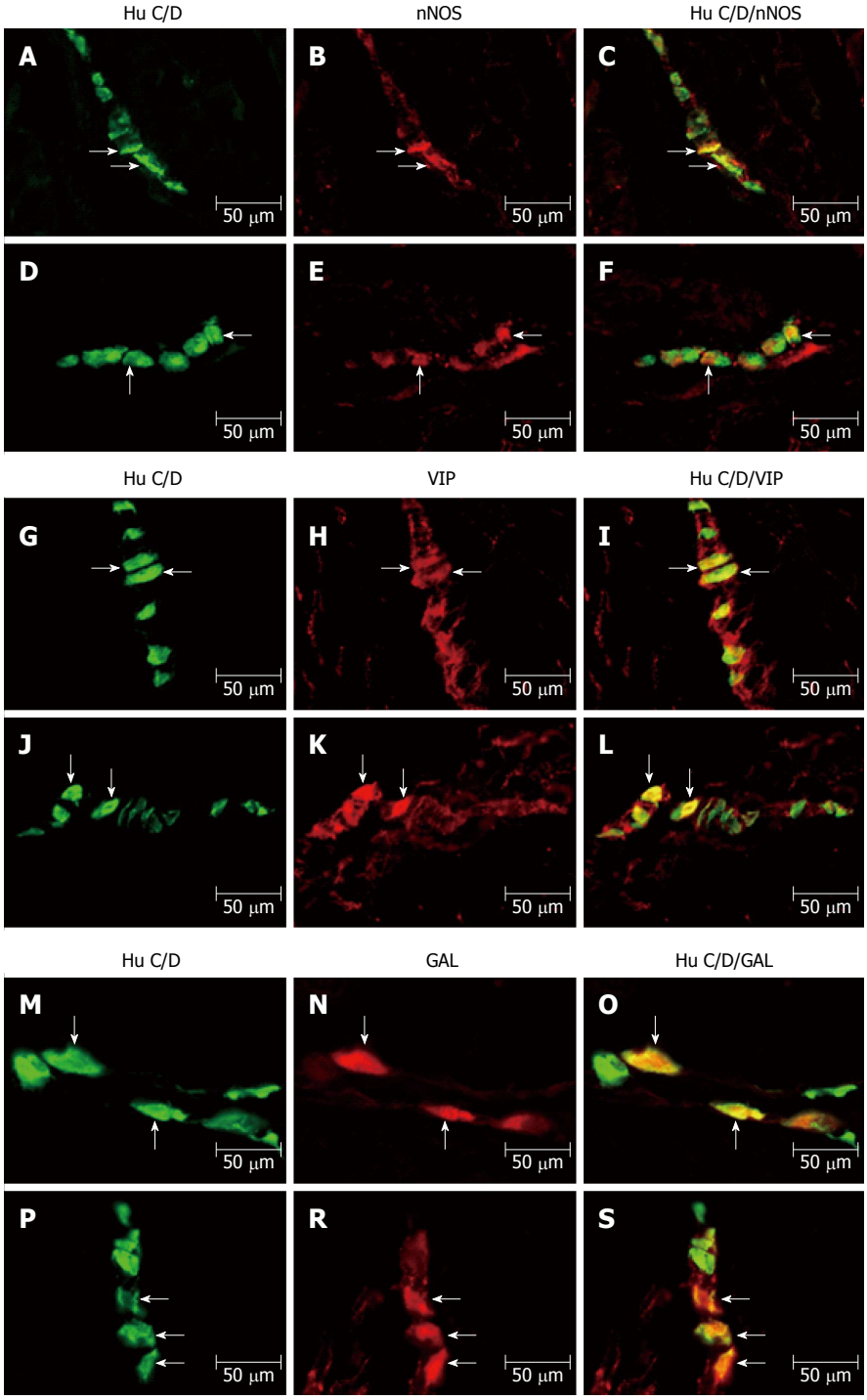


Figure 2 Submucosal ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to nNOS, VIP and GAL. A: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; B: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to nNOS; C: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and n NOS; D: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; E: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to nNOS; F: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS; G: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to VIP; I: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and VIP; J: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to VIP; L: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP; M: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; N: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to GAL; O: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and GAL; P: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; R: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to GAL; S: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. nNOS: Neuronal isoform of nitric oxide synthase; VIP: Vasoactive intestinal peptide; GAL: Galanin; Hu C/D: Pan-neuronal marker.

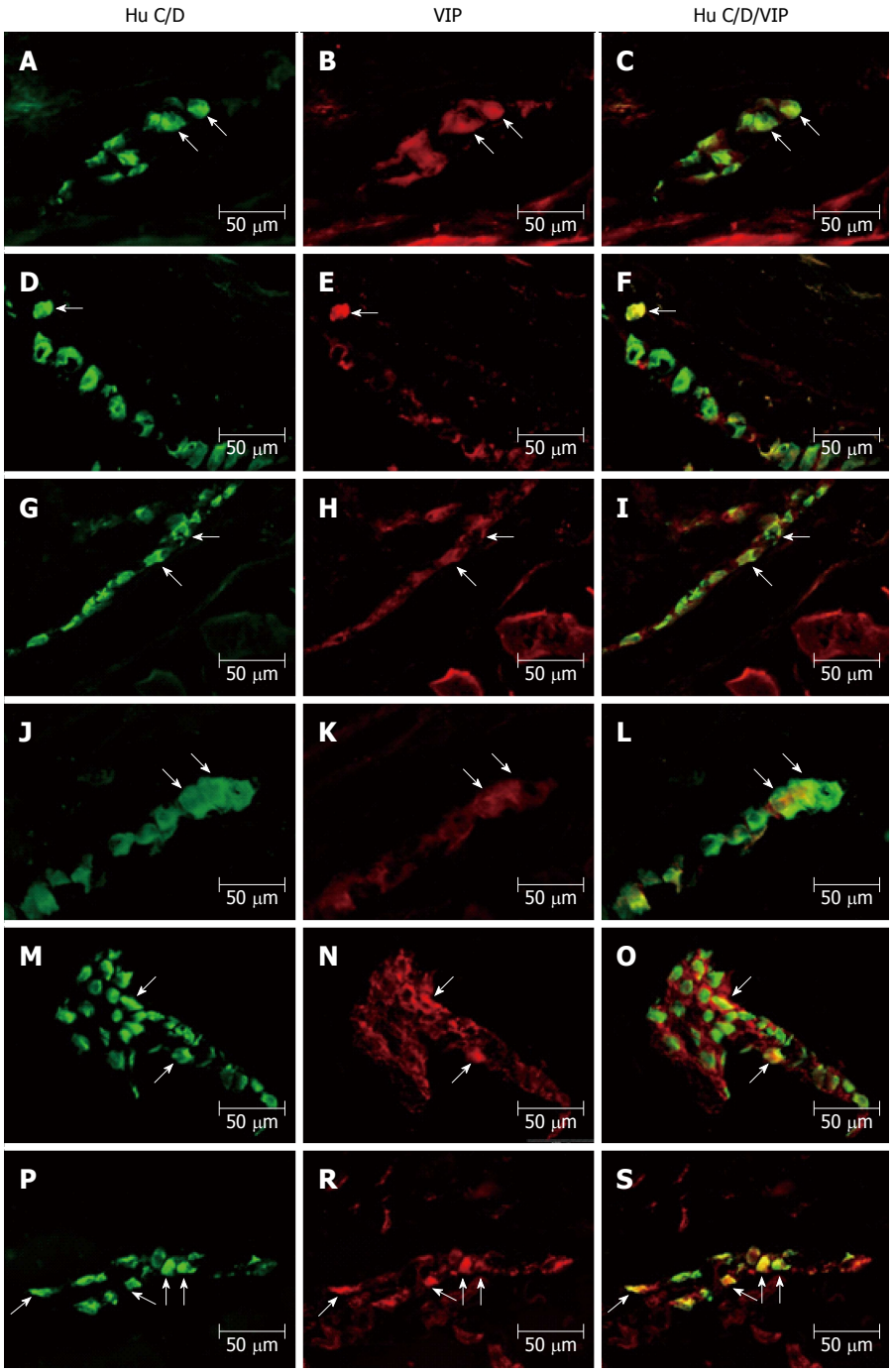


Figure 3 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to VIP. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to VIP; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and VIP; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to VIP; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and VIP; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to VIP; I: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and VIP; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to VIP; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to VIP; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and VIP; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to VIP; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. VIP: Vasoactive intestinal peptide; Hu C/D: Pan-neuronal marker.

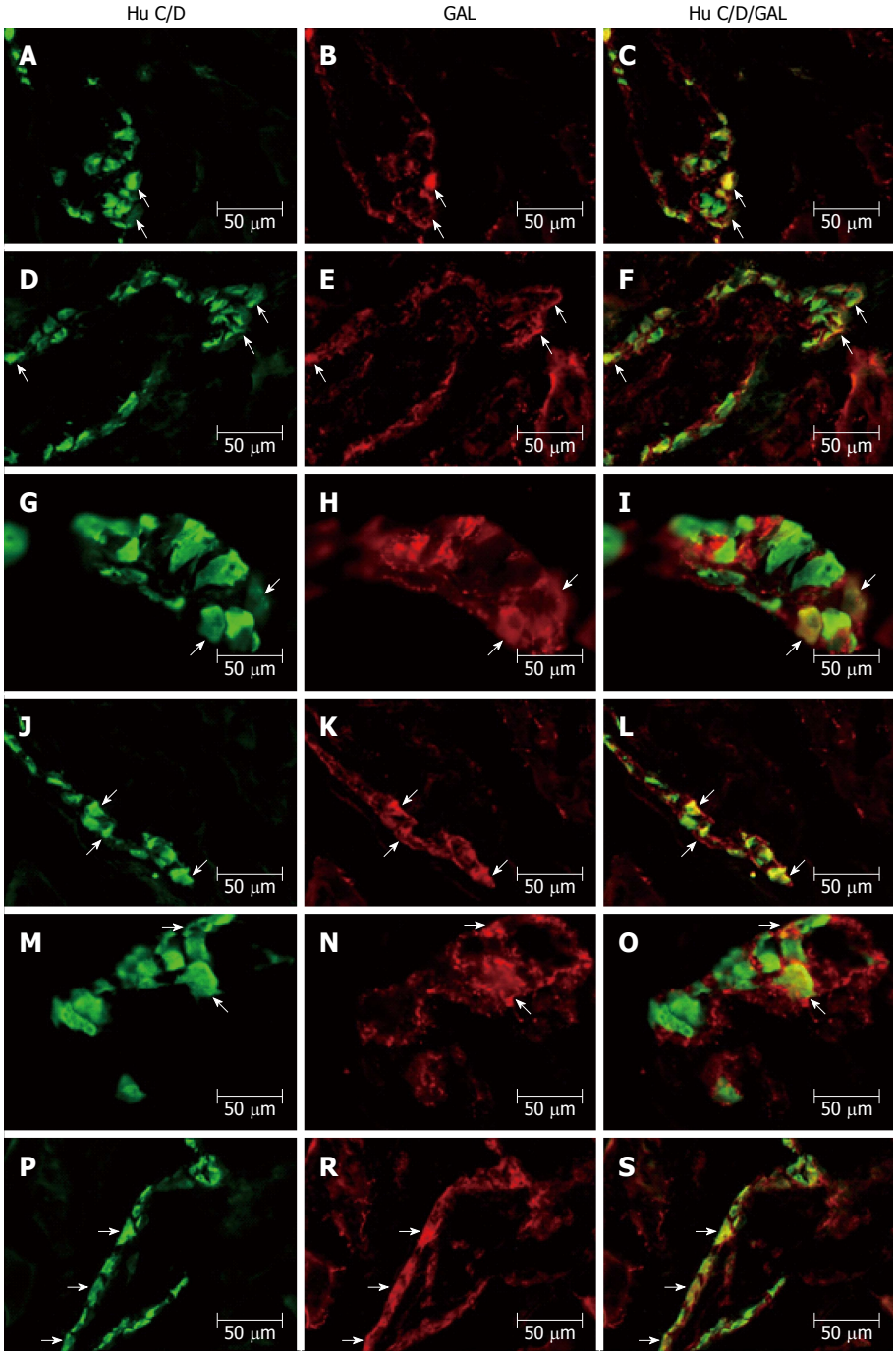


Figure 4 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to GAL. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to GAL; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and GAL; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to GAL; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and GAL; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to GAL; I: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and GAL; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to GAL; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to GAL; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and GAL; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to GAL; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. GAL: Galanin; Hu C/D: Pan-neuronal marker.

not presently used[25]. Therefore, in the present study we used streptozotocin administered intravenously in order to induce diabetes. This method is widely-accepted and used in many studies to determine the side-effects of elevated blood glucose[26]. In our study, the choice of streptozotocin as a diabetogenic substance also turned out to be the opposite, because all animals developed hypoglycemia during the first week of experiment and we did not note mortality falling during the study. Moreover, it is worth underlining that the type 1 STZ-induced hyperglycemic/diabetic pig model has been predominately validated in studies of the cardiovascular complications as well as in peripheral neuropathy[27,28]. On the other hand, diabetic gastroenteropathy, especially concerning the chemical phenotyping of enteric neurons in this model is unclear. The available data describing neurochemical changes in the ENS of hyperglycemic/diabetic animals are limited to the studies on small animal models, mainly rat and mice[29-34].

Our results demonstrated that the porcine ENS, exhibits alterations in the chemical coding of the inhibitory neurons after six weeks of sustained hyperglycemia. It is generally recognized that NO, together with a VIP, acts as inhibitory neurotransmitter in muscle and submucosal layer of gastrointestinal tract[14,15]. As shown in our results, a visible increase in nNOS expression was presented in the myenteric ganglion of the corpus and pylorus. Contrary to these results, in the MG of the antrum and in the SG of the corpus, changes in the synthesis of nNOS have not been noted. As mentioned above, the previous studies, of the chemical coding of enteric neurons were conducted mainly on rodents. The obtained results indicate a differential response of enteric neurons, depending on the duration time of hyperglycemia and the gastrointestinal tract region studied. Belai *et al*[29,30] described a decrease in nNOS expression in the antrum, while in the small intestine and colon the same authors did not find statistically important changes compared to the control group. Meanwhile, another study revealed an increased level of nNOS in the antrum during streptozotocin-induced diabetes in rats[32]. In turn, hyperglycemia evoked a visible increase in the number of myenteric neurons in all investigated areas and a slight decrease in VIP-LI immunoreactivity in the submucosal ganglia in the corpus. As previously reported in diabetic rats, chronic hyperglycemia caused a marked increase in the immunoreactivity of VIP, in both the submucosal and myenteric ganglion. However, in the myenteric plexus, the increase was preceded by a slight decrease in the intensity of VIP immunoreactivity[31,34]. It is worth underlining that since these results focused on the ileum, we cannot directly relate them to the stomach.

Galanin is a neurotransmitter which can play an inhibitory or excitatory role depending on the investigated area of gastrointestinal tract as well as animal species. Galanin is also a modulator regulating the activity of other neurotransmitters, including VIP and NO. In this study, although we have shown an essential increase of galanin expression in myenteric ganglion neurons in the antrum and pylorus, in the corpus of the stomach we did not observe statistically significant changes compared to the control group. To date, an increase in galanin immunoreactivity has only been observed in the colon of non-obese diabetic mice (NOD) and the ileum of 12-wk-old diabetic rats[31,35]. Only in obese mice with coexisting diabetes has a decrease of galanin in the colon been noted[35]. Taking into account our results and the available data on the expression of neuroactive substances in enteric neurons (especially on inhibitory properties) the common pattern of changes is generally acceptable, i.e. inhibitory neurotransmitters such as nNOS, VIP and GAL appear to decrease in the early stage of diabetes with an increase in later stages, possibly secondary to regeneration[7]. Our results clearly indicate that the neurotoxic effect of chronic hyperglycemia in pigs leads to neuronal regeneration, which is reflected by an up-regulation of the synthesis of NO, VIP and GAL. It is well-known that NO and VIP have strong neuroprotective activity[36-40]. Therefore, the ENS adapts to changes in environmental conditions, increasing the synthesis of biologically active substances which may play a protective role against cellular oxidative stress as a consequence of high glucose serum levels. Moreover, we have noted down-regulation of VIP expression in the corpus of the submucosal ganglion. The different reaction of submucosal neurons probably results from another function played by neurons which leads to regulation of secretory activity[16]. It is worth noting that since other investigated substances within the submucosal ganglion in the corpus of the stomach did not show statistically significant changes, we can conclude that these neurons have a different sensitivity to high glucose level.

Our results may partly explain the reason of gastrointestinal motility disorders observed in people with long-term diabetes. Increase of the expression of inhibitory substances may impair the function of the pyloric sphincter relaxation and the antrum contraction. Which, in turn, contributes to gastric emptying which has been observed in humans[11].

In conclusion, this is the first report describing changes in the expression of inhibitory substances in the intramural neurons of the stomach in hyperglycemic STZ-induced diabetic pigs. Our results indicate that after six weeks of high glucose serum level expression of inhibitory substances (such as NO), vasoactive intestinal polypeptide and galanin undergo significant changes (described as chemical phenotyping), which suggests that STZ-injected pigs might serve as a good model in early studies of autonomic nerve changes under hyperglycemic conditions. Furthermore, our data provide a background for more detailed studies on the contribution of enteric neurons to the pathogenesis

of gastrointestinal disturbances as well as reference data for further, pre-clinical studies on hyperglycemia-related autonomic neuronal changes in a species more closely related to humans.

COMMENTS

Background

Diabetes mellitus is associated with several changes in gastrointestinal (GI) tract motility that lead to nausea, bloating, abdominal pain, diarrhoea and constipation. Up to 75% of patients with diabetes can experience these symptoms. The pathogenesis of altered GI functions in diabetes is multifactorial and the role of the enteric nervous system (ENS) in this respect has gained significant attention. The neuronal remodelling affects the ratio of inhibitory neurons, which in turn leads to impaired of nerve mediated muscle responses and can contribute to the motility dysfunction seen in diabetes patients.

Research frontiers

Streptozotocin is widely used to induce experimental diabetes in animals. Its application leads to blood insulin levels decrease and result in development of hyperglycaemia. Long term elevated glucose level cause diabetic neuropathy.

Innovations and breakthroughs

This is the first study utilizing pig as a model species for human diabetes induced gastrointestinal complications. The results suggest that swine due to close physiological similarity to human especially concerning gastrointestinal tract function seems to be better model for biomedical research than rodents.

Applications

The results provide evidence that acute hyperglycaemia changes properties of neuromodulators/neurotransmitters in GI. Pharmacological modulation of selected biologically active substances can be potentially new approach for treatment of the diabetes evoked gastrointestinal complications.

Terminology

Streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action B cells undergo the destruction by necrosis.

Peer-review

The authors have provided evidence that long term episodes of artificially initiated hyperglycaemia induced changes of expression of neuronal isoform of nitric oxide synthase, vasoactive intestinal peptide and galanin in inhibitory neurons inside the stomach ENS. Those changes might be responsible for broad spectrum of gastrointestinal disturbances.

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ORIGINAL ARTICLE

Basic Study

HOX transcript antisense intergenic RNA represses E-cadherin expression by binding to EZH2 in gastric cancer

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Data sharing statement: No additional data are available.

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Abstract

AIM

To clarify the mechanisms of HOX transcript antisense intergenic RNA (HOTAIR) in gastric cancer (GC) migration and invasion.

METHODS

Quantitative real-time polymerase chain reaction (qPCR) was used to detect the expression level of HOTAIR in GC tissues. The correlation of its expression with clinicopathological features was analyzed. Area under receiver operating characteristic curve (AUC_{ROC}) was constructed to evaluate the diagnostic value of HOTAIR. Wound-healing assay and Transwell assay were performed to detect the biological effects of HOTAIR in GC cells. qPCR, western blot and

immunohistochemistry were used to evaluate the mRNA and protein expression of E-cadherin. RNA-binding protein immunoprecipitation was used for the analysis of EZH2 interactions with HOTAIR. Chromatin immunoprecipitation assay was performed to investigate direct interactions between EZH2 and E-cadherin.

RESULTS

The expression of HOTAIR was up-regulated in GC tumorous tissues compared with the para-tumorous tissues ($P < 0.001$). Its over-expression was correlated with tumor-node-metastasis (TNM) stage ($P = 0.024$), tumor invasion ($P = 0.018$), lymph node metastasis (P = 0.023), and poor prognosis ($P < 0.001$). Multivariate Cox regression analysis confirmed expression of HOTAIR as an independent predictor of overall survival ($P = 0.033$), together with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$). The AUC_{ROC} was up to 0.709 (95%CI: 0.623-0.785, $P < 0.001$). Knockdown of HOTAIR by siRNA in GC cells suppressed the migration and invasion of GC cells. Significantly negative correlation between HOTAIR and E-cadherin was found in GC tissues and cell lines, and HOTAIR contributed to the regulation of E-cadherin through binding to EZH2 with the E-cadherin promoter.

CONCLUSION

HOTAIR may play a pivotal role in tumor cell migration and invasion. It can be used as a potential diagnostic and prognostic biomarker for GC.

Key words: Long noncoding RNA; HOX transcript antisense intergenic RNA; Gastric cancer; Migration and invasion; E-cadherin

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Core tip: In this study, we found that HOX transcript antisense intergenic RNA (HOTAIR) expression was up-regulated in gastric cancer (GC) tissues. High expression of HOTAIR was associated with clinicopathological characteristics and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly inhibited the invasion and migration of GC cells. We also tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. The findings from our study will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

Chen WM, Chen WD, Jiang XM, Jia XF, Wang HM, Zhang QJ, Shu YQ, Zhao HB. HOX transcript antisense intergenic RNA represses E-cadherin expression by binding to EZH2 in gastric cancer. *World J Gastroenterol* 2017; 23(33): 6100-6110 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6100.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6100

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide, having high rates of both incidence and mortality[1]. Even though remarkable progress has been made in surgical resection and chemotherapy, which are the main therapeutic approaches for GC, the prognosis continues to be dismal[2]. The main cause of treatment failure is cancer recurrence and metastasis[3,4].

Tumor metastasis is a complex process, in which cancer cells leave the primary tumor site and migrate to distant secondary organs. Metastatic cancer cells fractionally retain their epithelial properties and obtain mesenchymal characteristics that give them the ability to invade or distract[5]. This process is called epithelial-to-mesenchymal transition (EMT). EMT is characterized by E-cadherin expression loss and N-cadherin and vimentin up-regulation[6]. It is regulated by many signaling pathways, transcriptional factors and post-transcriptional factors[7]. Post-transcriptional regulatory networks include the miRNA and long noncoding (lnc)RNA families[8]. Emerging evidence shows that lncRNAs have an important role in regulating EMT and cancer metastasis[5,9]. Several important lncRNAs are reported to induce EMT, including highly upregulated in liver cancer (HULC)[10], metastasis-associated lung adenocarcinoma transcript (MALAT)-1[11], H19[12], and HOX transcript antisense intergenic RNA (HOTAIR)[13].

HOTAIR is identified as an oncogene involved in many kinds of cancers, including breast cancer[14], esophageal squamous cell carcinoma[15], colorectal cancer[16], and GC[13]. HOTAIR acts mainly through the polycomb repressive complex (PRC)2 (EZH2, RbAp46, RbAp48 and SUZ12)[17], which trimethylates histone H3 lysine-27 (H3K27) of the HOXD locus and repress HOXD gene expression[14]. EZH2 which is the core catalytic component of the PRC2, can affect cancer progression by altering H3K27 trimethylation and silencing transcription[17].

A previous study showed that silencing of HOTAIR inhibits GC cell migration, invasion and metastasis, and reverses the EMT in GC cells[13]. HOTAIR in combination with PRC2 could epigenetically inhibit miR34a, and control C-Met (HGF/C-Met/Snail pathway) and Snail, thus promoting the EMT process of GC cells and tumor metastasis[13]. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis have not yet been fully investigated.

Up-regulation of HOTAIR expression in GC tissues was found in the present study. We also found that over-expression of HOTAIR was related to the clinicopathological characteristics of and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly repressed migration and invasion. Finally, we tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. These studies

Table 1 Relationships between the expression of HOTAIR and clinicopathological characteristics in 65 patients with gastric cancer

Characteristic	Expression of HOTAIR		*P* value
	Low *n* = 32	High *n* = 33	
Sex			0.408
Female	7	11	
Male	25	22	
Age in yr			0.455
≤ 60	17	21	
> 60	15	12	
Histological grade			1.000
Well/moderate	14	15	
Other	18	18	
Tumor invasion depth, T			0.018
Tis, T1,T2	15	6	
T3 or above	17	27	
Lymph node metastasis, N			0.023
N0	12	4	
N1 or above	20	29	
TNM stage			0.024
I/II	23	14	
III/IV	9	19	
Tumor location			0.102
Antrum	8	15	
Cardia	10	9	
Angulus	13	5	
Body	1	3	
Full stomach	0	1	
Laure's classification			0.373
Intestinal	17	15	
Diffuse	12	17	
Mixed	3	1	

will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

MATERIALS AND METHODS

Tissue collection and ethics statement

A cohort of 65 primary GC patients was enrolled in this study, each of who underwent surgery at the First Affiliated Hospital of Nanjing Medical University and Jining NO.1 People's Hospital between 2008 and 2009. None received chemotherapy or radiotherapy prior to surgery. This study was approved by the University Ethics Committee. All patients participated after providing informed consent. Tumor stage was evaluated in accordance with the tumor-node-metastasis (TNM) classification system (UICC/AJCC 2002). Clinical characteristics are shown in Table 1. Patients discharged from hospital were followed up routinely and according to a scheduled program, at least once a year.

Cell lines and culture conditions

Human GC cell lines SGC-7901and BGC-823 were obtained from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank. Cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco-BRL, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Shanghai, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) in an incubator at 37 ℃ with 5% CO_2.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the frozen tissues using TRIzol reagent (Invitrogen). RNA concentrations were estimated by spectrophotometer absorbance readings of 260 nm. One microgram of total RNA was used for reverse transcription to cDNA with a Reverse Transcription Kit (TaKaRa, Shiga, Japan). An ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, United States) was used to quantify HOTAIR. Ten microliters of SYBR Premix Ex*Taq* (TaKaRa) was mixed. Primer sequences are listed in Supplementary Table 1. The relative expression was calculated using the equation: ΔCt = Ct (target gene) − Ct (GAPDH); Fold change = $2^{-[\Delta Ct(tumour) - \Delta Ct(nomal)][18]}$.

Cell transfection

HOTAIR small interfering (si)RNAs and negative control siRNA (si-NC) were purchased from Invitrogen. The siRNA sequences are listed in Supplementary Table 1. BGC-823 or SGC-7901 cells were grown in 6-well plates until confluent, then transfected with Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were harvested for qPCR or western blot analysis.

Wound-healing assay

Cells were seeded in 6-well plates in normal cell growth medium and incubated to confluence. A 20-μL tip was used to make a straight scratch, simulating a wound. The medium was changed to medium containing 1% FBS. At different time points, images of the plates were acquired using a microscope.

Cell migration and invasion assays

For the migration assays, at 48 h after transfection, 5×10^4 cells in serum-free medium were placed into the upper chamber of an insert (8-μm pore size; Millipore, Bedford, MA, United States). For the invasion assays, 10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, St. Louis, MO, United States). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged and counted in five random fields per well using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were repeated independently three times.

Western blot assay and antibodies

Cell protein lysates were separated by 15% SDS-PAGE, transferred to 0.22-μm nitrocellulose membranes

(Sigma-Aldrich) and incubated with specific antibodies. GAPDH antibody was used as a control. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad, Hercules, CA, United States). Anti-E-cadherin was purchased from Abcam (Cambridge, United Kingdom). Anti-GAPDH was purchased from Cell Signaling Technology (Danvers, MA, United States).

RNA-binding protein immunoprecipitation assay

Binding protein immunoprecipitation assay (RIP) experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Antibody for EZH2 RIP assays was from Abcam.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) assays were performed using an EZ-CHIP KIT (Millipore). EZH2 antibody was obtained from Abcam. H3 trimethyl Lys 27 antibody was from Millipore. The ChIP primer sequences are listed in Supplementary Table 1. Quantification of immunoprecipitated DNA was performed using qPCR with SYBR Green Mix (TaKaRa). ChIP data were calculated as a percentage relative to the input DNA by the equation of $2^{(\text{Input Ct - Target Ct})} \times 0.1 \times 100$.

Immunohistochemistry

The immunohistochemical analysis of E-cadherin was performed as described previously[19]. To quantify E-cadherin protein expression, both the intensity and extent of immunoreactivity were evaluated and scored. In the present study, staining intensity was scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The scores of the extent of immunoreactivity ranged from 0 to 3, and were determined according to the percentage of cells that showed positive staining in each microscopic field of view (0, < 25%; 1, 25%-50%; 2, 50%-75%; 3, 75%-100%). A final score ranging from 0 to 9 was achieved by multiplying the scores for intensity and extent.

Statistical analysis

SPSS version 21.0 software was used for all statistical analysis. Significance of the differences between groups was estimated by Student's *t*-test, χ^2 test, or Mann-Whitney test. Survival curves were estimated by the Kaplan-Meier method. The log-rank test was used to estimate the statistical difference between survival curves. Cox proportional hazards analysis was performed to calculate the hazard ratio (HR) and the 95% confidence interval (CI) to evaluate the association between HOTAIR expression and overall survival (OS) time. Multivariate Cox regression was performed to adjust for other covariates. Spearman correlation analysis was performed to investigate the correlation between HOTAIR and E-cadherin mRNA expression. Receiver operating characteristic (ROC) curve was produced to evaluate the diagnostic value for differentiating between GC and adjacent non-tumor tissues. A two-tailed *P* value of $\leqslant 0.05$ was considered statistically significant. All the graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, United States).

RESULTS

HOTAIR was up-regulated in GC tissues

HOTAIR showed markedly higher expression in GC tissues as compared with matched adjacent non-tumor tissues ($P < 0.001$; Figure 1A).

Diagnostic value of using HOTAIR as a marker

An ROC curve was generated by comparing HOTAIR expression in GC tissues to expression in matched adjacent non-tumor tissues. With a cut-off value of 9.31, the area under the ROC curve (AUC_{ROC}) reached 0.709 (95%CI: 0.623-0.785, $P < 0.001$; Figure 1B). The sensitivity was 64.62%, the specificity was 75.38%, and Youden's index was 0.4.

HOTAIR expression and clinicopathological factors in GC

Patients with GC were divided into two groups based on the cut-off ratio of HOTAIR expression (2.35-fold) in tumor tissues: high-expression group ($n = 33$) and low-expression group ($n = 32$) (Figure 1C). Table 1 showed that HOTAIR over-expression was significantly correlated with tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$) and higher TNM stage ($P = 0.024$). However, there was no correlation between HOTAIR expression and age, sex and histological grade ($P > 0.05$).

Up-regulation of HOTAIR associated with poor survival of GC

To assess the impact of HOTAIR expression on OS of GC patients, Kaplan-Meier analysis and log rank test were used. The results revealed that patients in the high-expression group had a shorter OS (median OS: 25.9 mo) than those in the low-expression group (median OS: 42.5 mo, $P < 0.001$; Figure 1D). Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 2. Further analysis in a multivariate Cox proportional hazards model showed that HOTAIR expression ($P = 0.033$), along with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$), were strongly associated with OS. These results revealed that HOTAIR expression was an independent prognostic indicator of OS.

Knockdown of HOTAIR suppressed migration and invasion of GC cells

The wound-healing assay showed that HOTAIR knockdown cells were significantly slower than control

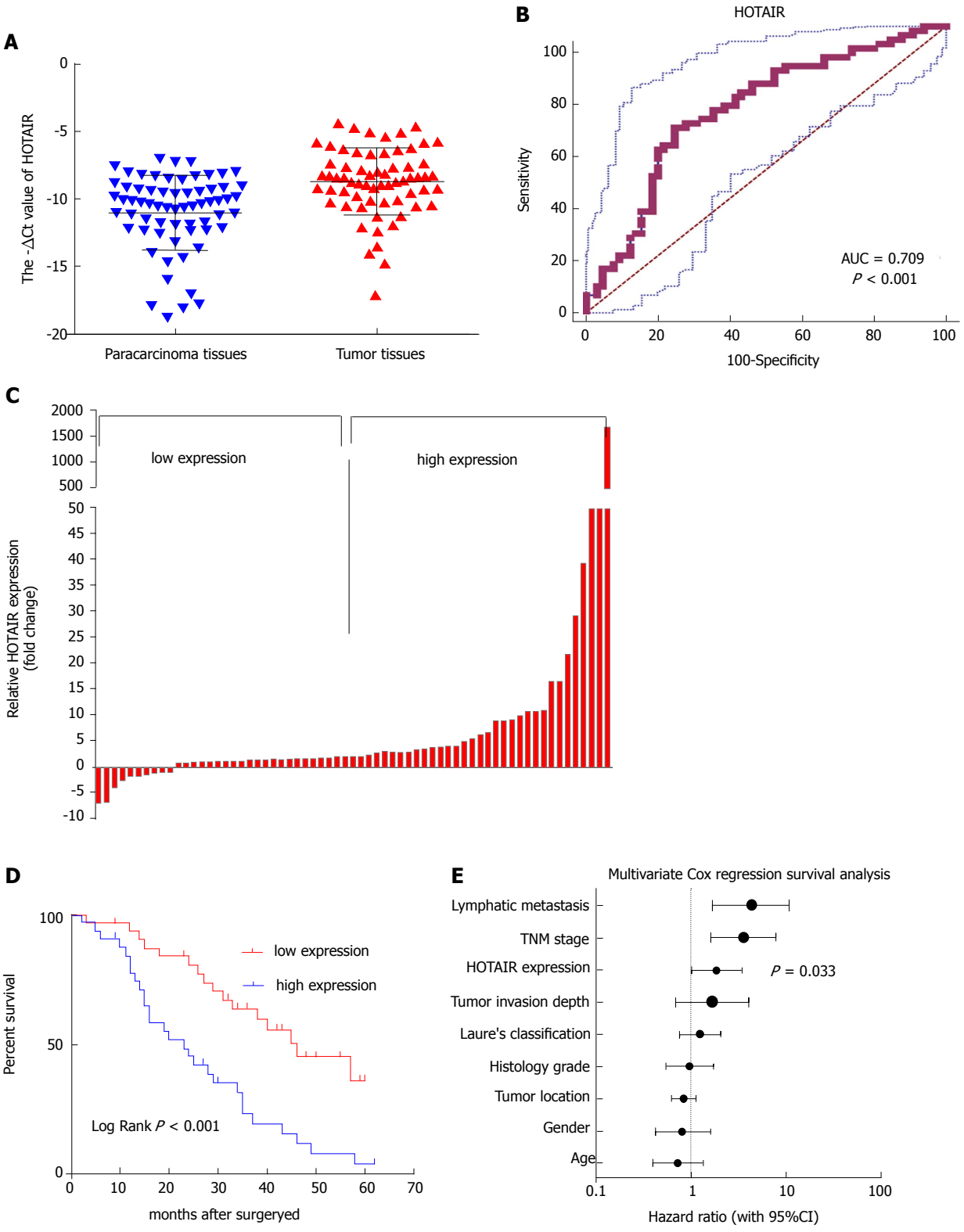


Figure 1 HOTAIR up-regulation correlates with poor survival in patients with GC. A: HOTAIR expression was significantly up-regulated in tumor tissues compared with their corresponding para-carcinoma tissues (shown as -ΔCt); B: ROC curves for observing the diagnostic value of HOTAIR; C: HOTAIR expression was classified into two groups according to HOTAIR expression levels (median split); D: Kaplan-Meier analysis of OS was analyzed according to HOTAIR expression levels; E: Different factors (including HOTAIR, tumor invasion depth, lymph node metastasis, TNM stage, histological grade, gender, tumor location, Lauren's classification and age) were analyzed for their association with patient survival using Cox regression model. The hazard ratio and 95%CI are plotted for each factor. CI: Confidence interval; GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; OS: Overall survival; ROC: Receiver operating characteristic; TNM: Tumor-node-metastasis.

cells (Figure 2A-D). Transwell assay and invasion assay demonstrated knockdown of HOTAIR notably reduced the number of BGC-823 or SGC-7901 cells migrating across the membrane (Figure 2E-H). These results

Table 2 Univariate and multivariate analysis of clinical pathological characteristics and overall survival of 65 patients with gastric cancer

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
HOTAIR expression	2.943	1.580-5.483	0.001	1.998	1.058-3.772	0.033
Tumor invasion depth, T1, T2/above	2.651	1.316-5.341	0.006	1.676	0.691-4.067	0.254
Lymphatic metastasis, absent/present	3.470	1.459-8.250	0.005	4.324	1.701-10.993	0.002
TNM stage, I/II, III/IV	4.494	2.351-8.592	< 0.001	3.598	1.624-7.975	0.002
Histology grade, well, moderate/others	0.967	0.535-1.750	0.913			
Age, < 60/> 60	0.730	0.396-1.344	0.312			
Sex, male/female	0.822	0.428-1.581	0.557			
Tumor location	0.839	0.623-1.131	0.250			
Lauren's classification	1.256	0.757-2.084	0.378			

confirmed that knockdown of HOTAIR inhibited the migration and invasion of GC cells.

HOTAIR was inversely correlated with E-cadherin in GC tissue and cell lines

The HOTAIR and E-cadherin expression levels in GC tissues were detected by qPCR and immunohistochemistry. E-cadherin expression was significantly down-regulated in tumor tissues (P = 0.001), as compared with the para-tumorous tissues (Figure 3A and B). E-cadherin mRNA levels were negatively associated with HOTAIR expression in GC tissues (r^2 = -0.298, P = 0.016) (Figure 3C). HOTAIR silencing strikingly enhanced the expression of E-cadherin at transcript (Figure 3D and E) and protein (Figure 3F and G) levels in BGC-823 and SGC-7901 cells. These results demonstrated that HOTAIR promoted EMT in GC tissues and cell lines, and that HOTAIR over-expression was significantly associated with migration and invasion.

HOTAIR recruited EZH2 to inhibit the expression of E-cadherin

HOTAIR plays its role in the epigenetic regulation of gene expression mainly by binding to PRC2 complex. We firstly used RIP assay to investigate whether HOTAIR could bind to EZH2, which is the core catalytic component of the PRC2. The results showed that endogenous HOTAIR was abundant in the anti-EZH2 RIP fraction (Figure 4A), validating that HOTAIR can bind to EZH2 in GC cells. Then we conducted ChIP assay to determine whether HOTAIR regulates E-adherin *via* recruiting EZH2 in GC cell lines. ChIP assay demonstrated that knockdown of HOTAIR significantly reduced the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4B). The rescue experiments showed that up-regulation of HOTAIR increased the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4C). These results indicated that HOTAIR inhibits E-cadherin expression partly through combining with EZH2.

DISCUSSION

LncRNAs are non-protein-coding transcripts > 200 nucleotides. Dysregulation of lncRNAs has been found in GC cells and their aberrant expression is correlated with tumorigenesis, metastasis, prognosis or diagnosis[20,21].

We found that HOTAIR was markedly up-regulated in GC tissues, which was correlated with the invasion of primary tumor, lymph node metastasis, and TNM stage. The ROC curve used for distinguishing between GC tissues and normal tissues showed that HOTAIR is a promising cancer biomarker. Univariate and multivariate Cox regression analyses demonstrated that HOTAIR is a valuable prognostic factor independent of major clinicopathological features. We also found that decreased HOTAIR expression inhibited migration and invasion of tumor cells.

To clarify the molecular mechanism of HOTAIR action contributing to GC metastasis, we also investigated potential target genes involved in cell migration and invasion. Previous studies have revealed that the expression of epithelial markers (such as E-cadherin) are increased following HOTAIR knockdown in GC cells[13,22,23]. Further mechanistic studies have shown that by recruiting and binding to PRC2, HOTAIR could epigenetically silence miR34a expression to promote GC cell EMT and metastasis[13]. However, there are still some issues that need to be clarified. For one thing, E-cadherin is a target gene of EZH2. Because EZH2 can mediate transcriptional silencing of E-cadherin through trimethylation of H3 lysine 27[24,25], and HOTAIR binds to EZH2[14]. Furthermore, a significantly negative correlation was found between HOTAIR and E-cadherin in GC tissues in the present study, and knockdown of EZH2 also up-regulated E-cadherin expression in GC cells as shown by another study[26]. So, why does HOTAIR not regulate expression of E-cadherin directly by binding to EZH2? We speculate that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin *via* EZH2 directly. To confirm this, we performed ChIP analysis in HOTAIR-silenced GC cell lines. Not unexpectedly, HOTAIR contributed to the regulation of E-cadherin *via* recruiting EZH2 and H3K27me3 to the E-cadherin promoter. Our findings provide additional insight into the mechanisms by which HOTAIR promotes GC migration and invasion.

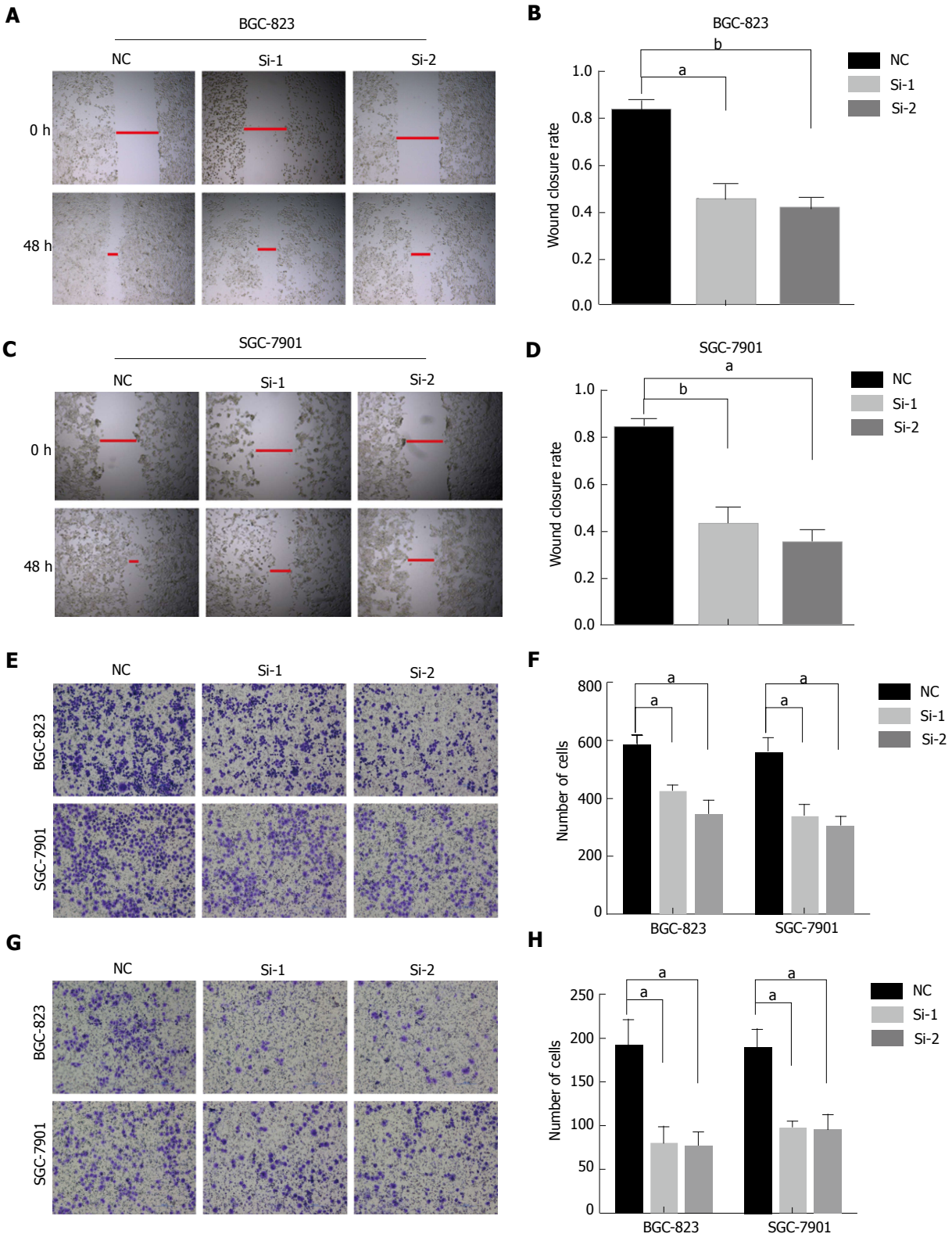


Figure 2 HOTAIR knockdown decreases migration and invasion of gastric cancerGC cells. A and B: HOTAIR knockdown samples exhibited slower scratch closure rate by the wound-healing detection in BGC-823 cells; C and B: Wound-healing assay in SGC-7901 cells with HOTAIR silencing; E and F: Representative pictures of cell migration across the membrane in BGC-823 and SGC-7901 cells with HOTAIR reduction; G and H: Representative pictures of Transwell invasion assay in BGC-823 and SGC-7901 cells with HOTAIR depletion. ($^{a}P < 0.05$, $^{b}P < 0.01$). GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA.

In conclusion, we demonstrated that HOTAIR was significantly up-regulated in GC tissues, and its overexpression was correlated with tumor progression and poor prognosis. We can distinguish between

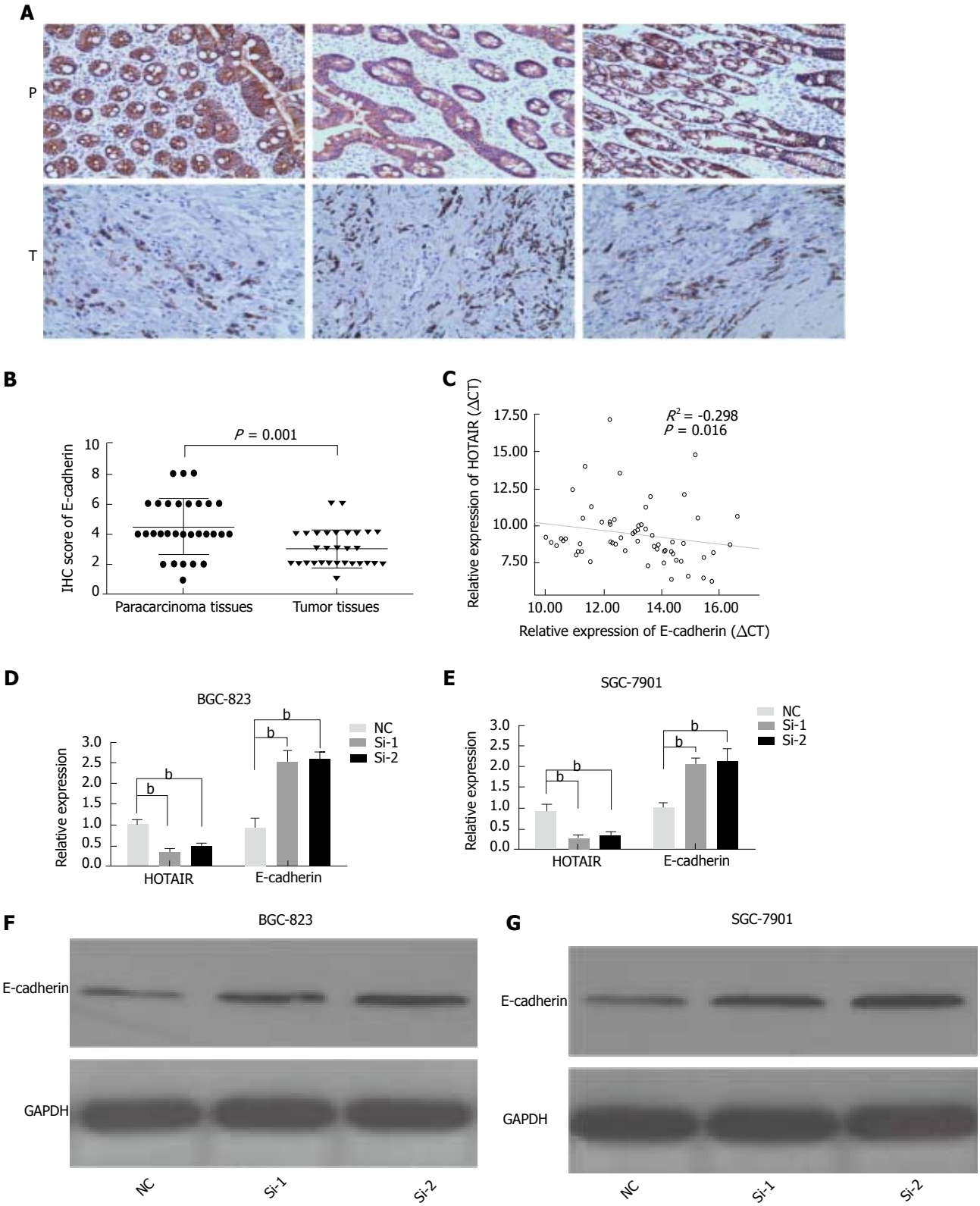


Figure 3 HOTAIR negatively correlates to E-cadherin expression in GC tissues and cell lines. A: E-cadherin immunostaining in para-carcinoma tissues (denoted as '*P*') and GC tissues (denoted as 'T'); B: Representative E-cadherin protein levels and scores in GC tissues and para-carcinoma tissues was analyzed by immunohistochemistry (n = 30); C: Negative correlation between E-cadherin mRNA levels and HOTAIR levels in 65 GC samples; D, E: Relative expression of HOTAIR and E-cadherin in BGC-823 and SGC-7901 cells treated with siRNA for 48 h; F, G: Western blot analysis of E-cadherin after HOTAIR-siRNA (Si1 and Si2) treatment for 48 h in BGC-823 and SGC-7901 cells. $^{a}P < 0.05$, $^{b}P < 0.01$. GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; si: Small interfering.

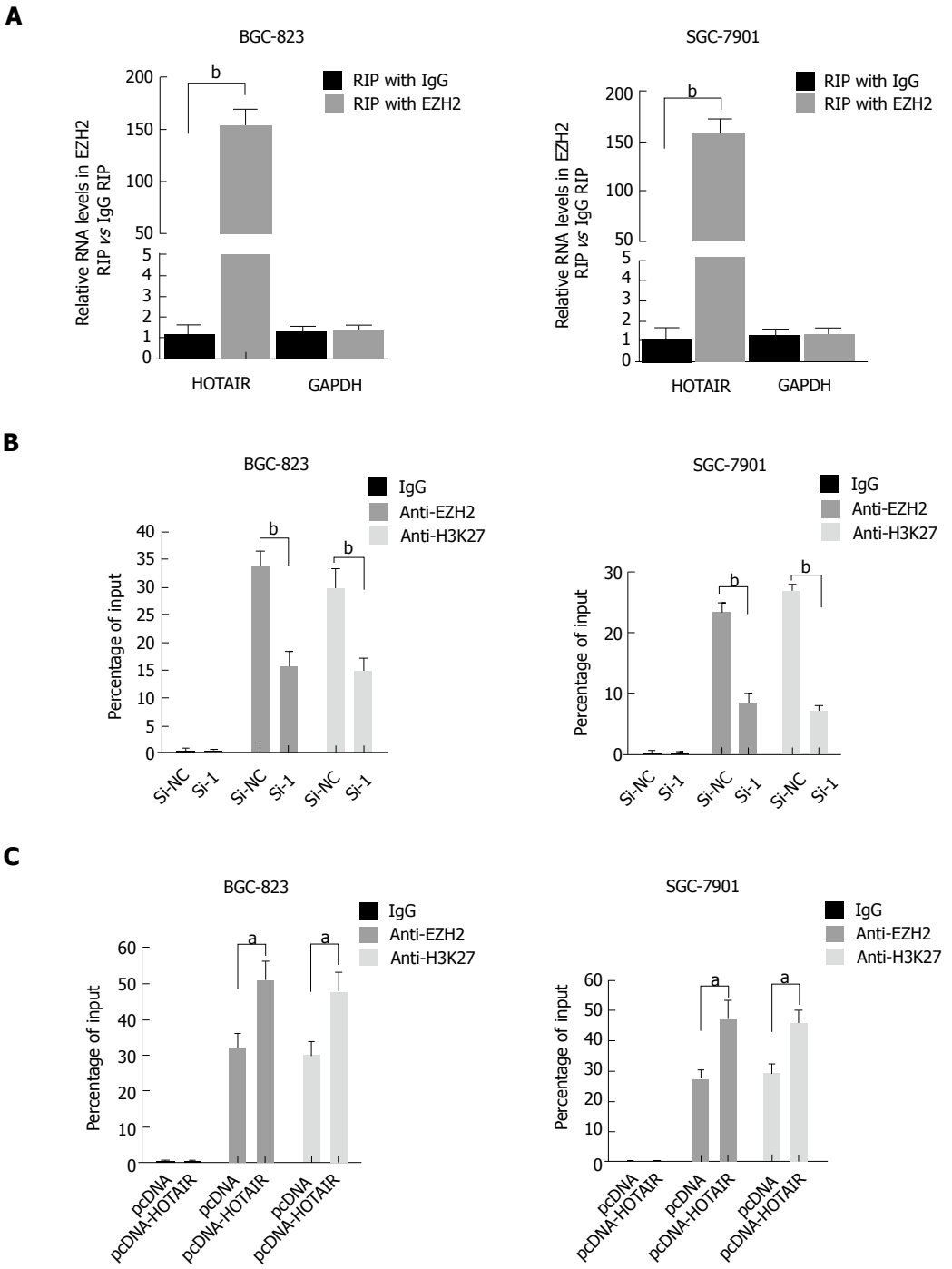


Figure 4 HOTAIR represses E-cadherin expression by associating with EZH2. A: RIP experiments were performed in BGC-823 and SGC-7901 cells, and HOTAIR levels were analyzed in co-precipitated RNA using qPCR. The fold-enrichment of HOTAIR in EZH2 RIP is relative to its matching IgG control RIP ($^{a}P < 0.05$, $^{b}P < 0.01$); B: ChIP of EZH2 and H3K27me3 in the E-cadherin promoter regions after siRNA treatment targeting si-NC or HOTAIR-siRNA-1 in BGC-823 and SGC-7901 cells. qPCR was performed to quantify ChIP assay results. Enrichment was quantified relative to input controls. Antibody directed against IgG was used as a negative control. Results are represented as the average ± SD based on three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$; C: ChIP of EZH2 and H3K27me3 in the E-cadherin promoter regions after transfected with empty vector (denoted as 'pcDNA') or pcDNA-HOTAIR in BGC-823 and SGC-7901 cells. qPCR was performed to quantify ChIP assay results. Enrichment was quantified relative to input controls. Results are represented as the average ± SD based on three independent experiments ($^{a}P < 0.05$, $^{b}P < 0.01$). ChIP: Chromatin immunoprecipitation; GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; qPCR: Quantitative polymerase chain reaction; RIP: RNA-binding protein immunoprecipitation; SD: Standard deviation; si: Small interfering.

cancerous and non-cancerous lesions based on the expression of HOTAIR. HOTAIR may regulate the invasive ability of GC cells, partially *via* EMT regulation. Our findings have provided new insight into the GC pathogenesis, which benefits diagnosis and therapy in cancer.

COMMENTS

Background

Gastric cancer (GC) is one of the most aggressive malignancies with high morbidity and mortality worldwide. Treatment options are limited due to the lack of knowledge of the molecular and genetic bases of gastric carcinogenesis. A deeper understanding of the molecular mechanisms of GC will shed light on its

pathogenesis, and identification of new biomarkers for diagnosis and prognosis may improve individualized treatment strategies in the future.

Research frontiers

Emerging evidence suggest that long non-coding (lnc)RNA may play a role in gastric carcinogenesis. HOX transcript antisense intergenic RNA (HOTAIR) is one of the well-documented lncRNAs, which is aberrantly expressed in several tumors and plays a crucial role in cancer development. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis has not yet been well investigated.

Innovations and breakthrough

The authors demonstrated that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin *via* EZH2 directly. Their findings have provided additional insight into the mechanisms of gastric carcinogenesis.

Applications

The results from this study provide novel clues for further investigation of HOTAIR as a potential biomarker and therapeutic target for GC.

Terminology

RNA-binding protein immunoprecipitation assay was used for the analysis of EZH2 interactions with HOTAIR. ChIP assay was performed to investigate direct interactions between EZH2 and E-cadherin.

Peer-review

The study aimed to investigate the clinical significance and the mechanism behind lncRNA HOTAIR in GC. The overall study is solid and well designed. The results are consistent with the proposed hypothesis.

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ORIGINAL ARTICLE

Basic Study

Ca^{2+}/calmodulin-dependent protein kinase II regulates colon cancer proliferation and migration *via* ERK1/2 and p38 pathways

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Abstract

AIM

To investigate the role of calmodulin-dependent protein kinase Ⅱ (CaMKⅡ) in colon cancer growth, migration and invasion.

METHODS

CaMKⅡ expression in colon cancer and paracancerous tissues was evaluated *via* immunochemistry. Transcriptional and posttranscriptional levels of CaMKⅡin tissue samples and MMP2, MMP9 and TIMP-1 expression in the human colon cancer cell line HCT116 were assessed by qRT-PCR and western blot. Cell proliferation was detected with the MTT assay. Cancer cell migration and invasion were investigated with the Transwell culture system and wound-healing assay.

RESULTS

We first demonstrated that CaMKⅡ was over-expressed in human colon cancers and was associated with cancer differentiation. In the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. Furthermore, KN93 also significantly

prohibited HCT116 cell migration and invasion. The specific inhibition of ERK1/2 or p38 decreased the proliferation and migration of colon cancer cells.

CONCLUSION
Our findings highlight CaMKⅡ as a potential critical mediator in human colon tumor development and metastasis.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II; Colon cancer; Proliferation; Migration

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Core tip: In the present study, we demonstrated that calmodulin-dependent protein kinase Ⅱ (CaMKⅡ) was over-expressed in human colon cancers and was associated with cancer differentiation. We investigated the role of CaMKII in human colon cancer proliferation and migration. The results revealed that in the human colon cancer cell line HCT116, the CaMKⅡ-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. KN93 also significantly prohibited colon cancer cell migration and invasion. Additionally, we found that ERK1/2 and p38 were the targets of CaMKⅡ regulation. These findings highlight CaMKⅡ as a potential critical mediator in human colon tumor development and metastasis.

Chen W, An P, Quan XJ, Zhang J, Zhou ZY, Zou LP, Luo HS. Ca^{2+}/calmodulin-dependent protein kinase II regulates colon cancer proliferation and migration *via* ERK1/2 and p38 pathways. *World J Gastroenterol* 2017; 23(33): 6111-6118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6111.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6111

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer death globally. Researchers estimate that roughly 1.4 million new cases of CRC will be diagnosed and 694000 deaths will result from CRC per year[1]. Despite improvements in multimodal anticancer strategies, the prognosis of advanced CRC is still poor, with 5-year survival rates for stage III and stage IV colon cancer at 65.4% and 12.8%, respectively[2]. A detailed understanding of the biological processes that modulate the progression and metastasis of CRC may provide new molecular pathogenesis targets for CRC diagnosis and benefit anti-tumor therapy.

The calcium ion (Ca^{2+}) is a ubiquitous intracellular signal responsible for a broad range of cellular events, such as cell growth, cytoskeletal organization, regulation of synaptic transmission, and Ca^{2+} homeostasis[3]. The Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKⅠ, CaMKⅡ and CaMKⅣ) are multifunctional serine/threonine kinases whose activity is activated upon Ca^{2+}/CaM binding[4]. The upstream CaMK kinases (CaMKKs) phosphorylate a critical Thr 200 in the activation-loop to activate CaMKIV, whereas CaMKII is fully activated by the autophosphorylation of its own Thr 286[5]. Studies have revealed that the calcium signal is involved in cancer cell proliferation, invasion, tumor growth and metastasis[6-9]. However, fewer studies have focused on CaMKII, one of the most important sensors and regulators of the Ca^{2+} signal, in digestive cancers, especially in CRC.

In the present study, we first demonstrated that CaMKII was over-expressed in human colon cancers and was associated with cancer differentiation. We further sought to investigate the role of CaMKII in human colon cancer proliferation and migration. The results revealed that in the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. KN93 also significantly prohibits HCT116 cell migration and invasion. Additionally, we determined that CaMKII inhibition decreased the phosphorylation of ERK1/2 and p38. The specific inhibition of ERK1/2 or p38 decreased the proliferation and migration of colon cancer cells. These findings highlight CaMKII as a potential critical mediator in human colon tumor development and metastasis.

MATERIALS AND METHODS

Reagents

Antibodies against CaMKIIα, ERK1/2, phospho (p)-ERK1/2, p38, p-p38, MMP2, MMP9, TIMP-1 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). KN-93 (2-[N-(2-Hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)] amino-N-(4- chlorocinnamyl)-N-methylbenzylamine), KN-92 (2-[N-(4-Methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), PD98059 and SB203580 were obtained from Calbiochem (La Jolla, CA, United States). Other chemicals of the highest purity were purchased from Sigma (St. Louis, MO, United States).

Tissue samples

Colon tissue samples were obtained from the Department of Gastrointestinal Surgery at Renmin Hospital of Wuhan University in accordance with local ethics committees. All samples were obtained from the primary colon sites of pretreatment cases of paracancerous lesions (n = 5), well-differentiated colon cancer tissues (n = 6) and poorly differentiated colon cancer tissues (n = 6). All samples were analyzed and assessed by two histological specialists working blindly.

Cell culture and treatment

The human colon cancer cell line HCT116 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured

in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD, United States) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Life Technologies, Rockville, MD, United States) at 37 ℃ in a humidified atmosphere with 5% CO_2. For conditioned media, 3 × 10^5 HCT116 cells were seeded in 6-well plates and then stimulated for the indicated amount of time.

Immunostaining

Immunohistochemistry (IHC) for CaMKII was performed on paraffin-embedded tissues from paracancerous tissues, well-differentiated colon cancers and poorly differentiated colon cancers. Specimens were fixed in a 4% formaldehyde solution for 12-48 h and embedded in paraffin. IHC was performed on 3.5 μm tissue sections on slides. Heat-induced epitope retrieval was carried out. For immunofluorescent staining for CaMKII, all slides were blocked with serum solution and incubated with the primary antibody overnight at 4 ℃. After rinsing again, the slides were incubated for 45 min at room temperature with secondary antibodies. Visualization was achieved with the DAB (3,3'-diaminobenzidine) detection kit, and the cell nuclei were counterstained using hematoxylin. After staining, five fields (at a magnification of × 100) were randomly selected in each slide. Staining was quantified with Image J software and evaluated by two investigators following the "blinded" principle.

MTT assay

Cell proliferation was measured with the MTT assay (ATCC, Manassas, VA, United States). A total of 5 × 10^3 cells were seeded at the well bottoms of 96-well culture plates and treated with TGFβ1 or conditioned medium from fibroblasts or colon cancer cells in triplicate. After treatment, MTT solution [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazoliumbromide] was added to the culture medium (0.5 mmol/L), and plates were incubated for 2 h at 37 ℃ with 5% CO_2. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 540 nm with a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, United States).

Western blot analysis

Protein from unstained paraffin-embedded samples was deparaffinized and rehydrated *via* successive washes in xylene/ethanol and water. After incubation in extraction buffer at 100 ℃ for 20 min followed by a 2 h incubation at 80 ℃, proteins were obtained by centrifugation. Whole cell lysates were prepared with RIPA lysis buffer containing protease and phosphatase inhibitor cocktail. Protein concentrations of cell lysates were determined with the bicinchoninic acid protein assay. A total of 30-40 μg of lysate were loaded onto a 10% SDS-PAGE gel and subjected to gel electrophoresis. Resolved proteins were transferred to a polyvinylidine fluoride membrane. Membranes were then blocked in 5% non-fat dry milk in Tris-buffered saline with Tween (TBS-T) for 1 h and then incubated with antibodies to GAPDH, CaMKIIα (Cell Signaling Technology), ERK1/2, phospho-ERK1/2, p38, p-p38, MMP2, MMP9 and TIMP-1 at 4 ℃ overnight. Membranes were washed with TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blotting bands were visualized with enhanced chemiluminescence. GAPDH was used as a loading control.

Quantitative real-time PCR

Cells were seeded at 10^6 cells per 10 cm dish and were allowed to grow to 80% confluency in complete media. Cells were removed with 0.025% ethylene-diaminetetraacetic acid and centrifuged for 5 min at 1100 rpm. Cell pellets were resuspended in 1 mL of TRIzol (Life Technologies, Carlsbad, CA, United States), and RNA was extracted according to the manufacturer's protocol. Total RNA in unstained paraffin-embedded samples was extracted with the RNeasy FFPE Kit (Qiagen, Hilden, Germany). The SuperScript First-Strand Kit (Life Technologies) was used to synthesize cDNA from 5 μg of total RNA. Quantitative PCR was established with the RT2 SYBR Green Flour FAST Mastermix (Qiagen, Venlo, the Netherlands) and run on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad). Data were analyzed with Bio-Rad CFX Manager 3.0 software and are shown as relative fold-changes.

For all PCR reactions, GAPDH was used as an endogenous control, and CT values were normalized to levels of GAPDH expression. Primers were designed with Integrated DNA Technologies PrimerQuest software (Coralville, IA, United States). Sequences used to analyze RNA expression include the following: CaMKII, Forward: 5'-GAG AGC ACC AAC ACC ACC ATC G-3' and Reverse: 5'-AGG CTG ACT CGT CGC CCA TCA GG-3'; MMP2, Forward: 5'-TCT CCT GAC ATT GAC CTT GGC-3' and Reverse: 5'-CAA GGT GCT GGC TGA GTA GAT C-3'; MMP9, Forward: 5'-TTG ACA GCG ACA AGA AGT GG-3' and Reverse: 5'-GCC ATT CAC GTC GTC CTT AT-3'; TIMP-1, Forward: 5'-CTT CTG GCA TCC TGT TGT TG-3' and Reverse: 5'-GGT ATA AGG TGG TCT GGT TG-3'; GAPDH, Forward: 5'-ACA GTC CAT GCC ATC ACT GCC-3' and Reverse: 5'-GCC TGC TTC ACC ACC TTC TTG-3'. The results are averaged from three independent experiments.

Migration and invasion assay

A 24-well Transwell plate (Fisher Scientific, Hampton, NH, United States) was used to measure migratory and invasive ability *in vitro*. A total of 2 × 10^5 HCT-116 cells were plated on the bottom of each well. For the migration assay, 1 × 10^5 colon cancer cells were plated in the top chamber with a non-coated membrane. For the invasion assay, 2 × 10^5 HCT-116 cells were seeded in the top chamber coated with Matrigel (BD Biosciences, San Jose, CA, United States). In both

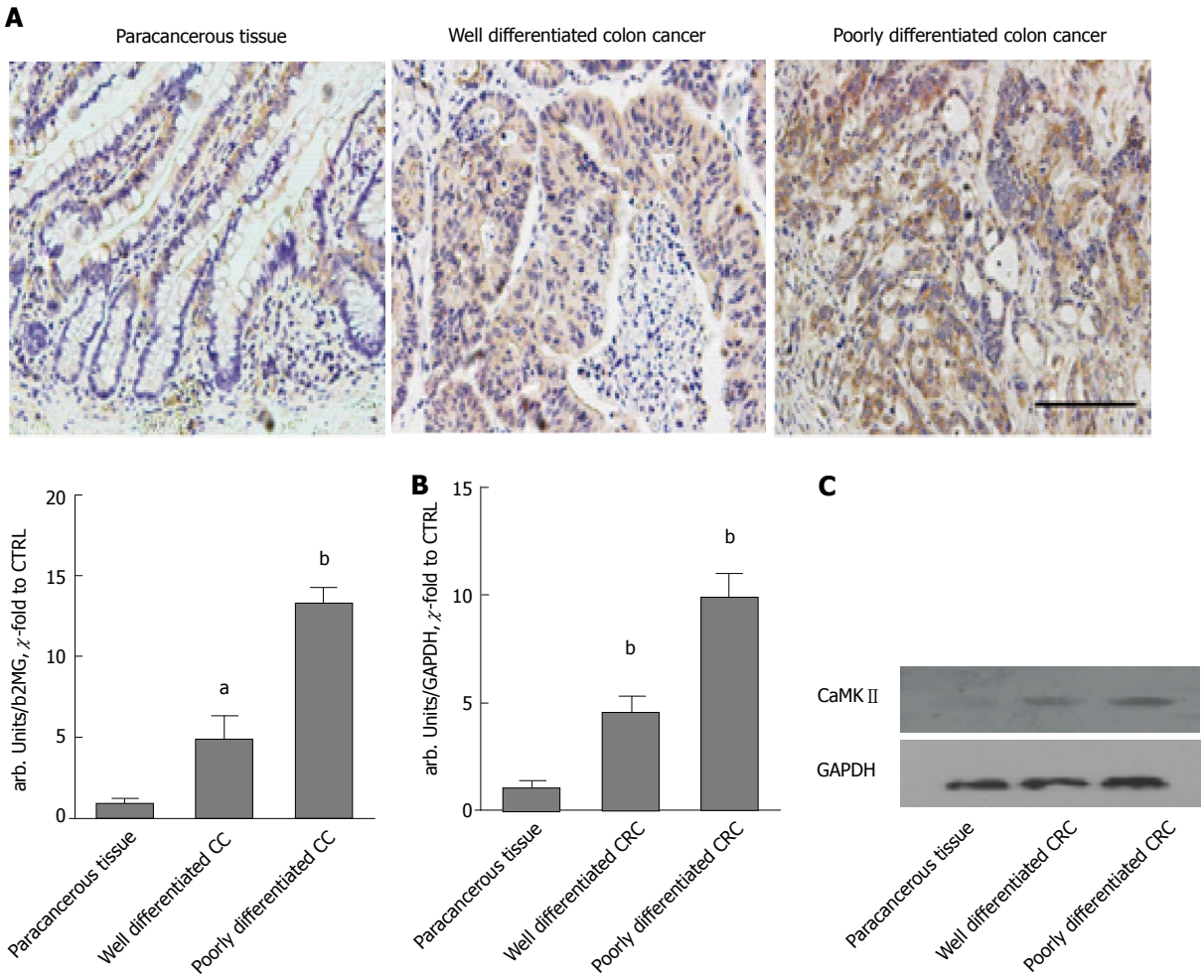


Figure 1 CaMKII over-expressed in colon cancer. A: Immunochemistry staining of CaMKII in paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples. Scale bar, 100 μm; B: qRT-PCR of CaMKII mRNA in paraffin-embedded paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples; C: Western blot of CaMKII protein in paraffin-embedded paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples. All data are presented as the mean ± standard error of at least three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* paracancerous tissue.

assays, cells were cultured in DMEM containing 10% FBS with the indicated treatment. After incubation at 37 °C for 24 h, migrated cells were fixed and stained with the Diff-Quik stain and counted in four random fields. The experiments were performed in triplicate wells, and each experiment was performed at least two or three times as indicated.

Wound-healing assay

A wound-healing assay was performed with 6-well plates. HCT116 cells were seeded at confluency 24 h before the experiment. The cell layers were carefully scratched with 200-mL sterile pipette tips and washed twice with fresh medium. The cells were photographed under a light microscope at × 200 magnification 24 h later.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as the mean ± SD. Differences between groups were calculated *via* one-way analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

CaMKII is over-expressed in colon cancers

The expression of CaMKⅡ was evaluated by IHC with a monoclonal CaMKⅡ antibody on sections obtained from formalin-fixed, paraffin-embedded samples of paracancerous tissue, well-differentiated colon cancer samples and poorly differentiated colon cancer samples. As expected, CaMKⅡ was significantly overexpressed in the poorly differentiated colon cancer samples and well-differentiated cancer samples compared to the paracancerous tissues (Figure 1A). A quantification assay suggested that the expression of CaMKⅡ was highest in poorly differentiated colon cancer tissues. We further evaluated CaMKII protein and mRNA levels in cancer and paracancerous

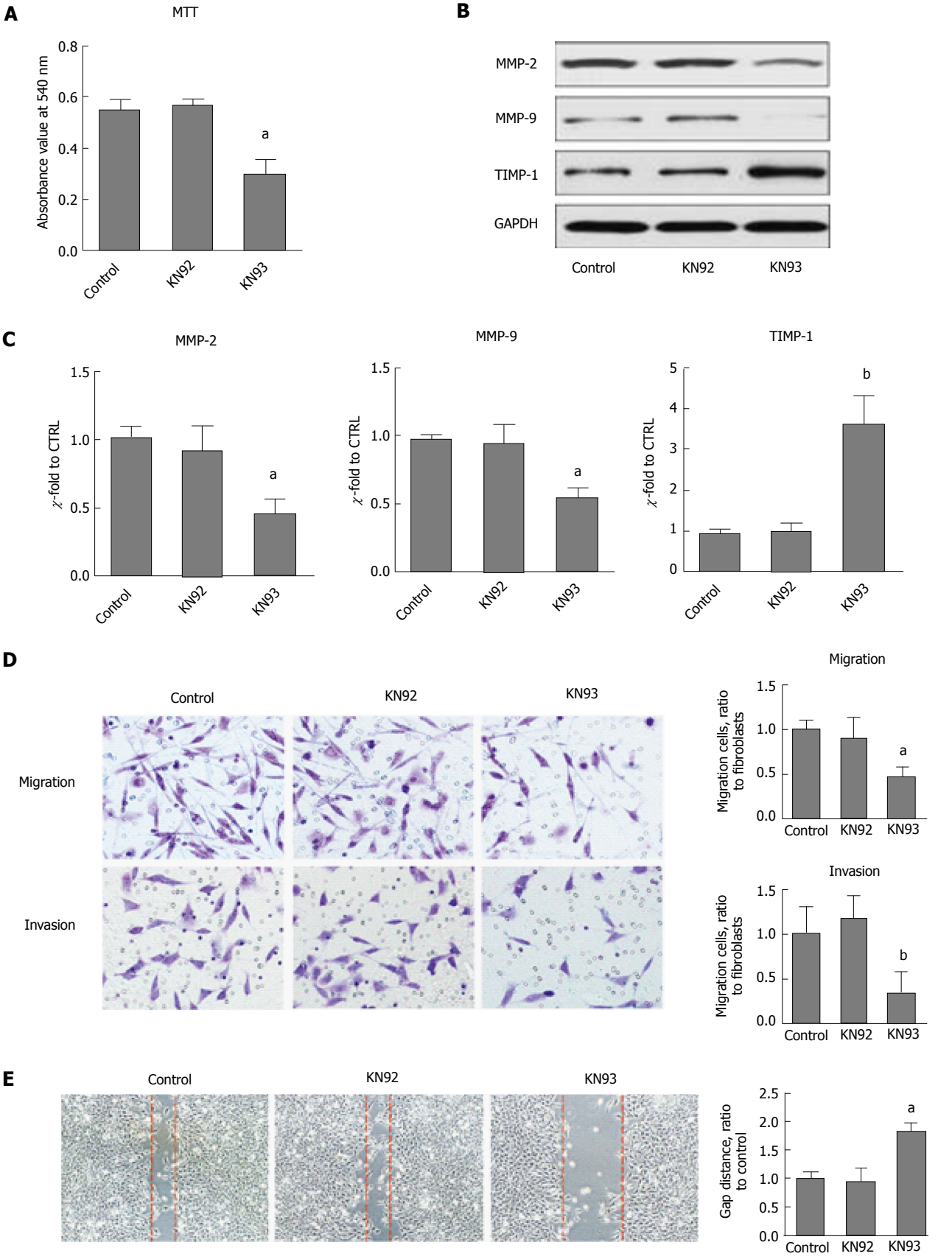


Figure 2 CaMKII is required for colon cancer proliferation and migration. Human colon cancer cells HCT116 were treated with or without KN93 or KN92 at 10 μmol/L for 24 h. A: MTT analysis for cell proliferation; B: Western blot of MMP2, MMP9 and TIMP-1; C: qRT-PCR assay of MMP2, MMP9 and TIMP-1; D: Immigration and invasion analysis by T transwell assay; E: Wound-healing test. All data are presented as the mean ± standard error SE of at least three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

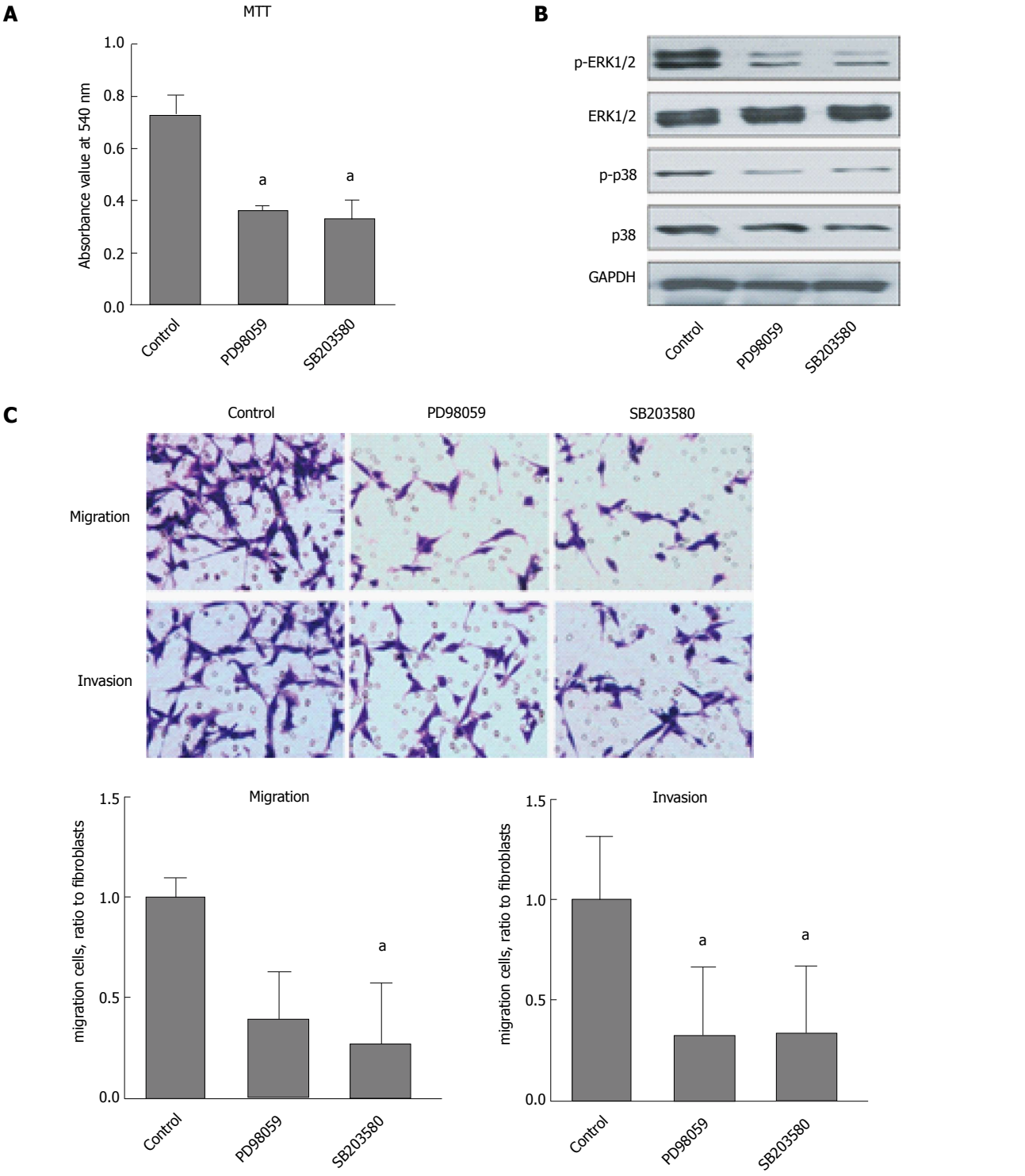


Figure 3 CaMKII regulates colon cancer cells proliferation and migration *via* ERK1/2- and p38-dependent pathways. Human colon cancer cells HCT116 were treated with or without PD98059 (50 μmol/L) or SB203580 (10 μmol/L) for 24 h. A: MTT assay for cell proliferation; B: Western blot analysis of ERK1/2, phosphor-ERK1/2, p38 and phosphor-p38; C: Immigration and invasion analysis by T transwell assay. All data are presented as the mean ± standard error SE of at least three independent experiments. $^{a}P < 0.05$, *vs* control group.

tissues. The results were similar to the findings of the above studies (Figure 1B and C). We deduced that CaMKII may be a potential regulator in colon cancer development.

CaMKII is required for colon cancer proliferation and migration

CaMKII has been shown to drive cancer growth and metastasis. To ascertain whether CaMKII regulates

colon cancer proliferation and migration, the CaMKII specific inhibitor KN93 was selected. Human colon cancer HCT116 cells were treated with KN93, and its inactive analogue, KN92, was used as an additional control. We found that KN93 inhibited colon cancer cell proliferation (Figure 2A). Importantly, in migration and invasion experiments, the motility of HCT116 cells was significantly attenuated (Figure 2B and C). Furthermore, CaMKII inhibition decreased MMP2 and MMP9 expression at both transcriptional and posttranscriptional levels. The opposite results were detected for TIMP-1 expression (Figure 2D and E). Thus, we concluded that CaMKII is required for colon cancer cell proliferation and migration.

CaMKII regulates colon cancer cell proliferation and migration via ERK1/2 and p38-dependent pathways

We further investigated the molecular mechanism of CaMKII regulation in human colon cancer cells. As shown in Figure 3, KN93, but not KN92, decreased the phosphorylation of ERK1/2 and p38, which are involved in colon cancer development. Pretreatment with specific inhibitors of ERK1/2 or p38 (PD98059, SB203580) decreased HCT116 cell proliferation, migration and invasion. These data demonstrated that the effects of CaMKII on human colon cancer are ERK1/2- and p38-dependent.

Taken together, these results indicate that CaMKII is an important regulator of human colon cancer growth and migration.

DISCUSSION

It is widely accepted that intracellular Ca^{2+} signaling pathways regulate various biological events, including cell proliferation, motility, activation and differentiation, during cancer development[10,11]. In the present study, we demonstrated that CaMKII is over-expressed in colon cancers and highly expressed in poorly differentiated colon tumors. We highlight the role of CaMKII in colon cancer growth and metastasis.

CaMKII inhibitors can inhibit CaMKII activity by interacting with the Ca^{2+}/CaM-binding site or interfering with its catalytic activities. Previous studies revealed that CaMKII-specific inhibitors such as KN-62, KN-93, and autocamtide 2-related inhibitory peptide inhibit CaMKII-dependent processes in tumor and normal cells, causing cell cycle arrest, cellular apoptosis, or the inhibition of cell proliferation[12-14].

Some studies have implicated CaMKII as an important player in cancer cell proliferation. CaMKII inhibition decreased ovarian cancer cell motility and decreased tumor growth and metastasis[15]. The *in vivo* administration of KN-93 to mice xenografted with human osteosarcoma cells significantly decreased intratibial and subcutaneous tumor growth[16]. In CRC, we demonstrated that CaMKII inhibition strongly inhibited human colon cancer cell proliferation (Figure 2A).

Recent studies support the important role of CaMKII in cancer invasion and metastasis. The up-regulation of CaMKIIα was found in primary osteosarcoma tissues from patients and in aggressive osteosarcoma cell lines[17]. CaMKIIα depletion decreased motility and invasion, whereas CaMKIIα over-expression increased the tumorigenic properties of osteosarcoma cells *in vitro*. Our current results revealed the important role of CaMKII in colon cancer migration and invasion. KN93 dramatically decreased the expression of MMP2 and MMP9, increased TIMP-1 levels and prohibited the invasive motility of colon cancer cells (Figure 2). CaMKII is a critical regulator of colon cancer progression.

The MAPK pathway is one of the important downstream targets in CaMKII regulation[4]. CaMKII directly or indirectly up-regulated multiple signaling pathways, such as ERK1/2, AKT1 and β-catenin, and is involved in regulating the survival and proliferation of non-small cell lung cancer cells[6]. We further investigated whether ERK1/2 and p38 were involved in CaMKII pathways. Our data showed that the inhibition of CaMKII by KN93 significantly decreased ERK1/2 and p38 phosphorylation (Figure 3). The specific inhibition of ERK1/2 or p38 decreased the proliferation, migration and invasion of HCT116 cells. Therefore, CaMKII mediates colon cancer growth and migration *via* ERK1/2- and p38-dependent pathways.

Taken together, the current study provided evidence for CaMKII as a pivotal regulator of colon cancer cell proliferation and migration. CaMKII is a potentially important target in CRC treatment.

COMMENTS

Background

Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) is one of the most important sensors and regulators of the Ca^{2+} signal which is involved in cancer cell proliferation, invasion, tumor growth and metastasis. Despite the critical role of CaMKII, fewer studies focus on it in digestive cancers, especially in colorectal cancer.

Research frontiers

Recently, studies have revealed CaMKII is an effective controller in ovarian cancer and osteosarcoma cell motility, cell growth and metastasis.

Innovations and breakthroughs

In the present study, the authors demonstrated that CaMKII was over-expressed in human colon cancers and was associated with cancer differentiation. Meanwhile, in the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. CaMKII inhibition also significantly prohibited colon cancer cell migration and invasion. Additionally, ERK1/2 and p38 were the targets of CaMKII regulation.

Applications

These findings highlight CaMKII as a potential critical mediator in human colon tumor development and metastasis. Its relationship to colon cancer

differentiation indicates CaMKII a potential target for clinical diagnosis and prognosis.

Terminology

CaMKII is a ubiquitous serine/threonine protein kinase. Autophosphorylation of CaMKII at threonine 286 switches the kinase from Ca^{2+}-dependent to Ca^{2+}-independent activity and leads to sustained CaMKII auto-activation. More importantly, this activation is thought to phosphorylate numerous different proteins and regulate diverse signaling pathways.

Peer-review

This is a brief study concerning the role of Ca^{2+}/calmodulin-dependent protein kinase II involved in invasion and migration of the colon cancer. The study is interesting and topical. The experiments were well designed. The manuscript is clear and structured very well.

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ORIGINAL ARTICLE

Basic Study

Aberrant DNA-PKcs and ERGIC1 expression may be involved in initiation of gastric cancer

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Abstract

AIM

To investigate the molecular mechanisms of gastric carcinogenesis.

METHODS

We used label-free quantification technology integrated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify differentially expressed proteins in 160 specimens of normal gastric mucosa, gastric mucosa with mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal gastric cancer (GC) collected at the Second Hospital of Lanzhou University from 2010 to 2015. Immunohistochemistry was used to verify the differentially expressed proteins detected by LC-MS/MS.

RESULTS

With a threshold of a 1.2-fold change and a *P*-value

< 0.05 between mild dysplasia, moderate dysplasia, severe dysplasia or early mucosal GC and matched normal gastric mucosa tissues, proteomic analysis identified 365 significantly differentially expressed proteins. ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by immunohistochemistry were consistent with the LC-MS/MS results.

CONCLUSION

The results suggest that aberrant ERGIC1 and DNA-PKcs expression may be involved in GC initiation.

Key words: DNA-PKcs; ERGIC1; Dysplasia; Proteomics; Gastric cancer

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Core tip: Using label-free combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), the expression of 365 proteins based on the tendency of fold change was revealed to be statistically different between the various stages of gastric cancer (GC) initiation. Furthermore, we observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by immunohistochemistry were consistent with the LC-MS/MS results. These data indicate that abnormal ERGIC1 and DNA-PKcs expression may play an important role in GC initiation.

Wang FR, Wei YC, Han ZJ, He WT, Guan XY, Chen H, Li YM. Aberrant DNA-PKcs and ERGIC1 expression may be involved in initiation of gastric cancer. *World J Gastroenterol* 2017; 23(33): 6119-6127 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6119.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6119

INTRODUCTION

Gastric cancer (GC) accounts for a large quantity of cancer-related deaths[1]. Over the decades, the prognosis of GC patients has improved, while overall survival rate of patients with GC has not been improved significantly yet, especially for advanced GC patients[2-4], which might be attributed to the poor understanding of mechanisms underlying the initiation and progression of GC. It is widely accepted that GC is a complex, heterogeneous, and multistep disease caused by various factors encompassing *Helicobacter pylori* infection, heredity, living habits, *etc*., which trigger series of molecular alterations, such as inactivation of tumor suppressor genes, activation of oncogenes, telomerase activation, DNA damage and even genome instability[5-8]. DNA-PKcs is the catalytic subunit of the DNA-dependent protein kinase and can combine with the Ku 70/Ku 80 heterodimer (Ku 70/80) into the DNA-dependent protein kinase (DNA-PK), which plays a crucial role in the activation of the non-homologous end joining (NHEJ) pathway[9,10]. Moreover, ERGIC1 has been identified as a cycling protein and participates in membrane trafficking and selective transport of cargo between the endoplasmic reticulum (ER), the intermediate compartment (ERGIC), and the Golgi apparatus in HepG2 cells, suggesting a possible role in protein secretion out of the ER[11].

Currently, high-throughput sequencing technologies, such as microarray assay and next-generation sequencing, have helped researchers obtain much more insight into molecular alterations[12]. These new tools have given a huge impetus to the discovery of novel intracellular molecular pathways and molecular subtypes of GC. For instance, some researchers have recommended that GC should be classified into Epstein-Barr virus-positive subtype, microsatellite instability subtype, genomically stable subtype, and chromosomally unstable subtype on the basis of the spectra of genetic alterations related with relevant clinical features, which is a totally new classification method[7,12].

However, it has been reported that GC is characterized by subtype heterogeneity and a lack of consistent genomic alterations across different individuals[13]. Furthermore, due to different translation regulation, posttranslational modifications and stability of proteins, genetic conditions cannot always provide reliable protein expression patterns, suggesting that it may be not sufficient to take advantage of the gene sequence-targeted tools to explore the molecular mechanisms underlying the GC initiation and progression[14]. Thus, proteomics has been thrust into the spotlight for its accurate and direct presentation of protein expression patterns that can provide much more information about the cellular function or dysfunction compared with genetic analysis[15]. It has been widely accepted that GC initiation can be divided into five stages including normal gastric mucosa, atrophic gastritis with mild dysplasia, atrophic gastritis with moderate dysplasia, atrophic gastritis with severe dysplasia, and mucosal GC. To date, abnormal expression of many proteins has been reported to be involved in GC carcinogenesis in several GC proteomic studies[12]. However, all of the previous studies only focused on identifying the differences between normal gastric epithelial tissues and GC rather than investigating the differences of protein among mild dysplasia, moderate dysplasia, severe dysplasia, and mucosal GC, which may contribute to more accurate insight into the molecular mechanisms underlying GC initiation. Thus, in this study we collected specimens of normal gastric mucosa, gastric mucosa with mild

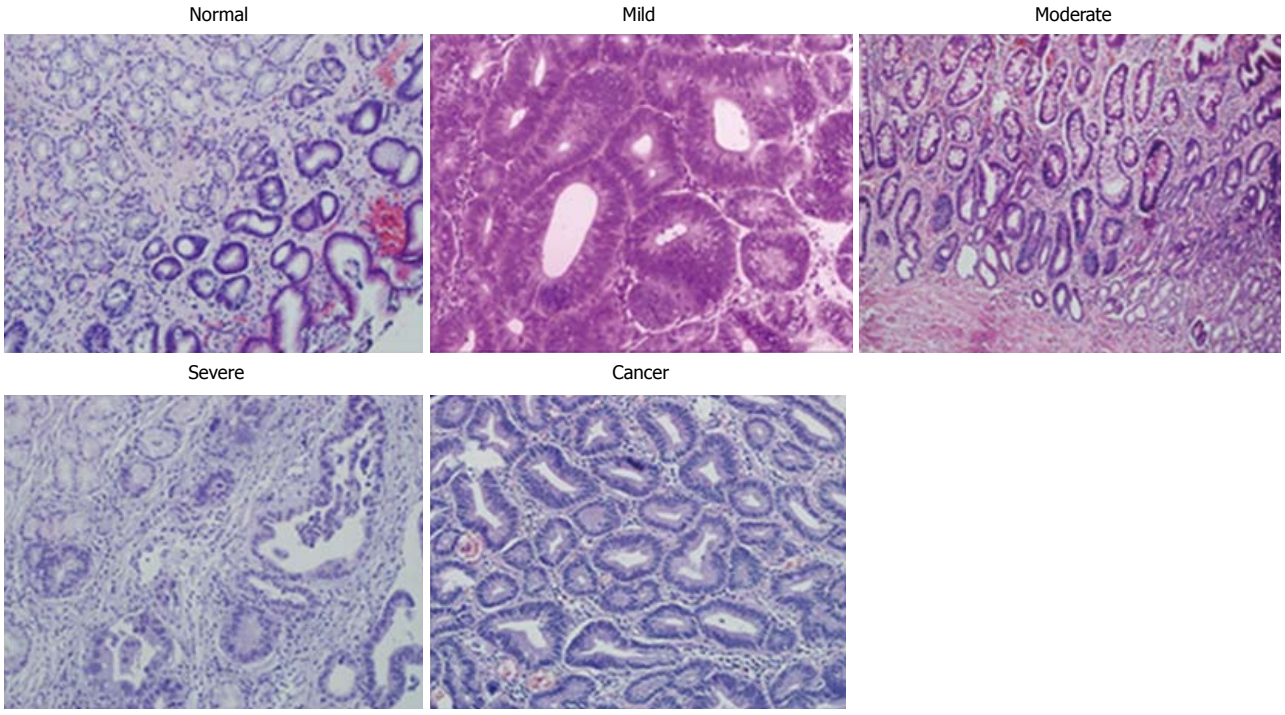


Figure 1 Representative graphs of HE staining (20 ×) of various gastric tissues including dysplasia.

Table 1 Basic demographic data of the study subjects

Group	*n*	Age (range) (yr)	Male/female
Healthy	30	43 (38-51)	16/14
Mild dysplasia	30	54 (42-67)	22/18
Moderate dysplasia	30	58 (40-65)	14/16
Severe dysplasia	30	63 (43-76)	19/11
Gastric cancer	40	61 (39-68)	23/17

dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC, and utilized label-free quantification technology integrated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify differentially expressed proteins in the five kinds of specimens in order to determine the molecular mechanisms underlying GC initiation, which may promote potential preventive and therapeutic strategies for GC. We also focused on ERGIC1 and DNA-PKcs proteins for their inverse expression patterns identified in the proteomic analysis.

MATERIALS AND METHODS

Clinical specimens

Clinical specimens were collected at the Second Hospital of Lanzhou University from 2010 to 2015. A total of 160 patients were enrolled in this study, including 30 patients with a normal gastric mucosa, 30 patients with atrophic gastritis accompanied by mild dysplasia, 30 patients with atrophic gastritis accompanied by moderate dysplasia, 30 patients with atrophic gastritis accompanied by severe dysplasia, and 40 patients with early intestinal-type GC (Table 1 and Figure 1). All patients underwent upper gastroduodenoscopy or gastrectomy and were pathologically reviewed and examined. This study was approved ethically by the Second Hospital of Lanzhou University. All of the patients provided written informed consent to participate in the study.

Proteomic analysis of gastric samples

Sample preparation: The five kinds of formalin-fixed, paraffin-embedded (FFPE) specimens were divided into three groups, respectively. Each FFPE specimen was cut into six pieces at a thickness of at least 4 μm, in order to ensure that the protein quantity to detect was no less than 40 μg. Fifteen groups of FFPE specimens were deposited in liquid nitrogen after conventional dewaxing, gradient alcohol, and hydration for proteomic analysis.

After 2 μL of SDT buffer was added, the sample was ground with a pestle and a mortar. The homogenate was sonicated and then boiled for 15 min. After centrifugation at 14000 *g* for 40 min, the supernatant was filtered with 0.22 μm filters. The protein content of the filtrate was quantified with the BCA Protein Assay Kit (Bio-Rad, the United States), and the sample was then stored at -80 ℃.

SDS-PAGE separation: Samples (40 μg proteins) were mixed with 5 × loading buffer and boiled for 5 min. The proteins were separated on a 12.5% SDS-PAGE gel at a constant current of 14 mA for 90 min. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining.

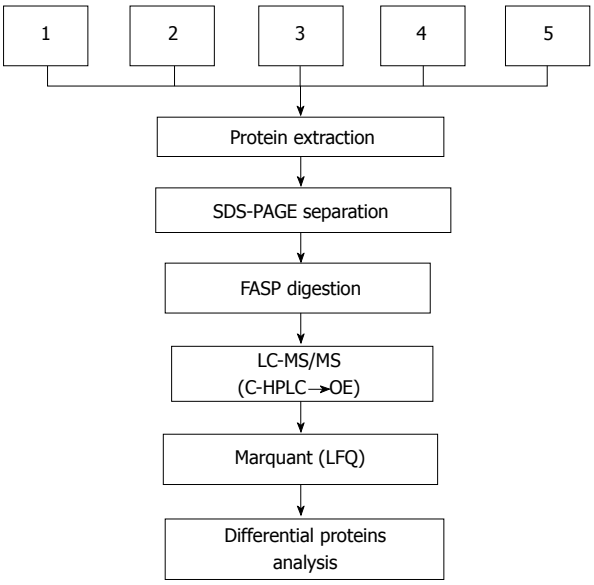


Figure 2 Outline of proteomic experimental workflow. SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; C-HPLC: Capillary-high performance liquid chromatography; QE: Q-exactive; LFQ: Label free quantitative.

Filter-aided sample preparation (FASP digestion): A total of 100 μg proteins for each sample were added into 30 μL SDT buffer (4% SDS, 100 mmol/L DTT, 150 mmol/L Tris-HCl, pH 8.0). The detergent, DTT and other low-molecular-weight components were removed using UA buffer (8 mol/L urea, 150 mmol/L Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 10 kDa). Then, 100 μL of iodoacetamide (100 mmol/L IAA in UA buffer) was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μL of UA buffer three times and then 100 μL of 25 mmol/L NH_4HCO_3 buffer twice. Finally, the protein suspensions were digested with 4 μg of trypsin (Promega) in 40 μL of 25 mmol/L NH_4HCO_3 buffer overnight at 37 ℃, and the resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 Cartridges (Empore SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma), concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1% (v/v) formic acid. The peptide content was estimated by UV light spectral density at 280 nm using an extinction coefficient of 1.1 of 0.1% (g/L) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

Mass spectrometry: Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18) connected to a C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 300 NL/min controlled by IntelliFlow technology (one hour gradient: 0%-35% buffer B for 50 min, 35%-100% buffer B for 5 min, and held in 100% buffer B for 5 min).

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 120 min. The mass spectrometer was operated in positive ion mode. MS data were acquired using a data-dependent top10 method that dynamically chooses the most abundant precursor ions from the survey scan (300-1800 m/z) for high energy collision dissociation (HCD) fragmentation. Automatic gain control target was set at 3×10^6, and maximum inject time set at 10 ms. Dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70000 at m/z 200. The resolution for HCD spectra was set at 17500 at m/z 200, and isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled (Figure 2).

Data analysis: The MS data were analyzed using MaxQuant software version 1.3.0.5 (Max Planck Institute of Biochemistry in Martinsried, Germany).

Immunohistochemistry

Primary antibodies were used in this study as follows: mouse monoclonal anti-human DNA-PKcs (GR190339-3; Abcam, Cambridge, United Kingdom) and anti-human ERGIC1 (GR104221-6; Anbobio, San Francisco, CA, the United States). The anti-human DNA-PKcs antibody was diluted 1:400, and the anti-human ERGIC1 antibody diluted 1:3500. The paraffin sections were mounted on slides, dewaxed in xylene, and sequentially dehydrated in 100%, 95% and 85% ethanol. The sections were stained using the PV-6000 Polymer Detection System (Zhongshan Goldenbridge, Beijing, China). Initially, endogenous peroxidase was blocked using 3% H_2O_2. After the sections were incubated with the primary antibody overnight at 4 ℃, they were washed with PBS and then incubated with polymer helper for 30 min and poly peroxidase-anti-mouse/rabbit IgG for 30 min. After that, the sections were washed with PBS, and then incubated with 3,3-diaminobenzidine (DAB, Zhongshan Goldenbridge). The sections incubated with PBS without primary antibodies were used as negative controls. Finally, the sections were counterstained with hematoxylin and examined under a light microscope. The cells that were stained a yellow or brown color in the nucleus and/or cytoplasm were defined as positive. Five randomly selected fields per section were analyzed. In a ran-

Table 2 Results of DNA-PKcs and ERGIC1 from proteomic analysis

Protein ID	Gene	Protein	Fold change			
			Mild/normal	Moderate/normal	Severe/normal	Cancer/normal
P78527	*PRKDC*	DNA-dependent protein kinase catalytic subunit	1.12	1.54	1.60	2.30
Q969X5	*ERGIC1*	Endoplasmic reticulum-Golgi intermediate compartment protein 1	1.18	0.84	0.73	0.46

Table 3 Results of DNA-PKcs and ERGIC1 from immunohistochemistry

Group	*n*	DNA-PKcs				*n*	ERGIC1			
		-	+	+ +	+ + +		-	+	+ +	+ + +
Normal	30	12	18	0	0	30	2	2	2	24
Mild	30	14	16	0	0	30	2	3	3	22
Moderate	30	5	3	23	0	30	2	3	25	0
Severe	30	3	2	25	0	30	5	5	20	0
Cancer	30	0	2	6	22	40	9	27	4	0

domly selected field from representative areas, the immunoreactive cells among 100 cells were assessed and quantified by percentage. Then, the average percentage of the five fields was used to assess the area of immunostaining (0 = 0%-5%; 1 = 6%-25%; 2 = 26%-50%; 3 =51%-75%; 4 = 76%-100%). In addition, the intensity of immunostaining was also semi-quantitatively assessed (0 = negative, 1 = weak, 2 = moderate, 3 = intense). Then, the scores from the "area × intensity" were calculated and used to describe the overall staining intensity that semi-quantitatively reflects the overall expression levels of proteins. The overall staining intensity was scored as follows: negative (-): 0-2; mild (+): 3-5; moderate (++): 6-8; and strong (+++): 9-12. All the sections were assessed and scored by two pathologists who were blinded to the clinical data of the patients.

Statistical analysis

All the data are expressed as mean ± SD or percentage. The χ^2 test (SPSS v.16.0 for Windows; SPSS, Inc, Chicago, IL, United States) was used to evaluate the difference between categorical variables. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Differentially expressed proteins detected by LC-MS/MS

To identify differentially expressed proteins in normal gastric mucosa, atrophic gastritis with mild dysplasia, atrophic gastritis with moderate dysplasia, atrophic gastritis with severe dysplasia, and early mucosal GC, we performed a study with label-free quantification technology integrated with LC-MS/MS. A total of 17443 peptides matching 2807 proteins were identified from tissue analysis. The expression of a total of 365 proteins, with a threshold of a 1.2-fold change and a *P*-value less than 0.05 between mild dysplasia, moderate dysplasia, severe dysplasia or early mucosal GC and matched normal gastric mucosa tissues, were considered to be statistically significantly different.

Aberrant expression of DNA-PKcs and ERGIC1 revealed by proteomic analysis

The fold changes in mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC compared to matched normal gastric mucosa tissues were 1.12, 1.54, 1.6, and 2.3 for DNA-PKcs, and 1.18, 0.84, 0.73, and 0.46 for ERGIC1, respectively (Table 2).

Aberrant expression of DNA-PKcs and ERGIC1 revealed by immunohistochemistry

In order to confirm the results of DNA-PKcs and ERGC1 from proteomic analysis, we further examined the expression of DNA-PKcs and ERGC1 by immunohistochemistry. The strongly positive (+++) rates in normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC were 80% (24/30), 73% (22/30), 0% (0/30), 0% (0/30), and 0% (0/40), respectively, for ERGC1, and for DNA-PKcs the strongly positive (+++) staining was observed only in the sections of early mucosal GC. The moderately positive (++) rates in normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC were 6.6% (2/30), 6.6% (3/30), 83% (25/30), 67% (20/30), and 10% (4/40), respectively, for ERGC1, and 0% (0/30), 0% (0/30), 76%(23/30), 83% (25/30), and 20% (6/30), respectively, for DNA-PKcs (Figures 3 and 4). The details on the rates of different-grade overall staining intensity are presented in Table 3.

In general, the average immunohistochemistry scores of the DNA-PKcs protein significantly increased, while those of ERGC1 protein significantly decreased along the sequence of normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC.

DISCUSSION

Using label-free quantification technology combined with LC-MC/MC, the expression of 365 proteins based on the tendency of fold change was revealed to be

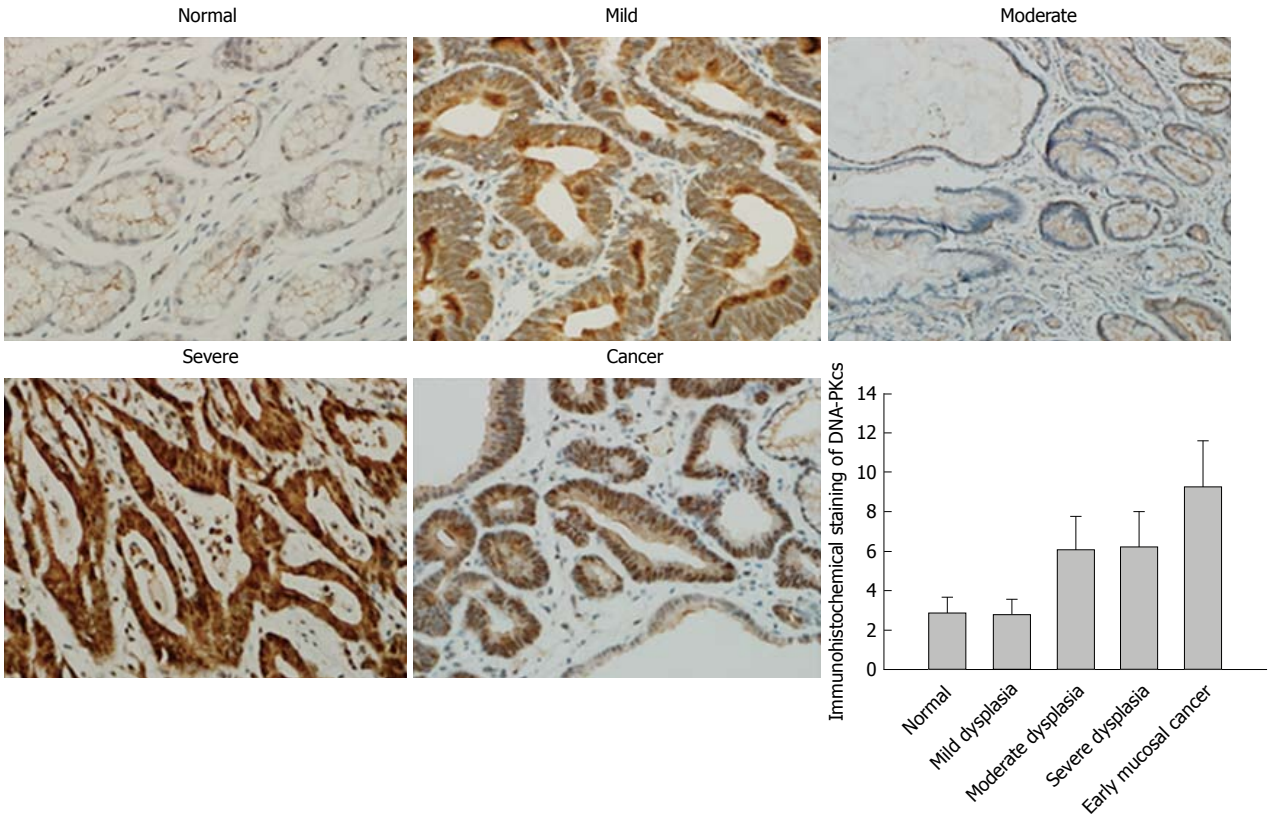


Figure 3 Immunohistochemical staining of DNA-PKcs (40 ×). A: Normal; B: Mild dysplasia; C: Moderate dysplasia; D: Severe dysplasia; E: Early mucosal cancer.

statistically different between various stages of GC initiation. Furthermore, we observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change, suggesting that abnormal ERGIC1 and DNA-PKcs expression may play an important role in GC initiation.

It has been reported that DNA-PKcs plays an important role in the activation of the NHEJ pathway[9,10]. When a DNA damage signal in cells is sent out, DNA-PKcs will recognize it and initiate a damage response in the first place, followed by the event that Ku 70/80 binds to the damaged DNA ends and induces DNA-PKcs to form DNA-PK that triggers NHEJ repair activities[16]. NHEJ is an error-prone and non-specific DNA repair mechanism and can be induced before homologous recombination, whose excessive activation has the capability of regulating cell cycle arrest, cell apoptosis, chromosome re-combination, and genome instability, all of which are closely related with carcinogenesis[17,18]. Dysregulation of DNA-PKcs has been reported to be associated with pathological processes in various cancers[19]. Consistent with the previous study indicating that DNA-PKcs expression in GC was up-regulated compared with normal gastric mucosa, and associated with GC progression[20], up-regulation of DNA-PKcs expression was also found in our study. Furthermore, our study showed more information that DNA-PKcs expression increased along with different stages of GC initiation. Hence, it is possible to hypothesize that enhanced NHEJ resulting from the overexpression of DNA-PKcs may contribute to GC initiation and progression.

ERGIC1, a cycling protein, plays a part in protein secretion out of the ER by participating in membrane trafficking and selective transport of cargo between the ER, the intermediate compartment, and the Golgi apparatus[11]. ER stress is defined as a disequilibrium between protein folding ability of the ER and protein load, causing the accumulation of misfolded and unfolded proteins[21]. Many factors, including hypoxia, starvation, infections, changes in secretary needs and so on, throw a challenge to the folding capacity of the cell and subsequently trigger ER stress[22]. ER stress has been speculated to be involved in many cancers including GC[23]. ER stress is capable of activating NF-κB, ROS, and JNK signal pathways[24-27], resulting in chronic inflammatory response that contributes to carcinogenesis. Besides, accumulating evidence indicated that ER stress response could facilitate cancer progression probably by promoting tumor angiogenesis and malignant cell autophagy, and mitigating apoptosis[28-31]. In this study, we observed that ERGIC1 expression decreased gradually along with different stages of GC initiation. Down-regulation of ERGIC1 expression may affect the protein secretion function of the ER and then disturb ER homeostasis,

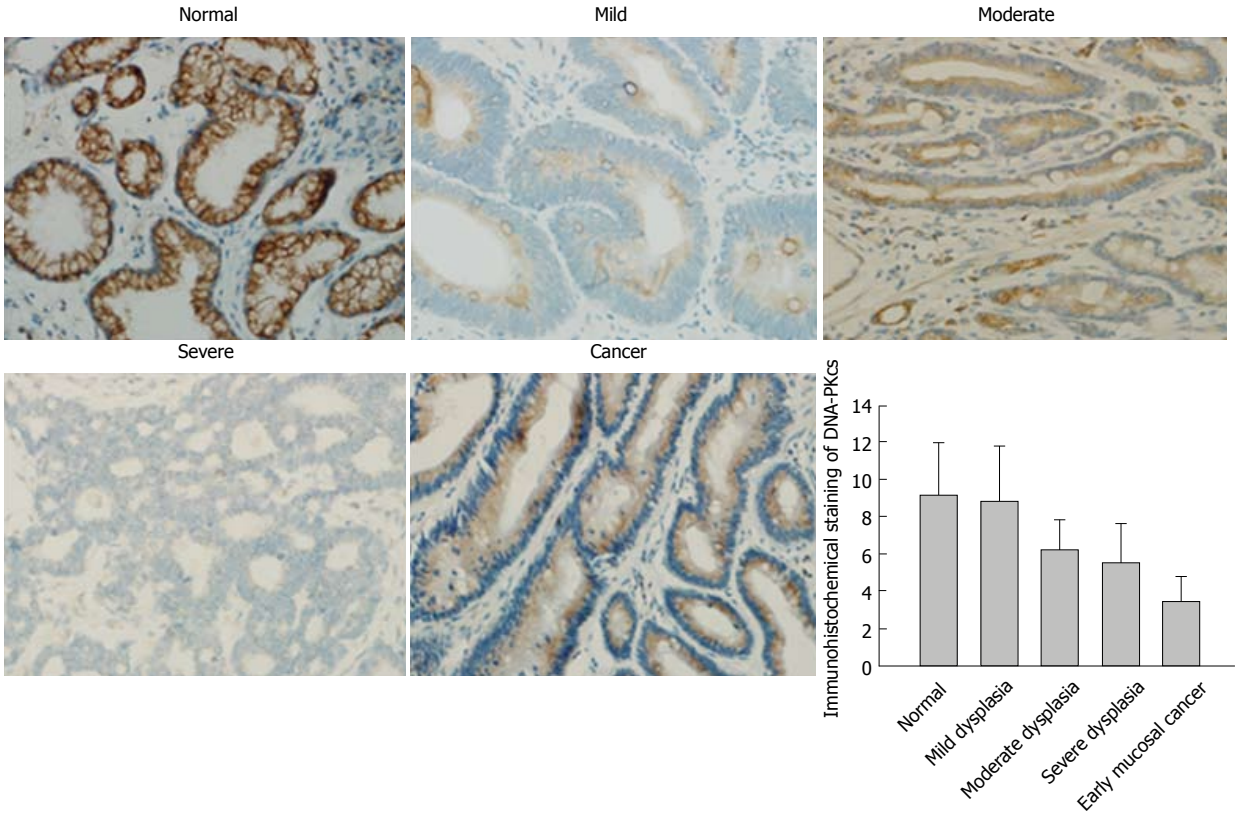


Figure 4 Immunohistochemical staining of ERGIC1 (40 ×). A: Normal; B: Mild dysplasia; C: Moderate dysplasia; D: Severe dysplasia; E: Early mucosal cancer.

which may cause the accumulation of unfolded and misfolded proteins, finally resulting in ERS. Thus, it is possibly reasonable to speculate that down-regulation of ERGIC1 expression may contribute to GC initiation by promoting ERS in gastric mucosal epithelial cells. Besides, some studies suggested that ER stress response can also induce the apoptosis of malignant cells[32-34], indicating that ER stress response may play a dual role in cancers. Furthermore, a study by Vainio *et al*[35] indicated that ERGIC1 expression was up-regulated in prostate cancer samples and knockdown of ERGIC1 could inhibit the ERG oncogene expression in prostate cancer cells *in vitro*, which suggested that the change tendency of ERGIC1 expression in cancers may be different, depending on the genomic or biological function of cancer cells. Therefore, another possible molecular mechanism responsible for the role of down-regulation of ERGIC1 in the GC initiation is that down-regulation of ERGIC1 perhaps disturbs the expression of some oncogenes or anti-oncogenes. Additionally, despite that there are some specific relationships between ERGIC1 and DNA-PKcs based on the inverse expression patterns observed in the current study, we did not provide adequate evidence to confirm that. Thus, further studies are required to elucidate the mechanisms by which down-regulation of ERGIC1 contributes to the initiation and progression of GCs and the internal link between ERGIC1 and DNA-PKcs.

In conclusion, our study conducted a systematic proteomic analysis of normal gastric mucosa, gastric mucosa with mild dysplasia, gastric mucosa with moderate dysplasia, gastric mucosa with severe dysplasia and early mucosal GC, which covered the whole sequential process of GC initiation, and suggested that the aberrant DNA-PKcs and ERGIC1 expression may be involved in the initiation of GC.

COMMENTS

Background

Gastric cancer (GC) is a global malignant disease with high incidence and mortality. The carcinogenesis of GC has been defined as a biological process involving polygene-driven, multi-step, and multi-stage events. However, the molecular mechanisms underlying the carcinogenesis of GC remain unknown.

Research frontiers

Using proteomics analysis, the current study aimed to investigate the molecular mechanisms of GC carcinogenesis.

Innovations and breakthroughs

The authors first report that aberrant ERGIC1 and DNA-PKcs expression may be involved in GC initiation.

Applications

Using label-free quantification technology combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), the authors observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by

immunohistochemistry were consistent with the LC-MS/MS results.

Peer-review

The authors demonstrated that that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation. A total of 160 specimens were enrolled in this study to identify differentially expressed proteins in normal gastric mucosa, gastric mucosa with mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC. These results are interesting and very valuable to verify the molecular mechanisms of GC carcinogenesis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Real-world treatment patterns of gastrointestinal neuroendocrine tumors: A claims database analysis

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Author contributions: All authors were equally involved in the design of the study; Chang E conducted the statistical analyses and all authors contributed equally in the interpretation of results and writing of the manuscript.

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Institutional review board statement: We conducted a retrospective cohort study using the Truven Health Analytics MarketScan Database and the IMS Health Pharmetrics Database, both commercial health insurance claims database for employer-insured beneficiaries in the United States. The databases are fully compliant with the Health Insurance Portability and Accountability Act and meets the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Conflict-of-interest statement: Cai B and Neary MP are employees of Novartis Pharmaceuticals Corporation; Benson III AB was paid as a research consultant for the study as a subject matter expert by Novartis and is an employee of Northwestern University; Broder MS, Chang E and Papoyan E are employees of Partnership for Health Analytic Research, LLC (PHAR, LLC), a health services research company paid by Novartis to conduct this research.

Data sharing statement: The study statistician, Eunice Chang, conducted all statistical analysis for this study using Health Insurance Portability and Accountability Act-compliant commercial-insurance secondary databases MarketScan and PharMetrics.

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Abstract

AIM

To describe real-world treatment patterns of gastrointestinal neuroendocrine tumors (GI NET).

METHODS

In this retrospective cohort study, we used 2009-2014 data from 2 United States commercial claims databases to examine newly pharmacologically treated patients using tabular and graphical techniques. Treatments included somatostatin analogues (SSA), cytotoxic chemotherapy (CC), targeted therapy (TT), interferon

(IF) and combinations. We identified patients at least 18 years of age, with ⩾ 1 inpatient or ⩾ 2 outpatient claims for GI NET who initiated pharmacologic treatment from 7/1/09-6/30/14. A 6 mo clean period prior to first treatment ensured patients were newly treated. Patients were followed until end of enrollment or the study end date, whichever was first.

RESULTS

We identified 2258 newly treated GI NET patients: mean (SD) age was 55.6 years (SD = 9.7), 47.2% of the patients were between 55 and 64 years, and 48.8% were female. All regions of the United States were represented. 59.6% started first-line therapy with SSA monotherapy (964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide), 33.3% CC, 3.6% TT, and 0.5% IF. The remainder received combinations. Mean follow up was 576 d. Overall mean first-line therapy duration was 361 d (449 d for SSA, 215 for CC, 267 for TT). 58.9% of patients had no pharmacological treatment beyond first line. The most common second-line was combination therapy with SSA. In graphical pattern analysis, there was no clear pattern visible after first line therapy.

CONCLUSION

In this study, 60% of patients initiated treatment with SSA alone or in combination. The relatively long time to discontinuation suggests possible sustained effectiveness and tolerability.

Key words: Gastrointestinal neuroendocrine tumors; Treatment patterns; Insurance claims; Somatostatin analogue; Targeted therapy; Chemotherapy

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Core tip: In this retrospective study of real-world treatment patterns, somatostatin analogues were the most common initial pharmacologic treatment in patients with gastrointestinal neuroendocrine tumors, and most of the remaining patients began treatment with chemotherapy. However, despite the many treatment options, over half of the patients discontinued treatments after first line and only less than 10% of patients received any second-line pharmacotherapy. Given limitations of claims data to elucidate reasons for this lack of continued treatment, a study using more detailed clinical information such as medical charts or physician surveys is warranted.

Benson III AB, Broder MS, Cai B, Chang E, Neary MP, Papoyan E. Real-world treatment patterns of gastrointestinal neuroendocrine tumors: A claims database analysis. *World J Gastroenterol* 2017; 23(33): 6128-6136 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6128.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6128

INTRODUCTION

Neuroendocrine tumors (NET) comprise a broad family of rare and often slow growing malignancies. NET can develop anywhere in the body and arise from neuroendocrine cells throughout the endocrine system[1,2]. Approximately two-thirds of NET tumors occur in the gastrointestinal (GI) tract. These sites include the stomach, small intestine, appendix, colon, and rectum[3]. NET secrete peptides and neuroamines that cause distinct syndromes (*e.g.*, carcinoid syndrome), in which case they are referred to as "functional" tumors. Clinical presentation depends on the site of the primary tumor and whether they are functional. Surgery may be curative in the early stages, but delayed diagnosis is typical.

While rare, the incidence and prevalence of NET appear to be increasing worldwide[4-8]. The incidence of NET in the United States increased from 10.9 cases per million person-years (PMPY) in 1973 to 52.5 PMPY in 2004, and to 69.8 PMPY in 2012 as reported using the United States Surveillance Epidemiology and End Results database[4,9]. Prevalence also increased and was reported as 216 per million per year for GI NET in the United States.

The management of GI NET is based on a variety of factors including stage, anatomic location, and the presence and type of symptoms. The most recent NCCN guidelines for unresectable and metastatic GI NET recommend somatostatin analogues (SSA) as first-line treatment, but do not recommend a particular treatment sequence for the remaining therapies[10]. Considering the heterogeneity of GI NET tumors and the resultant lack of specificity in guidelines, we aimed to describe the current real-world treatment patterns of GI NET in a large sample of patients.

MATERIALS AND METHODS

Data source

We conducted a longitudinal, retrospective cohort analysis of newly pharmacologically treated GI NET patients using two large United States commercial claims databases. Data from the Truven Health Analytics MarketScan database and the IMS Pharmetrics database (both using dates from January 1, 2009 to December 31, 2014) were combined to increase sample size. To prevent duplicate records, patients with the same age, gender, region, and date of first GI NET diagnosis in a calendar year found in both databases were randomly removed from one of the databases. Both databases are Health Insurance Portability and Accountability Act compliant administrative claims databases that contain de-identified adjudicated medical claims (*e.g.*, inpatient and outpatient services) and pharmacy claims (*e.g.*, outpatient prescriptions) submitted for payment by

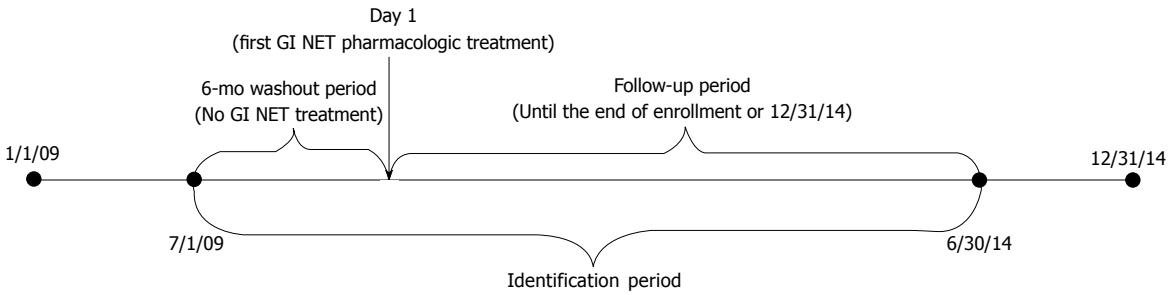


Figure 1 Study timeline. The first gastrointestinal neuroendocrine tumors (GI NET) pharmacologic treatment claim on or after the appearance of the GI NET diagnosis code and within the ID period (7/1/2009 to 6/30/2014) was considered to be the index date. Patients were required to be enrolled for a baseline period of at least six months before the index date. Patient follow-up was variable and continued until the end of enrollment or the study end date (12/31/14), whichever was first.

providers, healthcare facilities, and pharmacies. For both data sources, claims include information on each physician visit, medical procedure, hospitalization, drug dispensed, date of service, number of days of medication supplied, test performed, and complete payment information. Each medical claim has a principal diagnosis and secondary diagnoses codes associated with it. Available patient demographic information includes age, gender, and geographic region. Dates of enrollment and disenrollment are also recorded. As the data were fully de-identified, this study was considered exempt from approval by the Institutional Review Board.

Cohort selection

Patients at least 18 years of age were identified from each dataset if they had at least 1 inpatient or 2 outpatient claims with an International Statistical Classification of Disease-9-Clinical Modification (ICD-9-CM) for GI NET (209.00-209.03, 209.10-209.17, 209.23, 209.24-209.27, 209.40-209.43, 209.50-209.57, 209.62, 209.65-209.67) during the study period (1/1/2009-12/31/2014). The first GI NET pharmacologic treatment claim on or after the appearance of the GI NET diagnosis code and within the ID period (7/1/2009 to 6/30/2014) was considered to be the index date. Patients were required to be enrolled for a baseline period of at least six months before the index date. To ensure new treatment, patients with any evidence of pharmacologic treatment during this baseline period were excluded. In order not to include the same patient twice, we searched for any patients with the same age, gender, region, and date of GI NET diagnosis who could be found in both databases, but we found none. Patient follow-up was variable and continued until the end of enrollment or the study end date (12/31/2014), whichever was first (Figure 1).

Study variables and measures

The primary outcome measure was the use of pharmacologic or liver directed therapy. Pharmacotherapy was divided into four groups: SSA, TT, CC and IF. SSA included octreotide and lanreotide, TT included everolimus and sunitinib, and CC included temozolomide, streptozotocin, doxorubicin, liposomal doxorubicin, fluorouracil, capecitabine, dacarbazine, oxaliplatin and thalidomide. Pharmacologic therapy was identified in claims using both the Healthcare Common Procedure Coding System (HCPCS) and National Drug Codes (NDC). Liver directed therapies comprised liver resection, transplant, lesion ablation (using radiotherapy, cryotherapy, microwave and thermal energy, and including laparoscopic, open and percutaneous routes), embolization (including bland, radioisotope, and chemotherapy), and radiation therapy. Liver directed therapies were identified in claims using HCPCS, ICD-9-CM, and Current Procedural Terminology (CPT) codes. Chemotherapy observed only once and on the same date as embolization was considered chemoembolization and not part of a pharmacologic regimen.

First-line therapy was defined as the pharmacologic treatment regimen observed on, or within three months of, the index date. Therapy included monotherapy or combination therapies. A three-month period after the index date was used to identify pharmacologic therapy intended as first-line but not administered on the index date. This would include, for example, combination chemotherapy where the second agent is given after some delay. Second-line therapy was defined as beginning when treatment was switched from one category of pharmacotherapy to another (*e.g.*, from SSA alone to CC alone), or when a new category of treatment was added (*e.g.*, from SSA alone to SSA plus CC). Changes from one cytotoxic agent to another, or one SSA to another, were not considered a switch. The first day of treatment switch or addition was defined as the initiation date of second-line therapy.

Statistical analysis

Means and proportions were presented in tabular analyses. An inverse Kaplan-Meier curve was used to show duration of first-line therapy. All data transformations and statistical analyses were performed using SAS® version 9.4 (SAS Institute, Cary, NC). Graphical analyses were conducted using GRAPHx™, a proprietary graphics-based algorithm. The GRAPHx method uses multi-colored line segments to represent

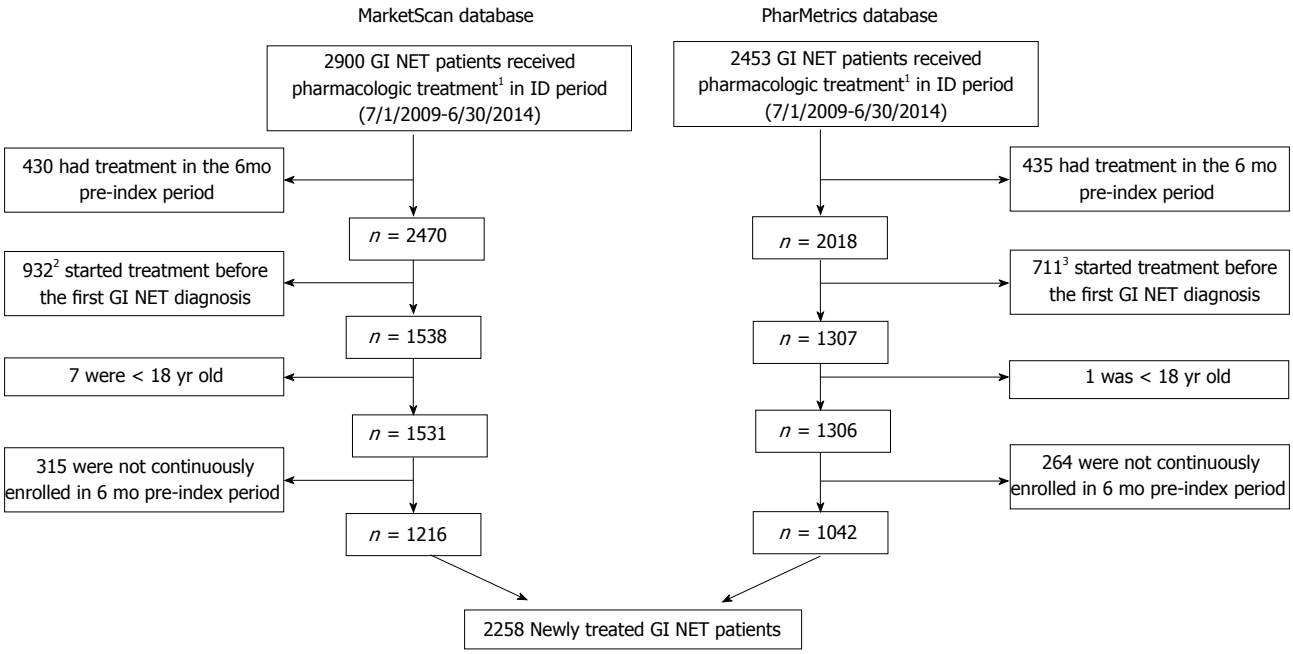


Figure 2 Patient identification. There were 2900 and 2453 gastrointestinal neuroendocrine tumors (GI NET) patients who also had a claim for pharmacologic treatment between 7/1/2009 and 6/30/2014 in the MarketScan and PharMetrics databases, respectively. After excluding patients who had treatment during a 6-mo pre-index period (and therefore were considered to be continuing, rather than initiating, treatment); received treatment before receiving a diagnosis of GI NET; were < 18 yr old; or were not continuously enrolled in the 6-mo pre-index period, there remained 2258 newly treated GI NET patients who were included in the study. [1]Somatostatin analogues (SSAs), targeted therapy, cytotoxic chemotherapy, or interferon; [2]324 (34.8%) within 3 mo, and 516 (55.4%) within 6 mo; [3]249 (35.0%) within 3 mo, and 380 (53.4%) within 6 mo.

various treatments, plotting them over time. The images are reviewed visually for the presence and length of segments and change in colors and patterns over time.

RESULTS

There were 2900 and 2453 patients meeting the definition of GI NET who also had a claim for pharmacologic treatment between 7/1/2009 and 6/30/2014 in the MarketScan and PharMetrics databases, respectively. After excluding patients who had treatment during a 6-mo pre-index period (and therefore were considered to be continuing, rather than initiating, treatment); received treatment before receiving a diagnosis of GI NET; were < 18 years old; or were not continuously enrolled in the 6-mo pre-index period, there remained 2258 newly treated GI NET patients who were included in the study (Figure 2).

Gender was evenly split with *n* = 1103 (48.8%) female patients and *n* = 1155 (51.2%) male. The average age was 55.6 years (SD = 9.7) and 47.2% of the patients were between 55 and 64 years. All regions of the United States were represented. More than half of patients, *n* = 1345 (59.6%), were treated with SSA as first-line monotherapy, 964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide. An additional 75 patients (3.3%) received SSA in combination with other either CC, TT or IF. The second largest group, *n* = 752 (33.3%), was treated with CC monotherapy, and *n* = 81 (3.6%) received TT monotherapy (Table 1). Mean duration of first-line therapy was 361 d (SD = 385) for all newly treated patients. The mean observed duration of treatment for first-line SSA monotherapy users was 449 d (SD = 434.2). It was 215 d (SD = 228.8) for first-line CC monotherapy and 267 d (SD = 325.7) for first-line TT monotherapy (Table 2). By 588 d of treatment (1.61 years), half of SSA initiators had discontinued treatment, compared to 182 d (0.498 years) for half of CC users and 171 d (0.47 years) for half of TT users to discontinue treatment (Figure 3). Liver directed therapy was used by 12.5% during first-line pharmacologic therapy; another 3.7% received it sometime after the first-line (Table 2).

By the end of the study follow-up period [mean (SD, median) of 576 d (447.1, 454)] 58.9% (*n* = 1331) patients had stopped pharmacologic therapy completely. These patients continued to be enrolled in one of the databases but no longer had claims for pharmacologic treatment. In Figure 4, these patients can be identified as colored line segments that terminate in gray segments of variable length, with the gray representing the period of no treatment. An additional 32.7% (*n* = 738) continued their initial therapy until the end of their enrollment; these patients were still receiving their first-line therapy at the time they left a covered plan or reached the end of study. This pattern is shown in Figure 4 as a colored segment terminating in white. The remaining 8.4% (*n* = 189) were observed to change pharmacologic treatment during the follow-up period (a colored segment terminating in different colored segment)

Table 1 Patient demographics by first-line treatment *n* (%)

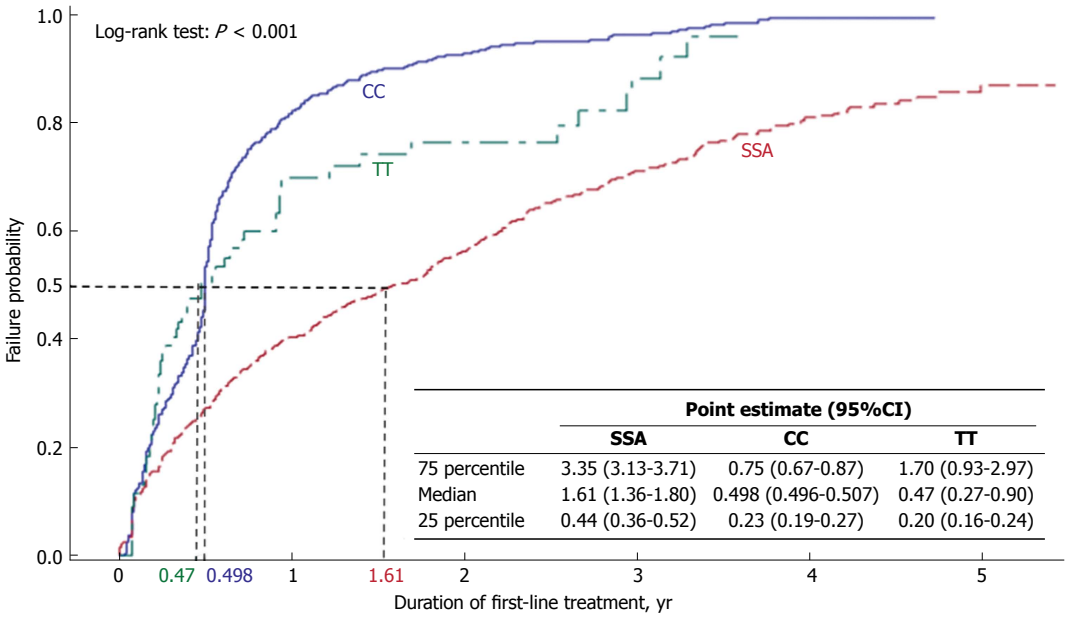
	First-line treatment									All newly treated patients
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	SSA + IF	SSA + TT + CC	
n	1345[1]	752	81	42	31	3	2	1	1	2258
	59.6%	33.3%	3.6%	1.9%	1.4%	0.1%	0.1%	0.0	0.0	100.0%
Age (mean ± SD, yr)	56.3 ± 9.5	54.7 ± 9.9	54.9 ± 10.5	53.5 ± 10.9	53.8 ± 10.1	59.3 ± 3.8	58.5 ± 3.5	N/A	N/A	55.6 ± 9.7
18-24	6 (0.4)	2 (0.3)	0 (0)	1 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (0.4)
25-34	20 (1.5)	25 (3.3)	4 (4.9)	3 (7.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	52 (2.3)
35-44	121 (9.0)	92 (12.2)	9 (11.1)	4 (9.5)	4 (12.9)	0 (0)	0 (0)	0 (0)	0 (0)	230 (10.2)
45-54	371 (27.6)	225 (29.9)	18 (22.2)	12 (28.6)	12 (38.7)	0 (0)	0 (0)	0 (0)	0 (0)	638 (28.3)
55-64	651 (48.4)	338 (44.9)	39 (48.1)	20 (47.6)	11 (35.5)	3 (100.0)	2 (100.0)	1 (100.0)	1 (100.0)	1066 (47.2)
65+	176 (13.1)	70 (9.3)	11 (13.6)	2 (4.8)	4 (12.9)	0 (0)	0 (0)	0 (0)	0 (0)	263 (11.6)
Female	677 (50.3)	341 (45.3)	40 (49.4)	26 (61.9)	17 (54.8)	1 (33.3)	1 (50.0)	0 (0.0)	0 (0.0)	1103 (48.8)
Region										
Midwest	321 (23.9)	183 (24.3)	20 (24.7)	13 (31.0)	7 (22.6)	0 (0)	1 (50.0)	0 (0)	0 (0)	545 (24.1)
Northeast	261 (19.4)	150 (19.9)	12 (14.8)	11 (26.2)	7 (22.6)	1 (33.3)	0 (0)	0 (0)	0 (0)	442 (19.6)
South	563 (41.9)	323 (43.0)	36 (44.4)	12 (28.6)	15 (48.4)	2 (66.7)	1 (50.0)	0 (0)	1 (100.0)	953 (42.2)
West	200 (14.9)	96 (12.8)	13 (16.0)	6 (14.3)	2 (6.5)	0 (0)	0 (0)	1 (100.0)	0 (0)	318 (14.1)
Year of treatment initiation	130 (9.7)	41 (5.5)	4 (4.9)	2 (4.8)	1 (3.2)	0 (0)	0 (0)	1 (100.0)	0 (0)	179 (7.9)
2009										
2010	271 (20.1)	122 (16.2)	4 (4.9)	11 (26.2)	7 (22.6)	0 (0)	1 (50.0)	0 (0)	0 (0)	416 (18.4)
2011	282 (21.0)	159 (21.1)	15 (18.5)	17 (40.5)	5 (16.1)	0 (0)	0 (0)	0 (0)	0 (0)	478 (21.2)
2012	270 (20.1)	168 (22.3)	33 (40.7)	4 (9.5)	10 (32.3)	0 (0)	1 (50.0)	0 (0)	1 (100.0)	487 (21.6)
2013	268 (19.9)	174 (23.1)	16 (19.8)	3 (7.1)	4 (12.9)	2 (66.7)	0 (0)	0 (0)	0 (0)	467 (20.7)
2014	124 (9.2)	88 (11.7)	9 (11.1)	5 (11.9)	4 (12.9)	1 (33.3)	0 (0)	0 (0)	0 (0)	231 (10.2)
Days of follow-up										
Mean	621	514	454	588	425	244	675	836	496	576
(SD)	(468.5)	(409.1)	(403.6)	(424.1)	(269.6)	(140.7)	(145.7)	N/A	N/A	(447.1)
Median	500	393	290	455	360	216	675	836	496	454

[1]964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide. SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

(Figure 4 and Table 2). Among these 189 patients who were observed to begin second-line therapy, 128 (67.7%) had initially been treated with SSA. Among these 128 first-line SSA users, 89 (69.5%) added additional therapy (*e.g.*, CC or TT) as their second-line treatment. In patients who did not begin therapy with SSA, most received SSA monotherapy or combination therapy as second-line (Table 3). Liver directed therapy (short, red segments) appears dispersed throughout periods of both pharmacologic treatment (colored segments) and periods of no pharmacologic treatment (gray segments) (Figure 4). Among 1331 patients who stopped pharmacologic treatment and did not begin a second-line treatment, 5.5% were treated with liver-directed therapy within 30 d before or after they stopped.

DISCUSSION

This study used two very large, nationally representative claims databases, which together represent up to 100 million covered lives, to describe real-world treatment of GI NET. Three findings were of particular interest. First, the most common initial pharmacologic treatment was with SSA, with average duration of use of just over 18 mo. Second, although 60% of patients initiated treatment with SSA alone or in combination, most of the remainder began treatment with CC, therapy recommended



	Point estimate (95%CI)		
	SSA	CC	TT
75 percentile	3.35 (3.13-3.71)	0.75 (0.67-0.87)	1.70 (0.93-2.97)
Median	1.61 (1.36-1.80)	0.498 (0.496-0.507)	0.47 (0.27-0.90)
25 percentile	0.44 (0.36-0.52)	0.23 (0.19-0.27)	0.20 (0.16-0.24)

No. of patients at risk

	Index date	0.5 yr	1 yr	1.5 yr	2 yr	2.5 yr	3 yr	3.5 yr	4 yr	4.5 yr	5 yr
SSA	1345	871	606	426	291	195	133	89	53	26	10
CC	752	320	107	52	31	21	13	5	1	1	0
TT	81	34	16	11	9	8	4	1	0	0	0

Figure 3 Time to discontinuation of first-line treatment. By 588 d of treatment (1.61 yr), half of SSA initiators had discontinued treatment, compared to 182 d (0.498 yr) for half of CC users and 171 d (0.47 yr) for half of TT users to discontinue treatment. SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy.

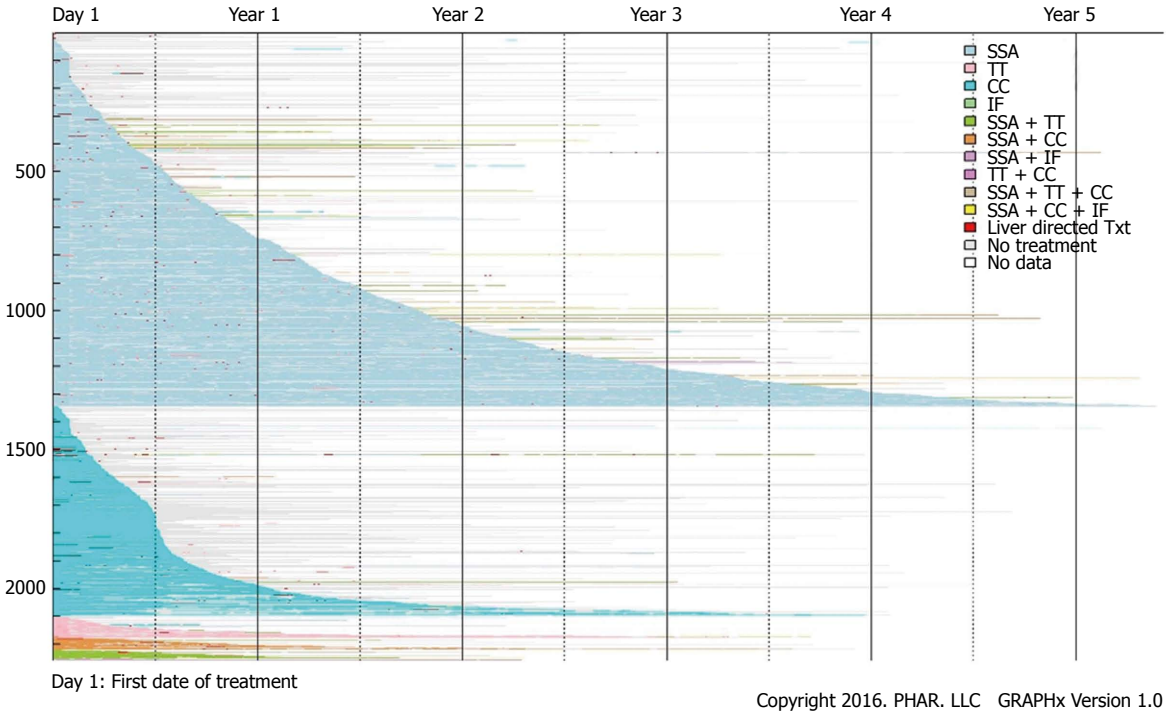


Figure 4 Pharmacologic treatment. By end of study follow-up [mean (SD, median) of 576 d (447.1, 454)], 58.9% (*n* = 1331) patients had stopped pharmacologic therapy completely. These patients (no pharmacologic treatment claims, but remained enrolled in one of the databases) can be identified as colored line segments that terminate in gray segments of variable length, with the gray representing the period of no treatment. An additional 32.7% (*n* = 738) continued their initial therapy until the end of their enrollment; these patients were still receiving their first-line therapy at the time they left a covered plan or reached the end of study. This pattern is shown as a colored segment terminating in white. The remaining 8.4% (*n* = 189) were observed to change pharmacologic treatment during the follow-up period (a colored segment terminating in different colored segment). Liver directed therapy (short, red segments) appears dispersed throughout periods of both pharmacologic treatment (colored segments) and periods of no pharmacologic treatment (gray segments). SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

Table 2 Use of first-line treatment *n* (%)

	First-line									All newly treated patients
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	SSA + IF	SSA + TT + CC	
n	1345	752	81	42	31	3	2	1	1	2258
	59.6%	33.3%	3.6%	1.9%	1.4%	0.1%	0.1%	0.0%	0.0%	100.0%
Duration of first-line treatment (mean ± SD, d)	449 ± 434.2	215 ± 228.8	267 ± 325.7	408 ± 327.9	276 ± 189.5	208 ± 165.6	251 ± 285.0	836 ± 0	426 ± 0	361 ± 385.0
First-line ending status	635 (47.2)	609 (81.0)	44 (54.3)	26 (61.9)	14 (45.2)	1 (33.3)	1 (50.0)	0 (0)	1 (100.0)	1331 (58.9)
Stop										
Switch	128 (9.5)	33 (4.4)	14 (17.3)	5 (11.9)	7 (22.6)	1 (33.3)	1 (50.0)	0 (0)	0 (0)	189 (8.4)
End of enrollment	582 (43.3)	110 (14.6)	23 (28.4)	11 (26.2)	10 (32.3)	1 (33.3)	0 (0)	1 (100.0)	0 (0)	738 (32.7)
Liver directed therapy timing										
During first-line	171 (12.7)	87 (11.6)	9 (11.1)	10 (23.8)	4 (12.9)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	282 (12.5)
After first-line	36 (2.7)	39 (5.2)	7 (8.6)	0 (0.0)	2 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	84 (3.7)
+/- 30 d after stopping first line therapy[1]	635	609	44	26	14	1	1	0	1	1331
	29 (4.6)	42 (6.9)	2 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	73 (5.5)

[1]Among patients who stopped.

by NCCN only if no other options (SSA, TT, or liver directed treatment) are feasible. Third, despite the many available treatment options, less than one in 10 patients was observed to receive treatment with second-line pharmacotherapy of any type.

Each of these findings must be considered in light of what is known about the disease as well as the inherent limitations of the data source. Treating NET patients is a complex process. Treatments are individualized based on tumor size, location, and pathology, as well as whether the tumor is functional, type and extent of symptoms, and speed of progression. Our data did not include this level of detail. Insurance companies aggregate information on inpatient and outpatient services (generally reported as ICD-9-CM or ICD-10), procedures (ICD-9 procedure codes and CPT codes) and pharmacy claims (NDC) as claims are submitted for payment by providers, healthcare facilities, and pharmacies. So for example, while GI NET can be identified by using a list of ICD-9-CM codes, presence of advanced disease must be inferred by observing the use of pharmacologic treatment. Another limitation of the date source is that the available length of time for analysis is relatively brief, so conclusions regarding average duration of use may not be representative of long-term treatment patterns.

Our primary finding that 60% of patients initiated pharmacologic therapy with SSA is consistent with the NCCN recommendation of these drugs for initial treatment of clinically significant and progressive NET. Consistent with the clinical observation that SSA tend to be well tolerated, patients initiating treatment with SSA remained on first-line therapy longer than the patients who began treatment with CC and TT. The second finding noted above, that 1/3 of GI NET patients began treatment with CC, is more surprising. CC is relatively ineffective in these patients, and as a result is recommended only if other options are not feasible.

There are several possible explanations for the frequent use of CC. Patients observed to initiate cytotoxic treatment may have been treated in the past with other agents and either progressed or were intolerant to those agents. We reviewed data for 6 mo before the first pharmacologic treatment, but treatments more than 6 mo in the past would have been missed. It may also be that some of the patients had a pathology finding suggesting chemotherapy would be beneficial, or a different type of GI tumor that was incorrectly coded as NET. Our data were de-identified, meaning we could not confirm the diagnosis in medical records, physician or patient surveys, or by other means. Finally, some clinicians may have been unfamiliar with either best practice recommendations or the available, albeit limited, data on GI NET treatment. Our findings are consistent with a recent large case series from a tertiary referral center that found SSA and CC were the two most common treatment strategies used for gastroenteropancreatic NET[11].

Previous studies have found significant divergence between clinical guidelines and treatment in other, more common, cancers. Chagpar and colleagues found that while Stage I colon cancer patients were treated according to guidelines by 95%, higher stage patients were less likely to be treated according to guidelines. Stage II

Table 3 Second-line treatment, stratified by first-line treatment *n* (%)

	First-line treatment							Patients with second-line treatment
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	
n	128	33	14	5	7	1	1	189
	67.7%	17.5%	7.4%	2.6%	3.7%	0.5%	0.5%	100.0%
Second-line treatment								
SSA + TT	51 (39.8)	4 (12.1)	4 (28.6)	3 (60.0)		0 (0)	1 (100.0)	63 (33.3)
SSA + CC	33 (25.8)	6 (18.2)	0 (0)		5 (71.4)	0 (0)	0 (0)	44 (23.3)
CC	24 (18.8)		4 (28.6)	0 (0)	1 (14.3)	0 (0)	0 (0)	29 (15.3)
SSA		16 (48.5)	5 (35.7)	0 (0)	0 (0)	1 (100.0)	0 (0)	22 (11.6)
TT	12 (9.4)	7 (21.2)		2 (40.0)	0 (0)	0 (0)	0 (0)	21 (11.1)
SSA + IF	3 (2.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.6)
IF	2 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.1)
TT + CC	1 (0.8)	0 (0)	1 (7.1)	0 (0)	0 (0)		0 (0)	2 (1.1)
SSA + TT + CC	1 (0.8)	0 (0)	0 (0)	0 (0)	1 (14.3)	0 (0)	0 (0)	2 (1.1)
SSA + CC + IF	1 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)

SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

high-risk patients showed the lowest concordance with only 36% being treated per recommendation[12]. In a prospective study of women with breast cancer, Giorano and colleagues found that 83% of patients 55-64 years old received care concordant with chemotherapy guidelines compared with 29% of patients 75 or older[13].

The finding that only a small proportion of patients are observed to receive second-line treatment is also surprising. The 5-year survival of patients with advanced GI NET is more than 70%. Most surviving patients would be expected to receive continued treatment, whether in the form of liver-directed treatment or pharmacotherapy. We considered multiple explanations for this finding. First, although median follow-up was over 15 mo, many patients were eventually lost to follow-up when they disenrolled from a plan included in our databases. One third of patients were continuing to use their index treatment when they were lost to follow-up. Whether (or when) these individuals progressed to second-line treatment cannot be determined using these databases. If these patients were systematically different from the ones who remained under observation, our results would be biased.

Despite this significant loss to follow-up, nearly 60% of patients were observed to continue enrollment but stop therapy. That is, they survived and remained in the data set, but no second-line pharmacotherapy use could be identified. We considered the possibility that these patients received some liver-directed treatment that alleviated their symptoms or controlled their disease, obviating the need for second-line treatment. However, we found no evidence of this: liver-directed treatment was observed in only 5.5% of patients around the time they stopped first-line treatment. We also considered whether some patients may have had a secondary source of payment, such that their claims for pharmacotherapy did not appear in our databases. Just over 11% of patients were 65 and older and would have been eligible for Medicare. Payment rules regarding patients with both commercial coverage and Medicare are complex[14] but generally require the commercial payer (for which we did have data) to be primarily responsible for payment. In cases where Medicare had primary responsibility, we would have missed claims for pharmacologic or liver-directed therapy and thus underestimated treatment. The magnitude of this problem is impossible to know using our current data source. A study using Medicare data and examining patients over 65 only might be less likely to suffer from this bias. Finally, it may indeed be the case that some patients stop therapy completely. Such patients may be terminal and choose not to undergo further treatment, or they may be relatively asymptomatic and decline to be treated on that basis. Further research with detailed clinical data would be needed to confirm which, if any, of these explanations is the most accurate.

In this large, claims-based, retrospective study of real-world pharmacologic treatment patterns, we found that 60% of GI NET patients began therapy with SSA and about one-third with CC. The relatively long time to discontinuation of SSA, as well as their use in combination with other agents, suggests they may be well tolerated and potentially have sustained effectiveness. We also found that over half of the patients discontinued treatment after first-line and only less than 10% of the patients received second-line treatment despite the availability of a number of different options. To address the limitations of this study and expand knowledge of real-world treatment patterns, a study using more detailed clinical information such as medical charts or physician surveys is warranted. In addition, future studies should consider using databases that would allow for greater longitudinal follow-up, such as registries, to assist in the further understanding of treatment patterns and length of therapy.

COMMENTS

Background

Neuroendocrine tumors (NET) comprise a broad set of rare tumors. Almost 2/3 arise in the gastrointestinal (GI) tract. The management of GI NET is based on a variety of factors including stage, anatomic location, and the presence and type of symptoms. The most recent NCCN guidelines for unresectable and metastatic GI NET recommend somatostatin analogues (SSA) as first-line treatment, but do not recommend a particular treatment sequence for the remaining therapies.

Research frontiers

This study's results add to the limited knowledge about real-world treatment patterns for GI NET, which is especially significant in light of the lack of treatment guidelines regarding treatment sequences beyond first-line therapy.

Innovations and breakthroughs

This study used two very large, nationally representative claims databases to describe real-world treatment of GI NET. The three key findings were: first, the most common initial pharmacologic treatment was with SSA, with average duration of use of just over 18 mo; second, although 60% of patients initiated treatment with SSA alone or in combination, most of the remainder began treatment with cytotoxic chemotherapy, therapy recommended by NCCN only if no other options (SSA, targeted therapy, or liver directed treatment) are feasible; and third, despite the many available treatment options, less than one in 10 patients was observed to receive treatment with second-line pharmacotherapy of any type. The authors findings are consistent with a recent large case series from a tertiary referral center that found SSA and CC were the two most common treatment strategies used for gastroenteropancreatic NET. Previous studies have also found significant divergence between clinical guidelines and treatment in other, more common, cancers.

Applications

This study suggests that there is frequent use of CC in GI NET treatment, although CC is relatively ineffective in these patients and recommended only if other options are not feasible. This may be a result of clinicians unfamiliarity with either best practice recommendations or the available, albeit limited, data on GI NET treatment. To address the limitations of this study and expand knowledge of real-world treatment patterns, a study using more detailed clinical information such as medical charts or physician surveys is warranted. In addition, future studies should consider using databases that would allow for greater longitudinal follow-up, such as registries, to assist in the further understanding of treatment patterns and length of therapy.

Terminology

NET arise from cells that release hormones in response to nerve stimulation. Insurance claims databases compile coded information related to charges for medical care for large populations, but they do not contain clinically detailed records.

Peer-review

The article aims to describe real-world treatment patterns of GI NET.

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ORIGINAL ARTICLE

Retrospective Study

Patients with inflammatory bowel disease have increased risk of autoimmune and inflammatory diseases

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Abstract

AIM

To investigate whether immune mediated diseases (IMD) are more frequent in patients with inflammatory bowel disease (IBD).

METHODS

In this population based registry study, a total of 47325 patients with IBD were alive and registered in the Danish National Patient Registry on December 16, 2013. Controls were randomly selected from the Danish Civil Registration System (CRS) and matched for sex, age, and municipality. We used ICD 10 codes to identify the diagnoses of the included patients. The IBD population was divided into three subgroups: Ulcerative colitis (UC), Crohn's disease (CD) and Both the latter referring to those registered with both diagnoses. Subsequently, odds-ratios (OR) and 95%CI were obtained separately for each group and their respective controls. The use of Bonferoni post-test correction adjusted the significance level to $P < 0.00125$. *P*-values were estimated using Fisher's exact test.

RESULTS

There were significantly more women than men in the registry, and a greater percentage of comorbidity in the IBD groups ($P < 0.05$). Twenty different IMDs were all significantly more frequent in the IBD group. Sixteen

were associated with UC versus twelve with CD. In both UC and CD ORs were significantly increased (P < 0.00125) for primary sclerosing cholangitis (PSC), celiac disease, type 1 diabetes (T1D), sarcoidosis, asthma, iridocyclitis, psoriasis, pyoderma gangrenosum, rheumatoid arthritis, and ankylosing spondylitis. Restricted to UC (P < 0.00125) were autoimmune hepatitis, primary biliary cholangitis, Grave's disease, polymyalgia rheumatica, temporal arteritis , and atrophic gastritis. Restricted to CD (P < 0.00125) were psoriatic arthritis and episcleritis. Restricted to women with UC (P < 0.00125) were atrophic gastritis, rheumatoid arthritis, temporal arteritis, and polymyalgia rheumatica. Restricted to women with CD were episcleritis, rheumatoid arthritis, and psoriatic arthritis. The only disease restricted to men (P < 0.00125) was sarcoidosis.

CONCLUSION

Immune mediated diseases were significantly more frequent in patients with IBD. Our results strengthen the hypothesis that some IMDs and IBD may have overlapping pathogenic pathways.

Key words: Immune mediated diseases; Ulcerative colitis; Risk; Prevalence; Registry; Chronic inflammatory diseases; Autoimmune diseases; Inflammatory bowel disease; Crohn's disease; Extraintestinal manifestations

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Core tip: Essential to inflammatory bowel disease (IBD) pathogenesis are environmental factors, altered gut microbiota and genetic susceptibility. The latter causing impairment of barrier function, autophagy, and Th1, 2 and 17 cell responses. Interestingly, these mechanisms are also thought important in other immune mediated diseases, as is the overlap of susceptibility genes. Besides the classic extraintestinal manifestations, we found a variety of immune mediated diseases to be more frequent in individuals with IBD. Physicians should be aware of this when treating these patients. Furthermore, these findings support the hypothesis that immune mediated diseases may have overlapping pathogeneses. Thus, understanding IBD might help us understand other immune mediated diseases and vice versa.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two distinct types of chronic inflammatory bowel diseases. The insight into etiology factors and the complex pathogenetic process is not yet fully understood. The diseases are often diagnosed in young individuals and recent studies report increasing incidences of both UC and CD, not only in Denmark but globally[1]. It has been suggested that inflammatory bowel disease (IBD) may be due to an inappropriate inflammatory response to the intestinal flora in genetically susceptible individuals. So far, several susceptibility genes have been identified[2]. Many of these are also found in other immune mediated diseases (IMDs), indicating overlaps between pathogenic pathways. The identified risk genes in IBD are involved in maintaining normal microbial gut homeostasis and adequate immune response[3,4]. Mutations in these may impair mechanisms essential to innate and adaptive immune response, *i.e.* weakened mucosal barrier, a decrease of antibacterial agents, impaired autophagy and antigen recognition. Mutations may also cause an imbalance of pro- and anti-inflammatory cytokines related to the regulation of Th1, 2 and 17 in particular[5]. CD is considered Th1 mediated thus characterized by interferon gamma, tumor necrosis factor alpha, and IL 12. UC is associated with a Th2 response where IL 4, 5, 10 and 13 are dominant. The Th17 response is present in both CD and UC but most pronounced in CD. It is characterized by IL 17 and 23 production. Th17 can also produce interferon gamma like Th1[5-8]. It is suggested that disturbances in these mechanisms may cause a loss of self-tolerance leading towards chronic inflammation or autoimmunity[9-12].

The gut microbiota of patients with IBD has been shown to contain less diversity, a reduced number of bacteria, and an altered microbial metabolite profile compared to healthy individuals[13]. Environmental factors, *i.e*. medication (antibiotics, non-steroid anti-inflammatory drugs and hormones), diet, geography, and previous infections might influence this[4]. A similar etiology is believed to exist in other IMDs, *i.e.* rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, type 1 diabetes (T1D), and celiac disease[14,15].

It has become clear, that patients with an existing IMD are more likely to develop other IMDs, this is more evident in females than in males[16]. Apart from the extraintestinal manifestations of IBD, little is known about the association between IBD and other IMDs.

Only a few large population based studies on the subject exist. The results of these suggest that IBD is associated with asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, autoimmune thyroiditis, T1D, and vasculitis[17-22]. Different study designs, varying validity of diagnoses, population sizes and confounders, *i.e.* ethnicity, economic and social status, all make the findings of these studies difficult to interpret.

In Denmark healthcare is free and all contacts to hospitals are registered on an individual basis based on a civil registration number together with diagnosis

Table 1 Participants' demographic data

Variables	IBD	Control	UC	Control	CD	Control	Both[1]	Control
n	47325	92839	31066	60951	13343	26172	2916	5716
Female	54%	55%	53%	53%	58%	58%	56%	56%
Male	46%	45%	47%	47%	42%	42%	44%	44%
Mean age at entry, yr	53	53	55	55	49	49	47	47
Mean age at onset of IBD, yr	42	-	44	-	37	-	34	-
Mean duration of IBD at entry, yr	10	-	9	-	10	-	11	-
Comorbidity 0[2]	77%	83%	76.50%	82.00%	77%	85%	82%	87%
Comorbidity 1-2	18%	13.50%	18.00%	14%	18%	12%	15%	10%
Comorbidity ⩾ 3	5%	3.50%	5.50%	4%	5%	3%	3%	3%

[1]Patients registered with both CD and UC; [2]No. of comorbidities at onset of IBD according to the Charlson comorbidity index. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

and procedural codes. This allows a unique access to information not confounded by economic and social status.

The aim of this study was to examine if IMDs are more frequent among patients with CD and UC compared to the background population.

MATERIALS AND METHODS

This was a cross-sectional study including all living patients with IBD who were matched with a control group to compare the point-prevalence of specific IMDs.

Identification of patients and controls

The Danish National Patient Registry include all contacts within the healthcare system both in-hospital, since 1977, and in outpatient settings since 1994. Data were retrieved on December 16, 2013 and included all patients alive registered with a diagnosis compatible with CD and UC. Patients were identified using the ICD 10 codes: CD K50.0-K50.9; UC, K51.0-K51.9). ICD 10 codes including "other" or "unspecified" were excluded to avoid inclusion of non-specific diseases and incorrect diagnosis codes.

The Danish Civil Registration System (CRS) includes all Danish inhabitants and each person has a unique 10-digit identification number. The CRS includes demographic data *e.g.* name, sex, date of birth, and death[23]. All IBD patients were paired (2:1) with random controls identified in the CRS and matched by sex, age (± 1 year) and municipality. Demographic data presented are based on data from the CRS.

The selected forty IMDs are all considered to be of either autoimmune or inflammatory origin. The same criteria were used for the IMDs. ICD 10 codes for the IMDs are listed in the Supplementary Table 1.

To assess comorbidity we used the Charlson comorbidity index which has been developed to estimate 1-year mortality in cancer patients. It is also useful in research to identify possible confounding diseases. It includes a number of systemic diseases associated with increased mortality, *i.e.* organ failure, AIDS, and cancer[24].

Ethics

This study was approved by the Danish Data Protection Agency (approval # 2013-41-1596). Approval from the Ethics Committee was not needed as this is a registry study.

Statistical analysis

The occurrence of IMDs was obtained separately for each group. Then OR and 95%CI were calculated. Fisher's exact test was used to calculate *P*-values.

We used the Bonferroni post-test correction to reduce the likelihood of false positives. We did 40 comparisons (the 40 IMDs investigated) and adjusted the significance level accordingly to $P < 0.00125$. Calculations was made using STATA version 13.0 (StataCorp LP, TX, United States).

RESULTS

A total of 47325 patients were alive and registered with IBD on December 16, 2013. A total of 92839 controls were identified.

CD was registered in 13343 patients, UC in 31066, and 2916 were registered with both diagnoses. A total of 92839 controls were found for the IBD group, 26172 for CD, 60951 for UC and 5716 for those with both diagnoses. Due to the matching criteria, five IBD patients had only one or no controls.

There was an excess of women in all IBD groups, most pronounced in CD ($P < 0.05$). The mean age at onset of disease was significantly higher in UC. Comorbidity was most frequent in those with either UC or CD ($P < 0.05$). See Table 1.

Twenty out of forty IMDs had significantly increased ORs in the IBD groups compared to their controls ($P < 0.00125$). Sixteen IMDs were associated with UC and twelve with CD. See Tables 2 and 3.

Seven of the IMDs were considered rheumatologic diseases, included ankylosing spondylitis, rheumatoid arthritis, psoriatic arthritis, polymyalgia rheumatic, temporal arteritis, polyarteritis nodosa, and Churg

Table 2 Number of immune mediated diseases

Disease	IBD	Control	CD	Control	UC	Control	Both[1]	Control
Primary sclerosing cholangitis	257	4	35	1	192	2	30	1
Pyoderma gangrenosum	193	8	60	1	97	7	36	0
Autoimmune hepatitis	124	35	15	11	96	22	13	2
Celiac disease	280	92	133	30	132	58	15	4
Ankylosing spondylitis	431	151	189	32	201	102	41	17
Churg Strauss syndrome	14	5	4	1	8	4	2	0
Primary biliary cholangitis	71	32	11	6	53	25	7	1
Episcleritis	56	33	25	9	23	21	8	3
Iridocyclitis	419	295	148	82	230	188	41	25
Atrophic gastritis	60	47	16	11	42	34	2	2
Psoriasis	378	345	148	99	200	229	30	17
Polyarteritis nodosa	42	38	15	9	24	27	3	2
Rheumatoid arthritis	446	401	119	110	250	311	32	25
Type 1 diabetes	1682	1464	359	431	1002	1180	103	71
Sarcoidosis	141	122	29	38	79	94	14	9
Asthma	1140	981	337	363	568	695	76	82
Giant cell arteritis	193	156	37	46	116	141	3	6
Psoriatic arthritis	316	249	81	93	147	206	21	17
Grave's disease	817	581	141	207	394	561	46	49
Polymyalgia rheumatica	468	320	72	122	242	324	6	22

[1]Patients registered with both CD and UC. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Table 3 Odds-ratios for immune mediated diseases, in patients with inflammatory bowel disease

Disease	IBD	95%CI	UC	95%CI	CD	95%CI	Both[1]	95%CI
Primary sclerosing cholangitis	126.7[a]	47.2-340.3	189.5[a]	47.0-763.4	68.8[a]	9.4-502.6	59.4[a]	8.1-436.2
Pyoderma gangrenosum	47.5[a]	23.4-96.4	27.3[a]	12.7-58.7	118.2[a]	16.4-853.3	36/0[a,2]	
Autoimmune hepatitis	7.0[a]	4.8-10.1	8.6[a]	5.4-13.6	2.7[b]	1.2-5.8	12.8[a]	2.9-56.8
Celiac disease	6.0[a]	4.7-7.6	4.5[a]	3.3-6.1	8.8[a]	5.9-13.0	7.4[a]	2.4-22.3
Ankylosing spondylitis	5.6[a]	4.7-6.8	3.9[a]	3.1-4.9	11.7[a]	8.1-17.1	4.8[a]	2.7-8.4
Churg Strauss syndrome	5.5[a]	2.0-15.3	3.9[b]	1.2-13.0	-[c]		-[c]	
Primary biliary cholangitis	4.4[a]	2.9-6.6	4.2[a]	2.6-6.7	3.6[b]	1.3-9.7	13.8[b]	1.7-111.9
Episcleritis	3.3[a]	2.2-5.1	2.1[b]	1.2-3.9	5.5[a]	2.5-11.7	5.2[b]	1.4-19.8
Iridocyclitis	2.8[a]	2.4-3.3	2.4[a]	2.0-2.9	3.6[a]	2.7-4.7	3.2[a]	2.0-5.4
Atrophic gastritis	2.5[a]	1.7-3.7	2.4[a]	1.5-3.8	2.9[b]	1.3-6.2	-[c]	
Psoriasis	2.2[a]	1.9-2.5	1.7[a]	1.4-2.1	3.0[a]	2.3-3.8	3.5[a]	1.9-6.5
Polyarteritis nodosa	2.2[a]	1.4-3.4	1.7[b]	1.0-3.0	3.3[b]	1.4-7.5	-[c]	
Rheumatoid arthritis	1.8[a]	1.5-2.0	1.6[a]	1.3-1.9	2.1[a]	1.6-2.8	2.5[a]	1.5-4.2
Type 1 diabetes	1.7[a]	1.6-1.9	1.7[a]	1.6-1.8	1.7[a]	1.4-1.9	2.9[a]	2.2-3.9
Sarcoidosis	1.7[a]	1.3-2.2	1.7[a]	1.2-2.2	-[c]		3.1[b]	1.9-4.8
Asthma	1.7[a]	1.6-1.9	1.6[a]	1.4-1.8	1.8[a]	1.6-2.1	1.8[a]	1.3-2.5
Giant cell arteritis	1.6[a]	1.3-2.0	1.6[a]	1.3-2.1	1.6[b]	1.0-2.4	-[c]	
Psoriatic arthritis	1.5[a]	1.3-1.8	1.4[b]	1.1-1.7	1.7[a]	1.3-2.3	2.4[b]	1.3-4.6
Grave's disease	1.4[a]	1.3-1.6	1.4[a]	1.2-1.6	1.3[b]	1.1-1.7	1.9[b]	1.2-2.8
Polymyalgia rheumatica	1.3[a]	1.2-1.5	1.5[a]	1.2-1.7	-[c]		-[c]	

[a]$P < 0.00125$; [b]$P = 0.00125$-0.05; [c]$P > 0.05$; [1]Patients registered with both CD and UC; [2]No. of cases in IBD cohort/No. of cases in control cohort. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Strauss Syndrome.

Five IMDs were gastrointestinal including celiac disease, atrophic gastritis, primary sclerosing cholangitis, primary biliary cholangitis, and autoimmune hepatitis.

The remaining IMDs were T1D, Grave's disease, pyoderma gangrenosum, psoriasis, iridocyclitis, episcleritis, sarcoidosis, and asthma.

There was a trend towards significance (P = 0.00125-0.05) for Wegener's granulomatosis, chorioretinitis, vitiligo, lichen ruber planus, scleroderma, and multiple sclerosis.

Seven IMDs were only significant in women. While only one was restricted to men. See Table 4.

In general, the same pattern is seen in those registered with both CD and UC.

We did not observe any OR below one, neither did we record any cases of Sjögren's syndrome, inclusion body myositis, eosinophilic esophagitis, or autoimmune adrenalitis.

DISCUSSION

In this study, we documented an increased frequency

Table 4 Odds-ratios for immune mediated diseases restricted to either gender

Disease	Females	95%CI	Males	95%CI
IBD				
Episcleritis	3.6[a]	2.1-6.1	2.9[b]	1.4-6.1
Atrophic gastritis	3.5[a]	2.1-5.9	-[c]	
Polyarteritis nodosa	2.6[a]	1.5-4.5	-[c]	
Rheumatoid arthritis	1.9[a]	1.6-2.2	1.4[b]	1.1-1.9
Giant cell arteritis	1.7[a]	1.3-2.2	-[c]	
Psoriatic arthritis	1.6[a]	1.3-2.0	1.4[b]	1.1-1.9
Polymyalgia rheumatica	1.5[a]	1.3-1.8	-[c]	
Sarcoidosis	1.5[b]	1.1-2.2	1.9[a]	1.3-2.6
UC				
Atrophic gastritis	3.1[a]	1.7-5.8	-[c]	
Rheumatoid arthritis	1.7[a]	1.4-2.1		
Giant cell arteritis	1.7[a]	1.3-2.3	-[c]	
Polymyalgia rheumatica	1.6[a]	1.3-2.0	-[c]	
CD				
Episclerit	5.9[a]	2.4-15.0	4.5[b]	1.2-17.5
Rheumatoid arthritis	2.3[a]	1.7-3.0	-[c]	
Psoriatic arthritis	2.0[a]	1.3-2.8	-[c]	
Sarcoidosis	-[c]		3.2[a]	1.6-6.6
Both[1]				
Iridocyklitis	3.6[a]	1.9-6.8	2.7	1.2-6.2
Celiac disease	6.0[a]	1.9-18.6	3/0[b,2]	
Autoimmune hepatitis	17.9[a]	2.3-141.5	7.8[b]	0.9-69.8

[a]$P < 0.00125$; [b]$P = 0.0125$-0.05; [c]$P > 0.05$; [1]Patients registered with both CD and UC; [2]No. of cases in IBD cohort/No. of cases in control cohort. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

of twenty IMDs in patients with IBD compared to matched cohorts.

Although most of the IMDs are considered to be Th1 mediated, UC was associated with more IMDs than CD. The presence of Th17 cells in UC and their ability to induce a Th1 response might explain this. Another explanation might be that certain susceptibility genes can act differently depending on the setting[25]. A gene might increase the risk of one disease while reducing the risk of others[25-27].

Extraintestinal manifestations

Ankylosing spondylitis, pyoderma gangrenosum, psoriasis, iridocyclitis, episcleritis, and primary sclerosing cholangitis (PSC) are all well described in IBD[28]. Thus the significant associations were expected. Except from PSC, these will not be discussed further.

Primary sclerosing cholangitis and gastrointestinal immune mediated diseases

PSC is predominant in men and most frequent in UC[29]. We found PSC to be associated with both types of IBD and both genders. Most striking is the association with CD which is less often described. Studies suggest that PSC is more frequent when colon is affected and a distinct subtype, PSC-IBD has been suggested[30-33]. This study does not include data on localization, severity or extension. Several PSC risk genes are shared with IBD and other IMDs[33,34]. Gene mutations influencing IL 10 signaling are identified in CD, UC and PSC. The absence of IL 10 can cause severe CD due to lack of Th1 and macrophage inhibition[33-35]. Interestingly, hepatobiliary inflammation is thought to be induced by microbial metabolites and changes in the microbiota and this inflammation is linked to the *FUT2* gene, which is also found in CD[33,34,36].

In contrast to most other studies[18,36,37], we found celiac disease to be more frequent in those with IBD regardless of type, as did another Danish study[16]. Other studies found IBD to be more common in patients with celiac disease but not vice versa[38-40]. Similarities and differences in pathogeneses might explain these conflicting results. Celiac disease is like IBD an inflammatory disorder of the intestine, often diagnosed in young individuals, more common in women, and Th1 mediated. Changes in microbiota and dysfunctional IL 18 receptor are also noted in both conditions[27,41]. Risk genes of celiac disease shared with CD relates to adaptive immunity while those shared with UC primarily relates to barrier function. Different from IBD is the absence of Th17 response, impaired autophagy and while important in celiac disease, IL 15 is not that important in IBD[41].

We found autoimmune hepatitis, primary biliary cholangitis, and atrophic gastritis to be more common in UC only. Again, results from previous studies conflict[16,18,21,42-45]. Little is known about the association with atrophic gastritis, which to our knowledge is unique to this study. Th1, 2 and 17 responses are important in IBD, PSC and primary biliary cholangitis pathogenesis. Primary biliary cholangitis and IBD have overlapping susceptibility genes, which is not the case with autoimmune hepatitis[46,47]. The pathogenesis of primary biliary cholangitis resembles those of autoimmune hepatitis and CD, dysfunctionalities in IL 12 signaling promotes a Th1 and possibly also a Th17 response, causing a granulomatous inflammation[47,48].

Endocrine diseases

UC is reported to occur more frequently in family members of patients with T1D[18,19,49]. However, three studies did not find any association[16,20,21]. This study found T1D associated with both UC and CD. Confounding due to treatment with corticosteroids is unlikely, as the mechanisms in steroid induced diabetes resemble those in type 2 diabetes[50,51]. Levels of IL 18 are elevated in CD and T1D, but not in UC. IL 18 causes a Th1 response and is likely to affect mucosal barrier function too[27,52]. PTPN2 is one of many shared risk genes[2,53]. It promotes beta cell apoptosis in T1D while causing intestinal barrier dysfunction, impaired autophagocytosis, and inhibition of Th17 in IBD[25,54]. Changes in the gut microbiota are also suggested to trigger T1D[27].

Data on autoimmune thyroiditis and IBD is sparse, similarities to IBD limited and only few risk genes overlap[55-57]. Restricted to UC only, we found OR significantly increased for Grave's disease. None was

detected for Hashimoto's thyroiditis. Similar results are reported in two other studies[18,58]. One study reports hypothyroidism more common in CD[19]. In addition, three other studies did not find any association at all[16,17,21].

Rheumatic diseases

Rheumatoid arthritis was associated with both UC and CD while psoriatic arthritis was restricted to CD. Previously published data support this[18,20,21,59]. The microbiome of the gut and skin are possible triggers in rheumatoid arthritis and psoriatic arthritis[60]. Both types of arthritis share characteristics with CD in particular. Th1 and 17 are essential in all three pathogeneses[2,61-64].

ORs for polymyalgia rheumatica and temporal arteritis were significantly increased in the IBD and UC group, not in CD. This is supported by one study while refuted by another[16,18]. Overlapping susceptibility genes suggest that Th1, Th17 and regulatory T cells are of importance to the pathogeneses[65].

ORs for Churg Strauss Syndrome and polyarteritis nodosa were significantly increased in the overall IBD group but not in the subgroups. The low number of cases calls for careful interpretation and future studies.

Other disorders

In this study, asthma was more common in both UC and CD. Both UC and allergic asthma are considered Th2 mediated. Also, a Th17 response is described in severe asthma[66]. Risk genes are associated with IL 13 and 17 production, dysfunctional regulatory T cells and regulation of Th1, 2 and 17 responses[26,66]. Studies have not found that asthma reduces the risk of IBD[67,68], rather the opposite seems more likely[17,18,20,21].

The association of sarcoidosis and IBD were restricted to UC and males with CD. Another study confirms the linkage to UC[18]. There is not much documentation for this association. The inflammation in sarcoidosis is similar to CD; granulomatous; Th1 and 17 driven; and mutations in NOD2 and IL 23 receptor gene are identified[2,69-71].

There were no cases of Sjögren's syndrome, inclusion body myositis, eosinophilic esophagitis, or autoimmune adrenalitis. This is unexpected. Some case reports have described the coexistence of Sjögren and primary adrenocortical insufficiency in IBD patients[16,72-74]. One case report describes eosinophilic esophagitis and CD[75]. While to our knowledge, no association between inclusion myositis and IBD has been reported. Although specific ICD 10 codes were used misclassification is still possible *e.g.* autoimmune adrenalitis might be registered as Addison's disease.

Strengths and limitations

The strength of this study is that it includes all patients alive with CD or UC in Denmark. The Danish population is homogenous regarding ethnicity and religion. Health care is free to all residents; thus, NPR is not biased by inclusion of specific hospitals, age groups, insurance policies, social, or financial status. As the general practitioners do not provide data, diseases not requiring hospital treatment could be underrepresented *i.e.* asthma, Grave's disease, Hashimoto's thyroiditis, and atrophic gastritis[76].

A limitation of the study is possible bias caused by varying validity of the ICD 10 codes. Only few Danish studies have addressed this issue. The average positive predictive value (PPV) of an ICD 10 diagnosis for any medical condition in the NPR varies from 65.5 % to 81%[76].

However, the completeness is 94% for both UC and CD while the PPV of UC and CD is 90% and 97% respectively[77].

The validity of T1D is like that of IBD, very high[24,78]. The PPV of asthma among hospitalized children is 85% while 65% among adults. However, a sensitivity analysis did not find the PPV in adults, low enough to nullify the hypothesis[79,80]. As a collective group the PPV of connective tissue diseases is reported as high[24]. The PPV of rheumatoid arthritis is low[81].

Despite varying validity of ICD 10 codes, most of our findings are in alignment with those of the studies using algorithms to increase the validity. Important to this study, is the occurrence of the classic extraintestinal manifestations which indicates that our results are not too biased.

Detection bias is another concern. Patients seen on regular basis by a physician such as those with IBD are more likely to be diagnosed.

To eliminate confounders like sex, age and geography in the IBD group, we used these as matching criteria. Information regarding smoking status was not available to us, thus no correction was made.

Another confounder is drug induced autoimmunity. A wide variety of drugs are suggested to induce autoimmunity. Among these are antibiotics, statins, methotrexate, thiopurines, and biological agents (anti-TNF-α agents)[82-88]. Biological agents, which are often used to treat IBD, ankylosing spondylitis, psoriasis, and rheumatoid arthritis, are paradoxically suggested to induce IMDs. No correction was made since we do not have data regarding patients' use of prescribed drugs.

While Bonferroni post-test correction reduced the risk of false positives, the risk of false negatives simultaneously increased. Knowing this, a low number of false positives were still preferred in this study.

In conclusion, our study emphasizes that immune mediated diseases are more frequent among patients with CD or UC. Our results strengthen the thesis of partially overlapping pathogeneses among some immune mediated diseases including IBD and emphasized the complexity of IBD pathogenesis. Our most important findings are the increased risk of

celiac disease and T1D in both UC and CD, but also the increased risk of primary sclerosing cholangitis in CD although not being limited to CD. Finally, when treating patients with UC or CD one should be aware of the strong association with other immune mediated diseases.

COMMENTS

Background

Extraintestinal manifestations in Crohn's disease (CD) and ulcerative colitis (UC) are well described. The authors aimed to investigate whether other immune mediated diseases were associated with inflammatory bowel disease (IBD).

Research frontiers

Most studies on the subject are small or case reports. Only few larger studies have been conducted. The authors aimed to estimate odds-ratios of developing an immune mediated diseases (IMD) in patients with IBD compared individuals without IBD.

Innovations and breakthroughs

This is one of few larger studies on the subject. It includes all patients alive with CD or UC in Denmark. Due to free health care to all residents the study is unbiased by inclusion of specific hospitals, age groups, insurance policies, social or financial status. The authors found several IMDs not considered classic extraintestinal manifestations to be significantly associated with IBD.

Applications

Physicians treating patients with IBD should obe aware of the increased risk of developing other IMDs than the classic extraintestinal manifestations. The findings support the hypothesis that shared pathogenic pathways among IMDs could exist.

Peer-review

It's a well-written and interesting manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Suspicious brush cytology is an indication for liver transplantation evaluation in primary sclerosing cholangitis

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Abstract

AIM

To investigate markers for high-grade dysplasia for the optimal timing of liver transplantation in patients with primary sclerosing cholangitis (PSC).

METHODS

Earlier data support a dysplasia-carcinoma sequence, even low- to high-grade dysplasia, in PSC-associated cholangiocarcinoma (CCA). Surveillance using endoscopic retrograde cholangiography (ERC) and brush cytology aims to detect cases of biliary dysplasia, and liver transplantation is an option in cases with suspicion of malignancy in brushing. This study investigated markers to identify patients with high-grade biliary dysplasia for optimal timing in early liver transplantation. Patients undergoing surveillance using ERC and brush cytology during 2008-2014 and who were diagnosed with biliary dysplasia in explanted liver or CCA until February 2016 were included in the study. Demographic data, cholangiography findings, laboratory values, cytological morphology and DNA ploidy were

analysed.

RESULTS

Thirty PSC patients had biliary neoplasia in the explanted liver during the study period. Sixteen of these patients had low-grade dysplasia, 10 patients had high-grade dysplasia, and 4 patients had CCA. Fifteen PSC patients diagnosed with CCA were not transplanted. Patients with low-grade dysplasia were younger. Alkaline phosphatase or carcinoembryonic antigen values did not differ between groups during surveillance, but carbohydrate antigen 19-9 was higher in CCA patients. No difference in PSC duration, ERC scores, suspicious cytology, or ploidy analysis was found between groups. No difference was observed between fibrosis stage in explanted livers. Low- and high-grade dysplasia could not be differentiated before liver transplantation based on liver enzymes, tumour markers, ERC scores, brush cytology or DNA ploidy.

CONCLUSION

Repeated suspicion of neoplasia in brush cytology should be an indication for evaluations of liver transplantation prior to the development of CCA.

Key words: Endoscopic retrograde cholangiography; Brush cytology; Cholangiocarcinoma; Biliary dysplasia

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Core tip: We investigated markers of high-grade dysplasia for the optimal timing of early liver transplantation (LT) in patients with primary sclerosing cholangitis (PSC). PSC patients in our unit undergo surveillance with endoscopic retrograde cholangiography (ERC) and brush cytology (BC) to identify evidence of dysplasia before progression to cholangiocarcinoma. Carbohydrate antigen 19-9 was higher in patients with cholangiocarcinoma, but no other differences between laboratory values, ERC scores, BC or ploidy analysis between the low-grade, high-grade or CCA groups were observed. Repeated suspicion of neoplasia in BC should be an indication for the evaluation for LT prior to the development of cholangiocarcinoma.

Boyd S, Vannas M, Jokelainen K, Isoniemi H, Mäkisalo H, Färkkilä MA, Arola J. Suspicious brush cytology is an indication for liver transplantation evaluation in primary sclerosing cholangitis. *World J Gastroenterol* 2017; 23(33): 6147-6154 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6147.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6147

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease that often presents in conjunction with inflammatory bowel disease (IBD). PSC leads to bile duct strictures and liver fibrosis. PSC also markedly increases the risk for cholangiocarcinoma (CCA), with a lifetime risk of 5%-10%[1,2]. Bile duct dysplasia is a precursor for CCA[3] in PSC. Brush cytology (BC) and imaging are used for the detection of malignancy in PSC.

The incidence of PSC is high in Nordic countries. The primary diagnosis of PSC in our hospital uses endoscopic retrograde cholangiography (ERC) with BC in all patients for individual risk stratification to estimate disease progression and risk for dysplasia[4]. PSC patients also undergo a regular surveillance programme using ERC and BC according to a previously described protocol[5], which demonstrated that the frequency of ERCs depends on disease severity in ERC and earlier results in BC and ploidy analysis. The indications for liver transplantation (LT) in PSC patients include end-stage liver disease or symptoms of the disease (*e.g.*, recurrent cholangitis). CCA is also an indication for LT in combination with chemoradiation in select centres[6]. CCA is generally a contraindication for LT in our centre. However, LT may be considered to prevent progression to CCA[4,7] in cases with biliary dysplasia.

This study evaluated risk factors in PSC patients with histologically confirmed biliary dysplasia or CCA.

MATERIALS AND METHODS

Patients

This study included PSC patients with verified biliary dysplasia or CCA. There were 588 PSC patients under surveillance at the Helsinki University Hospital during 2008-2014. PSC patients in the study population who were diagnosed with CCA or biliary dysplasia until February 2016 were included in this study. All explanted PSC livers were re-evaluated, and cases with biliary dysplasia or CCA (n = 30) were included. All PSC patients who were diagnosed with CCA during the aforementioned period who did not undergo liver transplantation were included (n = 15). At least one ERC was performed for each patient during the study period. Low-grade dysplasia (LGD) of the bile ducts was observed in 16 transplanted patients, and high-grade dysplasia (HGD), including carcinoma in situ, was observed in 10 patients with LT. CCA was observed in four patients with LT and 15 patients without LT. Suspicion of biliary dysplasia was considered an important decision criterion for evaluation for liver transplantation in 7/10 patients with HGD, 12/16 patients with LGD, and 4/4 patients with CCA. Thirty-two patients (71%) were male, and 13 patients were female.

Laboratory results

Laboratory parameters [carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), alkaline

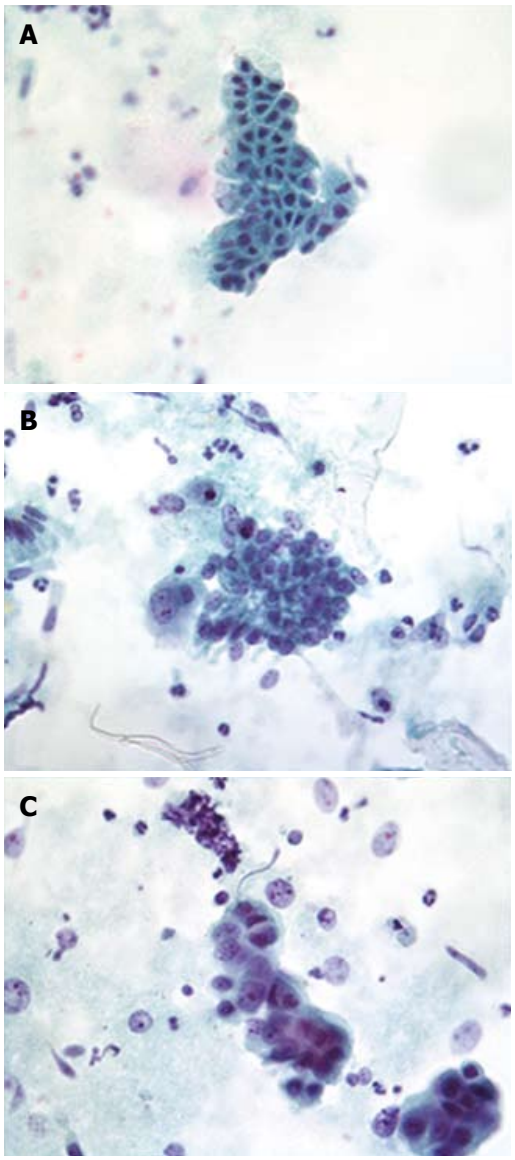


Figure 1 Representative examples of bile duct brushings. A: Benign brush cytology with inflammatory atypia; B: Brush cytology with suspicion of malignancy; C: Malignant brush cytology. Patient was diagnosed with cholangiocarcinoma.

phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, bilirubin, and international normalised ratio (INR)] were collected one day before the latest ERC.

Imaging studies

Images from the latest ERC were scored according to a modified Amsterdam score[4]. An MRI and/or CT were performed prior to LT.

Brush cytology and ploidy analysis

All brush samples and DNA flow cytometry specimens until LT or detection of CCA were included. Brush samples were re-evaluated and graded as benign (including atypical due to inflammation or regeneration), suspicious for neoplasia, or malignant (Figure 1).

Table 1 Histological findings in 30 explanted livers of primary sclerosing cholangitis patients with biliary low-grade dysplasia or high-grade dysplasia/cholangiocarcinoma

	LGD	HGD + CCA[2]	*P* value
n	16	14	
Early LT[1], *n*	12	11	1.000
Histology intrahepatic			
Liver weight, g (IQR)	1380 (1190-1717)	1408 (1262-1487)	0.910
Fibrosis stage 1-4 (IQR)	3.5 (3.0-4.0)	3.0 (2.0-4.0)	0.473
Stage 1-2, *n*	3	4	0.675
Stage 3-4, *n*	13	10	0.675
Histology extrahepatic, *n*			
Purulent cholangitis	12	6	0.142
Ulceration	11	6	0.224
Intestinal metaplasia	2	1	0.822
Squamous metaplasia	1	0	0.790

[1]In early LTs, suspicion of biliary neoplasia was an important decision criterion for LT; [2]CCA four patients; HGD ten patients. Values are median values unless otherwise indicated (IQR). *P* values: Mann-Whitney *U*-test for continuous variables, Fisher's exact test for dichotomous variables. CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile range; LGD: Low-grade dysplasia; LT: Liver transplantation.

Cytocentrifuge slides and slides from cellblocks were evaluated when cellblocks were available. Ploidy analyses were performed as previously described[7]. Briefly, a separate sample for DNA flow cytometry was obtained before the BC sample and added to RPMI 1640 medium (Gibco/Thermo Scientific, MA, United States) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 0.5% heparin and 0.5% human serum albumin. Cells were centrifuged, washed, treated with RNAse (Sigma Chemical Company, MO, USA) and stained with ethidium bromide (Sigma Chemical Company, MO, United States) in Tris-EDTA buffer. HL-60 cells were used as a normal control, and goose and salmon trout red blood cells were used as internal controls. DNA flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, Oxford, United Kingdom). The results were analysed using ModFit LT software (Becton Dickinson Immunocytometry Systems, Becton, Dickinson and Company, Franklin Lakes, NJ, United States), and a DNA index over 1.1 was considered aneuploidy.

Histology

Two pathologists, who were blinded to the BC or original interpretation, re-evaluated explanted livers and bile ducts or other histological samples (Table 1, Figure 2). Biliary dysplasia was graded as high-grade or low-grade. Patients were divided into groups (LGD, HGD, CCA) using the worst histological finding.

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistical software package version 24 (IBM, New York, NY, United States). The Jonckheere-Terpstra and Mann-Whitney *U*-tests were used to evaluate

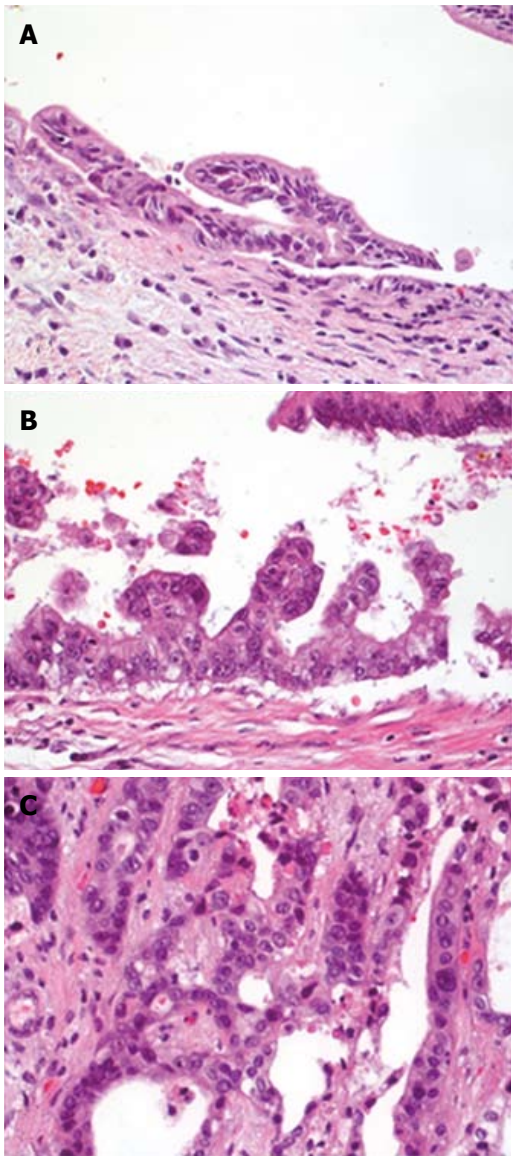


Figure 2 Histology from explanted livers diagnosed with biliary neoplasia. A: Bile duct from explanted liver with low-grade dysplasia; B: Bile duct from explanted liver with high-grade dysplasia; C: Cholangiocarcinoma from explanted liver.

differences in continuous variables between groups. Fisher's exact test was used for dichotomous variables. Bonferroni correction was used in pairwise tests, and adjusted values are presented.

Ethics
The local ethics committee approved the study protocol.

RESULTS

Demographics
There was no statistically significant gender difference between groups (Table 2), but all groups included a majority of male patients (32 males and 13 females in total). Patients with LGD were significantly younger than other patients (P = 0.003). There was no difference in PSC duration before dysplasia or CCA diagnosis. Thirty (67%) patients had IBD. Twenty-two of these 30 patients had ulcerative colitis, seven patients had Crohn's disease, and one patient had indeterminate colitis. The presence of IBD was not significantly different between groups.

Laboratory values
ALP, bilirubin, AST and ALT values were not significantly different between the three groups at the latest ERC before LT or detection of CCA. CA19-9 was higher in CCA patients (P = 0.002). CEA values did not significantly differ between groups (P = 0.081).

ERC findings
There were no significant differences in ERC scores between patient groups (Table 2). Intrahepatic disease without extrahepatic changes was observed in ERC in only two patients, and both of these patients exhibited LGD. Only mild extrahepatic ERC changes (modified Amsterdam ERC score 1-2) were observed in 11 patients, and five of these patients exhibited CCA. Abundant extrahepatic ERC changes (score $\geqslant$ 3, corresponding to dominant stricture) were observed in 32 patients. Fourteen (14/32) of these patients were diagnosed with CCA.

Brush cytology
All patients underwent ERC with BC (1-9 BC/patient, median 3 BCs). The number of available BCs did not significantly differ between groups (Table 3). Suspicious BC was observed in 11/16 patients with LGD, 8/10 patients with HGD and 4/4 patients with CCA in liver transplanted patients. Eleven of 15 patients without LT had suspicious or malignant BC (malignant 2 patients, suspicious 9 patients). Suspicious or malignant BC was observed in 34/45 patients (76%), with no difference between the neoplastic groups.

Ploidy analyses
Ploidy analysis of a brush sample was available for all patients (1-7/patient, median 3 DNA flow cytometry analyses). Aneuploidy was observed in 18/45 patients (40%), and there were no significant differences between groups. DNA indices were between 1 and 2.41 and did not significantly vary between the three groups. The median DNA index in HGD was 1.2.

Symptoms
Eighty percent of patients were symptomatic, with no difference between groups (Table 2). However, CCA patients were more often icteric than patients with LGD (P = 0.014).

Explanted livers
The median weight of explanted livers was 1399 g (range, 768-2195 g, IQR 1244-1526 g). The median

Table 2 Demographics, laboratory results and endoscopic retrograde cholangiography findings in patients with primary sclerosing cholangitis and low-grade dysplasia, high-grade dysplasia and cholangiocarcinoma

	LGD	HGD	CCA	*P* value	*P* value (LGD *vs* HGD)	*P* value (HGD *vs* CCA)	*P* value (LGD *vs* CCA)
n	16	10	19	.	.	.	.
Male/female	10/6	8/2	14/5	0.701	.	.	.
Age, yr	32 (26-48)	48 (35-59)	55 (37-60)	0.003	0.056	0.896	0.004
PSC duration, yr	4.1 (2.2-8.3)	4.4 (2.3-7.9)	4.0 (0.3-17.1)	0.948	.	.	.
Symptoms, *n*	12	8	16	0.890	.	.	.
Icterus, *n*	2	4	12	0.014	0.483	1.000	0.024
Pruritus, *n*	9	3	8	1.000	.	.	.
Cholangitis/fever, *n*	4	2	8	0.415	.	.	.
Fatigue, *n*	7	3	4	1.000	.	.	.
Pain, *n*	5	3	4	0.863	.	.	.
MELD score	7.6 (7.4-11.0)	7.6 (6.4-8.7)	7.7 (6.4-10.4)	0.883	.	.	.
IBD, *n*	10	5	15	0.307	.	.	.
ALP, U/L	175 (90-324)	89 (69-176)	237 (158-396)	0.214	.	.	.
AST, U/L	45 (32-118)	51 (34-64)	59 (38-94)	0.618	.	.	.
ALT, U/L	61 (31-113)	56 (30-102)	55 (46-112)	0.717	.	.	.
Bilirubin, μmol/L	23 (13-32)	11 (8-22)	25 (15-47)	0.354	.	.	.
CA19-9, KU/L	10 (3-22)	10 (5-16)	120 (14-415)	0.002	1.000	0.006	0.006
CEA, μg/L	1.3 (1.0-2.7)	2.4 (1.4-2.9)	2.1 (1.4-5.8)	0.081	.	.	.
mERC score							
Intrahepatic (IQR)	6 (4-6)	6 (5-7)	6 (6-6)	0.440	.	.	.
Extrahepatic (IQR)	3 (2-6)	4 (2-6)	4 (2-5)	0.613	.	.	.
Total score (IQR)	9 (7-11)	11 (8-12)	9 (8-11)	0.523	.	.	.

Values are presented as median (IQR) unless otherwise indicated. Jonckeere-Terpstra test was used to analyse differences of continuous variables, and Fisher's exact test was used for dichotomous variables. Significance values were adjusted using the Bonferroni correction for multiple tests. CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile rate; LGD: Low-grade dysplasia; mERC score: Modified Amsterdam ERC score; MELD score: Model for end-stage liver disease score; IBD: Inflammatory bowel disease; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PSC: Primary sclerosing cholangitis; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen.

Table 3 Brush cytology and DNA flow cytometry in patients with low-grade dysplasia, high-grade dysplasia or cholangiocarcinoma

	LGD	HGD	CCA	*P* value
Patients, *n*	16	10	19	
Liver transplanted, *n*	16	10	4	
BCs, *n* (IQR)	4.0 (2.3-5.0)	4.5 (1.8-5.3)	2.5 (1.8-5.3)	0.219
BC suspicious/malignant	11	8	15	0.746
Flow cytometry, *n* (IQR)	3.0 (2.3-4.0)	3.0 (1.8-5.0)	2.0 (1.0-4.3)	0.195
DNA-index (highest, IQR)	1.0 (1.0-1.2)	1.2 (1.0-1.8)	1.0 (1.0-1.2)	0.745
Aneuploid	6	5	7	0.795

Numbers are median (IQR) or *n*. *P* values: Jonckeere-Terpstra test for continuous variables, and Fisher's exact test for dichotomous variables. BC: Brush cytology; CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile rate; LGD: Low-grade dysplasia.

fibrosis stage was 3 (range 1-4[8]), with no difference between the groups. Only mild fibrosis (stage 1-2) was observed in 3/16 of the patients with LGD, and 4/14 patients with HGD or CCA. The remaining patients exhibited a fibrosis stage of 3-4. Histologically typical PSC morphology was observed in all liver samples, including onion-skin fibrosis around the bile ducts and cholangitis. No dysplasia in peripheric bile ducts was observed. All patients with HGD also exhibited LGD of the bile ducts. All CCA patients also exhibited HGD of the bile ducts. Hepatocellular carcinoma was observed in two patients, who also exhibited LGD.

Cholangiocarcinoma

Nineteen patients exhibited CCA. Four CCAs were diagnosed in explanted livers after LT. Two CCAs were diagnosed at laparotomy during a planned LT. Two CCAs were detected at autopsy. A histological sample from the main tumour or metastasis was obtained during surgery or core-needle biopsy in eight patients. Two CCAs were verified with imaging and (malignant) BC, and one CCA was verified using imaging only. CCAs were diagnosed a median of 4.0 years after PSC diagnosis (range, 0-19 years). Six CCAs were detected less than one year after PSC diagnosis, two of these CCAs were detected at a diagnostic ERC. The localisation of CCA was perihilar or extrahepatic in 14 cases and intrahepatic in four cases. The origin of the tumour could not be determined in one patient with peritoneal carcinosis. The median time from the latest BC until CCA diagnosis was 53 d. BC was malignant in two CCA patients, and it remained suspicious in 13 patients. BCs were benign in four CCA patients. BC was suspicious in two of the patients with intrahepatic CCA and benign in two of these patients.

DISCUSSION

Forty-two patients with histologically confirmed biliary neoplasia were found during ERC surveillance: 16 patients with LGD, 10 patients with HGD and 16 patients with CCA. An additional 3 CCAs were

detected without histological confirmation. Patients with LGD were younger. AST, ALT or CEA values did not differ between the groups. CA19-9 was significantly elevated only in patients with CCA. No difference in PSC duration, ERC changes, suspicious BC, ploidy analysis, presence of IBD or histological fibrosis was found between the three groups. It was impossible to differentiate LGD from HGD based on liver enzymes, tumour markers, or BC. Evaluations for LT should already be performed when BC is repeatedly suspicious.

The number of cytological samples suspicious for neoplasia was equal in all three neoplastic groups. The sensitivity of BC to detect biliary neoplasia was quite low (43%) in earlier studies[9]. The sensitivity to detect CCA with BC (neoplasia suspicion or malignant BC) in our previous study was up to 71% in a large, unselected patient population[5]. This high accuracy was thought to be a result of the sampling method[4] and the protocol used in our hospital. Two experienced gastroenterologists perform the ERCs, and the cellular yield is generally very high. A dedicated team of cytopathologists also perform the cytological interpretation. However, biliary neoplasia is not always found in the explanted liver, even with suspicious BC. Twenty-one patients exhibited benign biliary histology in the explanted liver in our earlier study, and four of these patients had suspicious BC[5]. It would be clinically relevant to differentiate the HGD group from the other two neoplastic groups before LT because the timing of an early LT would likely exhibit the most benefit. However, we report that cytology alone did not separate three groups with neoplastic biliary changes. Neoplastic biliary changes were also not detected in liver biopsy because biliary dysplasia was not observed in the peripheral bile ducts. However, HGD may be diagnosed with BC in select cases[10].

We also used DNA flow cytometry in addition to BC in selected cases (*e.g*., advanced ERC changes or previously suspicious BC). Based on earlier studies[7,11], aneuploidy was not more commonly observed in CCAs. This result may reflect the fact that cancer cells in CCA may not be reachable for brushing because of their growth pattern or desmoplasia. The median DNA index was higher in patients with HGD, but this difference was likely the result of the low number of cases, and statistical significance was not observed. The demand for more cases to reach significance is also based on the fact that only a portion of the dysplasias, or even CCAs, presented with aneuploid DNA. Better markers for HGD than aneuploidy are needed. The combination of genetic abnormalities with BC may help distinguish benign and malignant bile duct strictures[12,13]. However, there are no specific markers to detect HGD without histology.

CA19-9 levels often rise with CCA, but it is not specific for CCA[14,15]. CA19-9 may be markedly elevated in up to 32% of PSC patients in the absence of CCA[15]. CA19-9 was elevated mostly in CCA patients in the present study, which supports the use of CA19-9 as a tumour marker for CCA. However, it is not suitable for biliary dysplasia screening. Several surveillance algorithms of PSC recommend the use of CA19-9[2,16,17]. Elevated CEA values were also commonly observed in CCA patients but only immediately prior to the CCA diagnosis (data not shown). These findings suggest that CA19-9 and CEA are late markers, and they cannot be used for the detection of premalignant lesions. The elevation of CA19-9 generally indicates that the dysplasia surveillance programme has actually failed to find cases with dysplasia. CA19-9 is not expressed in 5%-10% of the population who have the Lewis a- b-genotype[18], and the fucosyltransferase genotype influences CEA levels[19].

ALP is generally elevated at PSC diagnosis, and normalisation of ALP is associated with a better prognosis[20-24]. ALP levels are not helpful in differentiating HGD from LGD, which was observed in this study. CCA patients were not younger than other patients, and there was up to 19 years of time between PSC and CCA diagnosis. One-third (6/19) of CCAs were detected within one year of PSC diagnosis in the present study. Ten of the 19 CCA patients in this study were diagnosed with PSC prior to the beginning of this study period (2008). Therefore, the effect of this surveillance programme could not be evaluated for the entire PSC population. Patients with LGD were significantly younger than patients with CCA or HGD. Retrospectively, some of these patients could have waited for the LT if there were no symptoms, but this study demonstrated that it is generally impossible to diagnose HGD prior to LT.

ERC findings were similar in all patient groups, which is likely due to several end-stage PSCs with cirrhosis in the LGD group. ERC with BC is routinely performed for the screening and detecting of biliary dysplasia before LT in our hospital, even LT due to end-stage cirrhosis or symptoms. Our results demonstrate that the ERC score is not helpful for the differentiation of these three groups. Our strategy significantly differs from the practice in many US transplantation centres, where pre-liver transplant screening for CCA in patients with PSC is routinely performed in only 30% of centres[25].

LGD was observed in conjunction with HGD in all cases, and HGD of the bile ducts was observed in all four CCA patients who received LT. These CCAs were discovered after LT in the explanted liver. These findings support the dysplasia-CCA sequence as described by Lewis *et al*[3]. This theory of a dysplasia-cancer sequence, which was indirectly demonstrated in this study, justifies the dysplasia surveillance programme of PSC. However, further tools are needed to differentiate premalignant cases.

All HGDs and 14/19 CCAs were located in perihilar or extrahepatic bile ducts, which supports earlier studies that demonstrated that most CCAs were located in perihilar or distal bile ducts[26]. CCA or dysplasia in

these locations is generally reachable using BC, and surveillance with BC may be justified. However, we observed 19 CCAs in these patients compared to 26 with premalignant lesions. One CCA was diagnosed in a patient who was dropped from the surveillance due to advanced age. Two CCAs were detected during the planned LT, and the LT was cancelled. Four CCAs were detected after LT, CCA was diagnosed in the explanted liver, and two of these patients had clean perioperative pathology samples. Two CCA patients were not under surveillance for years, but they returned with symptoms. The surveillance protocol was not optimal for all patients, and they entered the surveillance programme gradually. If HGD is considered the highest risk for CCA and patients diagnosed with CCA during the first year after PSC diagnosis are excluded, then at least 43% of CCAs were possibly prevented using early LT.

Early CCA is an indication for LT combined with chemo- and radiotherapy in select centres[6]. Surveillance of biliary dysplasia using BC provides another option, with LT being performed at an earlier phase to avoid oncological treatment. The indications of LT in PSC patients in most centres include end-stage liver disease or symptomatic disease (*e.g.*, recurrent cholangitis). CCA is generally a contraindication for LT in our centre. LTs due to PSC have been performed since 1984 in our unit, and dysplasia in ERC has been considered a partial indication for LT in PSC patients since 2001[27].

The limitation of this study is the small number of patients, which restrains the findings of this study, and some significant differences may be missed. Some patients who subsequently developed CCA did not follow the surveillance protocol until they became symptomatic. Our hospital is the only hospital in our country that performs organ transplantations. Therefore, no liver transplantations were missed during surveillance.

In conclusion, there is currently no reliable method to differentiate LGD from HGD prior to LT. CA19-9 rises with CCA but not in biliary dysplasia. Repeated suspicious BC, which suggests dysplasia and a high risk for future CCA, may be an indication for LT.

COMMENTS

Background

Primary sclerosing cholangitis (PSC) patients exhibit an elevated risk for cholangiocarcinoma (CCA), with a lifetime risk of 5%-10%. Biliary dysplasia is a precursor of CCA, and liver transplantation (LT) may be considered to prevent progression to CCA in cases with biliary dysplasia. A more common indication for LT is end-stage liver disease or disease symptoms. CCA develops via a low-grade dysplasia (LGD) to high-grade dysplasia (HGD) to CCA sequence. PSC patients in our centre undergo regular surveillance with endoscopic retrograde cholangiography (ERC) and brush cytology (BC) to detect biliary dysplasia, and patients with repeated suspicious findings are referred for evaluation of LT. The diagnosis of HGD prior to consideration for LT would be beneficial because of the high risk of progression to CCA. This study compared ERC, BC, ploidy analysis, and laboratory values between patients diagnosed with CCA, HGD or LGD.

Research frontiers

Biliary dysplasia is an established indication for LT in few transplantation centres. BC is used to detect biliary neoplasia, but HGD may be verified from brushing only in selected cases.

Innovations and breakthroughs

This study found that differentiating HGD and LGD prior to histological verification in the explanted liver is not practically possible. BC, laboratory values and ERC scores did not differ between patients with LGD, HGD and CCA, with the exception of CA19-9, which was elevated in CCA patients.

Applications

This study suggests that the patient should be referred for evaluation of LT when biliary neoplasia is repeatedly suspected in BC of a PSC patient to prevent progression to CCA.

Terminology

ERC - an endoscopic procedure that enables an evaluation of bile ducts by injecting contrast medium to the bile ducts for radiographic visualisation.

Peer-review

The paper is novel, and an interesting study evaluating clinical and laboratory characteristics of PSC patients in order to identify subjects with biliary dysplasia that could benefit from early liver transplantation. The figures are good and eye-catching.

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ORIGINAL ARTICLE

Retrospective Study

Management of gastric mucosa-associated lymphoid tissue lymphoma in patients with extra copies of the *MALT1* gene

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Abstract

AIM

To identify the clinical features of gastric mucosa-associated lymphoid tissue (MALT) lymphoma with extra copies of *MALT1*.

METHODS

This is a multi-centered, retrospective study. We reviewed 146 patients with MALT lymphoma in the stomach who underwent fluorescence *in situ* hybridization analysis for t(11;18) translocation. Patients were subdivided into patients without t(11;18) translocation or extra copies of *MALT1* (Group A, *n* = 88), patients with t(11;18) translocation (Group B, *n* = 27), and patients with extra copies of *MALT1* (Group C, *n* = 31). The clinical background, treatment, and outcomes of each group were investigated.

RESULTS

Groups A and C showed slight female predominance, whereas Group B showed slight male predominance. Mean ages and clinical stages at lymphoma diagnosis were not different between groups. Complete response was obtained in 61 patients in Group A (69.3%), 22 in Group B (81.5%), and 21 in Group C (67.7%). *Helicobacter pylori (H. pylori)* eradication alone resulted in complete remission in 44 patients in Group A and 13 in Group C. In Group B, 14 patients underwent radiotherapy alone, which resulted in lymphoma disappearance. Although the difference was not statistically significant, event-free survival in Group C tended to be inferior to that in Group A (*P* = 0.10).

CONCLUSION

Patients with t(11;18) translocation should be treated differently from others. Patients with extra copies of *MALT1* could be initially treated with *H. pylori* eradication, similar to patients without t(11;18) translocation or extra copies of *MALT1*.

Key words: Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; Gastric neoplasms; Esophagogastroduodenoscopy; t(11;18) translocation; Trisomy 18

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Core tip: We subdivided and retrospectively reviewed 146 patients with gastric MALT lymphoma into patients without t(11;18) translocation or extra copies of *MALT1* (Group A, *n* = 88, 60.3%), patients with t(11;18) translocation (Group B, *n* = 27, 18.5%), and patients with extra copies of *MALT1* (Group C, *n* = 31, 21.2%). Groups A and C exhibited similar clinical characteristics. *Helicobacter pylori* eradication alone resulted in complete remission in approximately half the patients in Group A and Group C. Consequently, patients with extra copies of *MALT1* could be treated similar to patients without t(11;18) translocation or extra copies of *MALT1*.

Iwamuro M, Takenaka R, Nakagawa M, Moritou Y, Saito S, Hori S, Inaba T, Kawai Y, Toyokawa T, Tanaka T, Yoshino T, Okada H. Management of gastric mucosa-associated lymphoid tissue lymphoma in patients with extra copies of the *MALT1* gene. *World J Gastroenterol* 2017; 23(33): 6155-6163 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6155.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6155

INTRODUCTION

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is one of the non-Hodgkin lymphomas, originating in tissues or organs outside of the lymph nodes. Typically, this neoplasm involves the gastrointestinal tract, thyroid, ocular adnexa, lungs, salivary glands, liver, and skin[1,2]. Of these organs, the stomach is the most frequently identified primary site. Approximately two-thirds of patients with gastric MALT lymphoma have chronic *Helicobacter pylori* (*H. pylori*) infection in the stomach, which is believed to be the causative organism. Clinically, eradication of *H. pylori* is the first-line management of choice, resulting in regression of lymphoma in 75% to 80% of patients[3]. Meanwhile, cases with chromosomal abnormalities of t(11;18)(q21;q21)/*API2-MALT1* translocation have been shown to be resistant to *H. pylori* eradication. Consequently, evaluation of t(11;18)(q21;q21) translocation at the initial workup is valuable to forecast the response to antibiotic therapy against *H. pylori*[4].

Previously, we reported a case of gastric MALT lymphoma with trisomy 18[5]. In that case, although there were no fusion genes of *API2-MALT1*, trisomy 18 was identified as extra copies of *MALT1*, using a fluorescence *in situ* hybridization (FISH) analysis for t(11;18)(q21;q21)/*API2-MALT1* translocation, since *MALT1* is located on chromosome 18q21. The patient required radiation therapy to treat the gastric lymphoma lesions, because the gastric lesions only partially improved after eradication therapy for *H. pylori*. Though radiation therapy resulted in complete remission, MALT lymphoma recurred in the stomach 16 mo later. Previous studies investigating chromosome aneuploidy in MALT lymphomas also reported that trisomy 18 might be indicative of progression or relapse in patients with gastric MALT

lymphoma[6-8]. Based on our experience and previous reports, we hypothesized that gastric MALT lymphoma with extra copies of the *MALT1* gene may be resistant to *H. pylori* eradication and may show more frequent progression or relapse than those without chromosomal aberrations. However, no studies have described the initial treatment and response in such patients. The purpose of this study is to reveal the clinical characteristics and outcomes of gastric MALT lymphoma in patients with t(11;18)(q21;q21)/*API2-MALT1* translocation or extra copies of *MALT1*, in order to determine initial treatment strategies for cases presenting with chromosomal aneuploidy.

MATERIALS AND METHODS

Letters of inquiry regarding patients with gastric MALT lymphoma were sent from the Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, to ten collaborating institutions. The inclusion criteria were: (1) patients with pathologically diagnosed MALT lymphoma in the stomach, and (2) patients who underwent fluorescence *in situ* hybridization analysis for t(11;18)(q21;q21)/*API2-MALT1* translocation on biopsy samples from the gastric MALT lymphoma lesions. FISH analysis was performed for all patients on fresh biopsy samples using dual-color, dual-fusion translocation probes for *API2*/*MALT1*. MALT lymphoma with a large-cell component was excluded from this study. Finally, a total of 146 patients diagnosed with gastric MALT lymphoma between October 1997 and November 2015 were identified. Thus, these patients were retrospectively registered in this study.

According to the FISH result for t(11;18) translocation, patients were subdivided into three groups as follows: (1) Group A: patients without t(11;18)(q21;q21)/*API2-MALT1* translocation or extra copies of *MALT1* (Figure 1A), (2) Group B: patients with t(11;18) translocation (Figure 1B), and (3) Group C: patients with extra copies of *MALT1* (Figure 1C). To determine the patient characteristics of gastric MALT lymphoma with or without chromosomal aberrations, we retrospectively examined the gender, age at diagnosis, endoscopic features, clinical stages according to Lugano classification[9], *H. pylori* infection status, treatments, response to treatments, and outcomes. *H. pylori* infection status was examined by urea breath tests, rapid urease tests, microscopic observations or culture tests on endoscopically biopsied specimens, stool antigen tests, serum or urine antibody tests, or a combination of these methods. Success of *H. pylori* eradication was confirmed by urea breath tests, rapid urease tests, microscopic observations or culture tests on endoscopically biopsied specimens, or stool antigen tests. The follow-up period was defined as the period from the lymphoma diagnosis to death from any cause or to the patient's last hospital visit.

Macroscopic features of gastric lymphoma lesions observed during esophagogastroduodenoscopy were classified into the following six subtypes[10]: (1) erosions/ulcers; (2) early gastric cancer-like lesion that formed a slightly depressed area; (3) whitish mucosa; (4) cobblestone appearance; (5) submucosal tumor-like lesion; and (6) mixed (a combination of these five subtypes). All cases were reviewed and their subtypes were classified by board certified endoscopists.

Event-free survival was measured from diagnosis until documented progression/relapse, death from primary disease, or commencement of the second treatment for any reason. For the comparisons of the two groups, statistical analyses including *t*-tests, χ^2 tests, and *F*-tests were performed using JMP 8.0.1 software (SAS Institute, Cary, NC, United States). Cumulative event-free probabilities were calculated by Kaplan-Meier analysis and log-rank tests were performed using the JMP 8.0.1 software. $P < 0.05$ was considered to indicate a statistically significant difference. The present study was approved by the Ethical Committee of the Okayama University Hospital and adhered to the Declaration of Helsinki.

RESULTS

In this study, clinical data of 146 patients (76 women and 70 men) were collected from nine institutions (Table 1). FISH analysis for t(11;18)(q21;q21)/*API2-MALT1* revealed that t(11;18) translocation and extra copies of *MALT1* were not identified in 88 patients (Figure 1A). Fusion genes of *API2-MALT1* indicating t(11;18)(q21;q21)/*API2-MALT1* translocation were detected in 27 patients (10 women and 17 men) (Figure 1B). Extra copies of *MALT1* were found in 31 patients; the ratio of *MALT1* signals to *API2* signals was 3:2 in 28 patients, suggesting trisomy 18 (Figure 1C), and 4:2 in one patient, suggesting tetrasomy 18, (Figure 1D). The remaining two patients had a combination of lymphoma cells with either trisomy 18 or tetrasomy 18. In one patient with extra copies of *MALT1*, several lymphoma cells had six *MALT1* signals with four *API2* signals, which is considered as trisomy 18 with tetraploidy (Figure 1E).

Patients without t(11;18) translocation or extra copies of *MALT1* (Group A) showed slight female predominance (48 women and 40 men), while patients with t(11;18)(q21;q21) translocation (Group B) showed slight male predominance (10 women and 17 men). However, there was no statistically significant difference between Groups A and B regarding sex ratio ($P = 0.11$). Patients with extra copies of *MALT1* (Group C) showed slight female predominance (18 females and 13 males) compared to Group A ($P = 0.73$). The mean ages at lymphoma diagnosis was 65.9 ± 12.3 years in Group A, 64.2 ± 11.2 years in Group B, and 65.0 ± 14.9 years in Group C and was not different between groups. Lymphoma lesions were localized in the stomach without lymph node or other organ

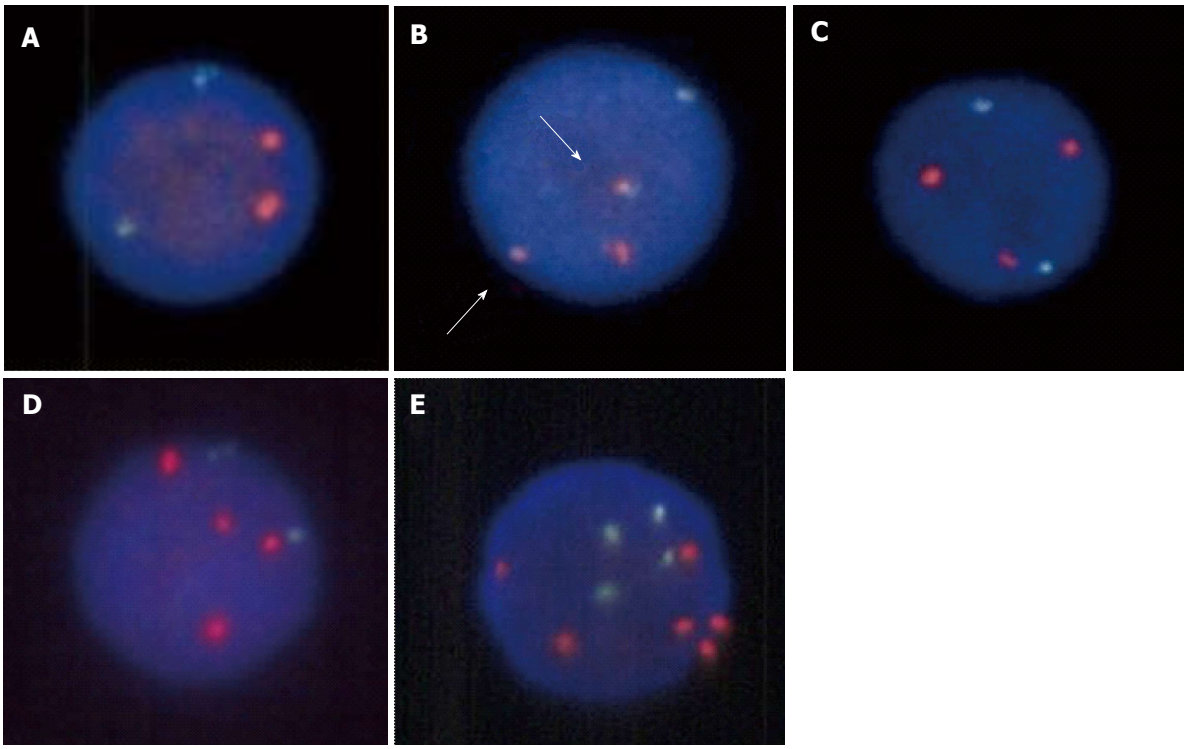


Figure 1 FISH images of the lymphoma cells. In the FISH analysis for t(11;18)(q21; q21) translocation, *API2* is visualized as a green signal and *MALT1* as a red signal. A: Two green signals and two red signals are seen in cases without t(11;18) translocation or extra copies of *MALT1*. B: Fusion genes of API2-*MALT1* are detected as yellow signals, indicating t(11;18) translocation (B, arrows). C: Although no fusion genes are visible, an extra copy of *MALT1* is noted, indicating trisomy 18. D: Two extra copies of *MALT1* are visible, indicating tetrasomy 18. E: In one patient, several lymphoma cells have six *MALT1* signals with four API2 signals, which is considered as trisomy 18 with tetraploidy.

Table 1 Clinical backgrounds of the study subjects *n* (%)

	Total	Group A: No chromosome aberration[1]	Group B: t(11;18) positive	Group C: Extra copies of *MALT1*	Group A *vs* B, *p* value	Group A *vs* C, *p* value
n	146	88	27	31		
Sex						
Male	70 (47.9)	40 (45.5)	17 (63.0)	13 (41.9)	0.11	0.73
Female	76 (52.1)	48 (54.5)	10 (37.0)	18 (58.1)		
Age (mean ± SD, yr)	65.4 ± 12.6	65.9 ± 12.3	64.2 ± 11.2	65.0 ± 14.9	0.53	0.75
Stage (Lugano system)						
I	131	81	24	26	0.92[2]	0.29[2]
II 1	3	1	1	1		
II 2	1	1	0	0		
IV	11	5	2	4		
H. pylori						
Positive	89 (61.0)	66 (75.0)	2 (7.4)	21 (67.7)	< 0.01	0.439
Negative	57 (39.0)	22 (25.0)	25 (92.6)	10 (32.3)		

[1]Patients without t(11;18) translocation or extra copies of *MALT1*; [2]stage I and II 1 *vs* II 2 and IV.

involvement in most of the patients (131/146, 89.7%), thus they were classified as stage I according to the Lugano system of staging[10]. *H. pylori* infection status was examined in all patients. In Group A, 75.0% of the patients were positive for *H. pylori*. Group C had similar positivity for *H. pylori* (67.7%). In contrast, only 7.4% of the patients in Group B had *H. pylori* infection ($P < 0.01$, *vs* Group A). Endoscopic features are summarized in Table 2. Overall, gross findings of the gastric lymphoma lesions were as follows: erosions/ulcers in 44 patients (30.1%), whitish mucosa in 42 patients (28.8%), submucosal tumor-like lesions in 29 patients (19.9%), cobblestone appearance in 17 patients (11.6%), early gastric cancer-like lesions in 10 patients (6.8%), and mixed lesions in four patients (2.7%). The ratio of each macroscopic morphology was similar between Groups A and C. Moreover, no patients in Group B presented with submucosal tumor-like lesions.

Treatments and outcomes are shown in Table 3. Follow-up periods were 3.9 ± 3.1 years in Group A, 5.1 ± 4.5 years in Group B, and 2.9 ± 2.2 years in

Table 2 Endoscopic features of the gastric lesions *n* (%)

	Total	Group A: No chromosome aberration[1]	Group B: t(11;18) positive	Group C: Extra copies of *MALT1*
Macroscopic feature				
Erosions/ulcers	44 (30.1)	23 (26.1)	9 (33.3)	12 (38.7)
Early gastric cancer-like	10 (6.8)	7 (8.0)	2 (7.4)	1 (3.2)
Whitish mucosa	42 (28.8)	25 (28.4)	12 (44.4)	5 (16.1)
Cobblestone appearance	17 (11.6)	12 (13.6)	3 (11.1)	2 (6.5)
Submucosal tumor	29 (19.9)	19 (21.6)	0	10 (32.3)
Mixed	4 (2.7)	2 (2.3)	1 (3.7)	1 (3.2)

[1]Patients without t(11;18) translocation or extra copies of *MALT1*.

Table 3 Treatment regimens and outcomes of the study subjects

	Total	Group A:No chromosome aberration[1]	Group B: t(11;18) positive	Group C: Extra copies of *MALT1*
Treatment				
Eradication alone	85	62	3	20
RT alone	23	8	15	0
Chemotherapy alone	8	3	2	3
RT and chemotherapy	2	1	0	1
Eradication RT	17	6	5	6
Eradication and chemotherapy	3	2	0	1
Eradication and RT and chemotherapy	2	1	1	0
None	6	5	1	0
Outcome				
Live without disease	103	61	21	21
Live with disease	33	20	5	8
Live, unknown disease status	6	5	1	0
Dead by other cause	3	1	0	2
Dead by MALT lymphoma	1	1	0	0
Follow-up period (mean ± SD, yr)	3.9 ± 3.3	3.9 ± 3.1	5.1 ± 4.5	2.9 ± 2.2

[1]Patients without t(11;18) translocation or extra copies of *MALT1*. RT: Radiotherapy; MALT: Mucosa-associated lymphoid tissue.

Group C. Only four patients died during the follow-up period: One patient in Group A died 2.4 years after lymphoma diagnosis because of concomitant advanced lung cancer, although complete response was obtained for gastric MALT lymphoma after *H. pylori* eradication. Two patients in Group C died of pneumonia 1.1 years and 2.0 years after lymphoma diagnosis, respectively. The other patient in Group A had stage IV lymphoma involving the stomach, ileum, colon, rectum, and lymphadenopathies of the neck, supraclavicular, subphrenic, and retroperitoneum. The patient underwent chemotherapy with cyclophosphamide, vincristine sulfate, and prednisone, followed by rituximab monotherapy and yttrium-90 ibritumomab tiuxetan monotherapy. However, the patient died of refractory lymphoma 1.4 years after the initial diagnosis due to intestinal perforation. Since pathological analysis was waived because of the patient's deteriorated condition, it was unknown whether histologic transformation occurred in the terminal stage. All patients, except for the four mortality cases, were alive at the patient's last visit to each institution. Five patients in Group A and one in Group B did not undergo esophagogastroduodenoscopy during the follow-up period, thus their lymphoma status, whether remitted or unchanged, was unknown. Complete response was obtained in 61 patients in Group A (69.3%), 22 in Group B (81.5%), and 21 in Group C (67.7%). On the other hand, lymphoma lesions were partially remitted or unchanged in 20 patients in Group A (22.7%), four in Group B (14.8%), and eight in Group C (25.8%).

Table 4 Treatment regimens resulted in complete remission

	Total	Group A: No chromosome aberration[1]	Group B: t(11;18) positive	Group C: Extra copies of *MALT1*
n	103	61	21	21
Treatment				
Eradication alone	58	44	1	13
RT alone	20	6	14	0
Chemotherapy alone	4	1	1	2
RT and chemotherapy	2	1	0	1
Eradication and RT	13	5	4	4
Eradication and chemotherapy	3	2	0	1
Eradication, RT and chemotherapy	1	0	1	0
None	2	2	0	0

[1]Patients without t(11;18) translocation or extra copies of *MALT1*. RT: Radiotherapy; MALT: Mucosa-associated lymphoid tissue.

With regard to treatment regimens, most of the patients without t(11;18) translocation or extra copies of *MALT1* (Group A) were treated with *H. pylori* eradication alone (*n* = 62, 70.5%). More than half of the patients with t(11;18)(q21;q21) translocation (Group B) were treated with radiotherapy alone (*n* = 15, 55.6%). Approximately two-thirds of the patients with extra copies of *MALT1* (Group C) were treated with *H. pylori* eradication alone (*n* = 20, 64.5%).

Table 4 shows treatment regimens that resulted in complete remission of MALT lymphoma lesions. Complete remission of lymphoma was observed in

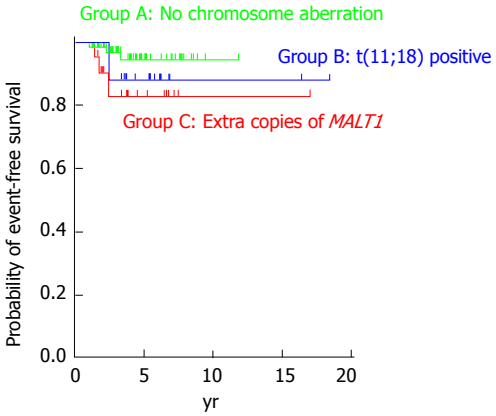


Figure 2 Cumulative event-free probabilities for the three groups. Although event-free survival of Group C patients appears to be inferior to that of the patients of the other two Groups, log-rank test revealed that the difference between Groups A and C was not statistically significant (P = 0.10).

44 (72.1%) and 13 (61.9%) patients in Groups A and C, respectively, by *H. pylori* eradication alone. In contrast, only one (4.5%) patient achieved complete remission after *H. pylori* eradication alone, whereas 14 (63.6%) patients achieved complete remission after radiotherapy alone in Group B. Figure 2 shows cumulative event-free probabilities between the three groups. Although event-free survival of Group C patients seems to be inferior than that of the Group A and Group B patients, log-rank test revealed that the difference between Groups A and C was not statistically significant (P = 0.10).

DISCUSSION

To our knowledge, the present report is the largest retrospective study investigating chromosome aberrations in gastric MALT lymphoma. Our review of 146 patients revealed that 31 patients (21.2%) had extra copies of *MALT1*. Prevalence of trisomy 18 in MALT lymphomas has been reported to range from 5% to 39%[11-17]. Other chromosomal abnormalities involved in MALT lymphomas are trisomies 3, 7 and 12. The incidence varies between reports in the literature: trisomy 3 can be detected in 6% to 24%, trisomy 7 in 3% to 15%, and trisomy 12 in 3% to 38%[17].

The clinical significance of these chromosomal numerical changes has been investigated in several studies[6-8,18-20]. Tanimoto *et al*[19] investigated 34 patients with primary ocular adnexal MALT lymphoma and found that disease recurrence was documented in five cases, all of which had trisomy 18. Statistical analysis revealed that the time to recurrence was significantly shorter in patients with trisomy 18 than in those without trisomy 18 (P = 0.05). We also reported a patient with gastric MALT lymphoma with trisomy 18 in whom only partial improvement was documented after eradication therapy for *H. pylori*, and lymphoma recurred in the stomach 16 months after radiotherapy[5]. On the other hand, Taji *et al*[17] retrospectively reviewed 13 patients with localized MALT lymphoma in the stomach, and reported that all patients with unresponsive or progressive disease had t(11;18) translocation or trisomy 3. Conversely, one patient with complete response had trisomy 18, and another patient with complete response had both trisomies 12 and 18. Based on these findings, the authors speculate that trisomy 3 is related to resistance to *H. pylori* eradication therapy and disease recurrence in gastric MALT lymphoma, whereas trisomies 12 and 18 are not. Nakamura and colleagues reviewed 90 cases of gastric MALT lymphoma and reported that extra copies of *MALT1* were found in 18 of 71 (25%) cases[6]. Although overall survival was not associated with the presence of extra copies of *MALT1*, disease progression or relapse of lymphoma was more frequently observed in patients with extra copies of *MALT1*. Possible correlations between extra copies of *MALT1* and progression or relapse of lymphoma have been described in other studies as well[7,8]. In the present study, although the difference was not statistically significant, log-rank test revealed similar tendencies (Figure 2). Consequently, patients with additional copies of *MALT1* may require more frequent clinical follow-up.

In the present study, the sex ratio of the patients with extra copies of *MALT1* (Group C) was similar to that of the patients without t(11;18) translocation or extra copies of *MALT1* (Group A), with the number of females being greater than males. On the contrary, although the difference was not statistically significant, patients with t(11;18) translocation (Group B) included more males than females. It was also noteworthy that Group C patients had similar prevalence of *H. pylori* infection to Group A. Other patient characteristics, such as the age at lymphoma diagnosis, clinical stages, and macroscopic morphology were not different between Groups A and C. The majority of Group A and Group C patients were treated with *H. pylori* eradication alone. Moreover, *H. pylori* eradication alone resulted in complete remission of lymphoma in 44 patients in Group A and 13 in Group C. Overall, the results observed in this study suggest that patients with trisomy and tetrasomy 18 require no distinct treatment from patients without aberrations. To our knowledge, the present study is the first to reveal clinical responsiveness to *H. pylori* eradication in gastric MALT lymphoma patients with extra copies of *MALT1*. In addition, in the present study, FISH analysis was performed by using fresh biopsy samples, whereas previous reports primarily involved analyses of archival pathologic specimens with the aim of clarifying the etiology of chromosomal aberrations. We believe that our results would be more readily interpreted in actual clinical settings for physicians responsible for gastric MALT lymphoma patient therapy.

The present study included 27 patients (18.5%)

with t(11;18)(q21;q21)/*API2-MALT1* translocation. t(11;18)(q21;q21)/*API2-MALT1* is the most frequent translocation among gastric, pulmonary, intestinal, and cutaneous MALT lymphomas, accounting for 13% to 35% of cases[3,21,22]. Other chromosomal aberrations detected in MALT lymphomas include t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(q27;q32), and t(3;14)(p14.1;q32)[3]. Several of such karyotypic alterations have been known to activate the nuclear pathway, which is considered to lead to lymphomagenesis. Thus, these translocation-positive MALT lymphomas arise independent of *H. pylori* infection and are unresponsive to antibiotic treatment[3]. Moreover, these patients often show a late response and lymphoma relapse during follow-up[18,23]. Regardless of the adverse clinical features, t(11;18) translocation is infrequently identified in transformed MALT lymphoma or diffuse large B-cell lymphoma, indicating that high-grade transformation is less likely to occur in translocation-positive MALT lymphomas than in those without chromosome aberration[3,8,18,24]. In this context, polymerase chain reaction or FISH analysis for t(11;18) translocation is essential during the initial workup to determine the appropriate treatment strategy[25]. Of additional clinical interest is that trisomy and tetrasomy 18 can be detected as extra copies of *MALT1* by FISH analysis, since *MALT1* is located on chromosome 18[18,20].

In our study, all patients with t(11;18) translocation (Group B), except two, were negative for *H. pylori* infection. With regard to the macroscopic morphology of the gastric lesions, no patients in Group B presented with submucosal tumor-like lesions (Table 2). We speculate that this result reflects the lower proliferative potential of MALT lymphoma cells with t(11;18) translocation, compared to that of cells without t(11;18) translocation or extra copies of *MALT1*. Since almost no patients in Group B had *H. pylori* infection, most of the patients were treated with radiotherapy and/or chemotherapy. These clinical features were concordant with previously reported characteristics of MALT lymphoma with t(11;18) (q21;q21)/*API2-MALT1* translocation[8,22-24].

There were several limitations associated with this study. First, the presence of extra copies of *MALT1* is often suggestive of partial or complete trisomy/tetrasomy 18; both are different. Trisomy/tetrasomy 18 should be determined only when extra copies are identified by FISH using centromere-specific probes for chromosome 18, or when gains of chromosome 18 are confirmed by array comparative genomic hybridization or by cytogenetic analysis (G-banding). However, these assays could not be performed because of the retrospective nature of this study. Second, not all chromosome alterations were investigated. As described above, trisomies 3, 7 and 12 and t(14;18) and t(3;14) translocations can be involved in MALT lymphomas[26,27]. Therefore, Group A may have been heterogeneous and included patients with chromosomal aberrations other than t(11;18) translocation and trisomy 18. Third, only patients who underwent FISH analysis for t(11;18) translocation were enrolled in this study. FISH analysis may be waived for patients in which *H. pylori* was eradicated immediately following the diagnosis of MALT lymphoma and lymphoma regression was achieved. Thus, selection bias is a possibility in this study. Fourth, because this is a retrospective, multi-centered study, treatment strategies of each patient may have varied according to the attending physician's preference and the availability of chemotherapy and/or radiotherapy at each institution. Fifth, methods to examine *H. pylori* infection status varied among the different institutions participating in this study. Prospective studies utilizing uniform protocols are required to definitively compare treatment responses and prognosis in each group.

In conclusion, we reviewed 146 patients, including 31 (21.2%) with extra copies of *MALT1* and 27 (18.5%) with t(11;18)(q21;q21)/*API2-MALT1* translocation. Patients with t(11;18) translocation should be treated differently from others. However, patients with extra copies of *MALT1* have similar clinical characteristics to cases without t(11;18) translocation or extra copies of *MALT1*. We propose that these patients can be initially managed in the same way as cases without chromosomal aberrations. However, since progression or relapse tended to be more frequently seen in patients with extra copies of *MALT1*, these patients may require closer clinical follow-up.

COMMENTS

Background

Previous studies investigating chromosome aneuploidy in extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) reported that trisomy 18 might be indicative of progression or relapse in patients with gastric MALT lymphoma.

Research frontiers

No studies have described the initial treatment and response in patients with extra copies of *MALT1*.

Innovations and breakthroughs

Patients with extra copies of *MALT1* have similar clinical characteristics to cases without t(11;18) translocation or extra copies of *MALT1*. The present study is the first to reveal clinical responsiveness to *Helicobacter pylori* eradication in gastric MALT lymphoma patients with extra copies of *MALT1*.

Applications

Patients with extra copies of *MALT1* can be initially managed in the same way as cases without chromosomal aberrations.

Terminology

MALT lymphoma is one of the non-Hodgkin lymphomas, originating in tissues or organs outside of the lymph nodes. Typically, this neoplasm involves the gastrointestinal tract, thyroid, ocular adnexa, lungs, salivary glands, liver, and skin. Of these organs, the stomach is the most frequently identified primary site.

Peer-review

This is a well-designed multicenter study on the role of extra copies of *MALT1*

in gastric MALT lymphoma. The conclusion can be supported by the results. It can add something interest to current knowledge.

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ORIGINAL ARTICLE

Retrospective Study

Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy

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Author contributions: Dai WK designed the study and Wang WJ managed the project; Zhou Q and Qiu CZ interpreted the data; Xie G and Zhou Q wrote the manuscript; Qiu CZ and Li YH conducted bioinformatics analysis; Wang HP and Ye JH collected sample information; Liao JX, Lu XG, Lin SF and Ma ZY contributed to the study design and patients' diagnoses; all authors read and approved the final manuscript. Xie G, Zhou Q and Qiu CZ contributed equally to this work.

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Abstract

AIM

To investigate whether patients with refractory epilepsy and healthy infants differ in gut microbiota (GM), and how ketogenic diet (KD) alters GM.

METHODS

A total of 14 epileptic and 30 healthy infants were recruited and seizure frequencies were recorded. Stool samples were collected for 16S rDNA sequencing using the Illumina Miseq platform. The composition of GM in each sample was analyzed with MOTHUR, and inter-group comparison was conducted by R software.

RESULTS

After being on KD treatment for a week, 64% of epileptic infants showed an obvious improvement, with a 50% decrease in seizure frequency. GM structure in epileptic

infants (P1 group) differed dramatically from that in healthy infants (Health group). Proteobacteria, which had accumulated significantly in the P1 group, decreased dramatically after KD treatment (P2 group). *Cronobacter* predominated in the P1 group and remained at a low level both in the Health and P2 groups. *Bacteroides* increased significantly in the P2 group, in which *Prevotella* and *Bifidobacterium* also grew in numbers and kept increasing.

CONCLUSION

GM pattern in healthy infants differed dramatically from that of the epileptic group. KD could significantly modify symptoms of epilepsy and reshape the GM of epileptic infants.

Key words: Ketogenic diet; Cronobacter; Seizures; Gut microbiota; Epilepsy

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Core tip: Many infants with epilepsy are refractory to current antiepileptic drugs, and ketogenic diet (KD) could help to moderate seizure frequency as an alternative treatment. A large number of reports have demonstrated that gut microbiota (GM) can affect children's neurodevelopment. Concurrently, GM could be dramatically affected by diet. KD could rapidly alter GM and alleviate seizure frequency in infants with refractory epilepsy. The GM structure of epileptic infants - comprising large numbers of pathogens, such as *Streptococcus* - differed from that of healthy controls. After KD therapy, GM of epileptic patients changed significantly, with fewer pathogens and more beneficial bacteria.

Xie G, Zhou Q, Qiu CZ, Dai WK, Wang HP, Li YH, Liao JX, Lu XG, Lin SF, Ye JH, Ma ZY, Wang WJ. Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. *World J Gastroenterol* 2017; 23(33): 6164-6171 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6164.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6164

INTRODUCTION

Pediatric epilepsy is widespread, with complications including cognitive impairment, delayed neurodevelopment and loss of bodily control[1,2]. Disequilibrium between excitation and depression of the central nervous system is acknowledged as the main factor in epilepsy incidence[3]. Prior reports have identified increased inflammatory reactions and pro-inflammatory cytokines, such as interleukin (IL)-6, IL-17 and interferon, in the cerebrospinal fluid (CSF)[4]. Anti-epileptic drugs (AEDs) and surgery are the main conventional treatments for infants with epilepsy[5]. However, there are still 30% of epileptic infants who suffered from therapeutic futility and recurrent attacks.

A growing number of reports indicated that KD is a promising therapeutic alternative for infants with refractory epilepsy, as it has been shown to ameliorate their clinical symptoms, including the frequency of seizures[6-10]. It remains unclear exactly how this occurs. Several reports implicated changed neurotransmitters after KD therapy, including γ-aminobutyric acid (GABA), monoamines and glutamate[7,11]. Dahin *et al*[12] and Freeman *et al*[13] also identified increased ketone bodies (KBs) and decreased dopamine and serotonin[12,13]. However, Sariego-Jamardo *et al*[14] found little change of neurotransmitters, pterins and amino acids in the CSF of KD responders as opposed to non-responders. These discrepant findings suggested a need for the further elucidation of the mechanisms of KD therapy.

Several studies showed that diet posed a significant effect on GM[8,15]. A high-fat diet induced selective enrichment of bile-metabolizing microbiota, such as *Bacteroides*[16], whilst high-fiber foods promoted the accumulation of plant-polysaccharide fermenting microbial organisms, including *Prevotella* and *Clostridium*[16]. A number of reports implicated involvement of GM in enteric nervous system, blood-brain barrier and glial cell development, all of which were pivotal to behavioral control and cognitive progression[17,18]. GM could produce neurotransmitters and gut hormones directly[19] or indirectly by producing signaling molecules to regulate host cells[20]. GM-derived short-chain fatty acids (SCFAs) could stimulate enterochromaffin cells to produce serotonin[21]. Wikoff *et al*[22] also documented decreased serotonin in peripheral serum in the absence of GM. Moreover, *Clostridium sporogenes* and *Ruminococcus gnavus* promoted decarboxylation of tryptophan to tryptamine, which modulated mood and appetite through amine-associated receptors[23]. Based on the involvement of GM in the gut-brain axis, increasing reports demonstrated imbalanced GM in neurogenic diseases (NDs), including autism-spectrum disorder, Parkinson's disease, and depression[24]. However, GM dysbiosis in childhood epilepsy remains unexplored.

Previous studies declared that short-term dietary intake could rapidly alter human GM[8,15]. In this study, we performed a comparison between diseased infants (before and after KD treatment) and healthy controls, to explore if and how GM of infants with refractory epilepsy differed with that of age-matched healthy subjects. We also evaluated the therapeutic effect of KD on refractory epilepsy and the changes in GM after treatment. It is hoped that this research will help to bridge some gaps in the current understanding of refractory childhood epilepsy.

MATERIALS AND METHODS

Sample collection

We enrolled 14 pediatric patients with refractory

epilepsy (aged 1.95 ± 3.10 years, 11 male and 3 female) in Shenzhen Children's Hospital, according to the following inclusion criteria: Convulsion more than four times per week after treatment with ⩾ 3 AEDs; no antibiotic exposure for at least 1 mo; no known genetic metabolic disorders or severe systemic illnesses; and successive KD therapy for at least 1 wk. KD was provided by Zeneca (Shenzhen, China), including Qitong ketogenic liquid milk (3.4 g protein, 8.0 g lipid and 0.6 g carbohydrate per 100 g milk), Qitong ketogenic cookies and Qitong ketogenic set-meal packages[25].

Healthy subjects (aged up to 3 years, 15 male and 15 female) were also recruited based on the following criteria: No antibiotic exposure for at least 1 mo before this study, no disease symptoms for at least 1 mo following recruitment, and no history of seizures (Supplementary Table 1). Fisher's exact test was used to evaluate the effect of gender and age on GM composition.

DNA extraction, library construction and sequencing

The genomic DNA of microbiota was extracted from stool samples using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad) following the manufacture's protocol. The hypervariable V3-V4 region of the 16S rRNA gene was amplified using PCR kit (TransGenAP221-02, Peking), and DNA products were quantified by gel electrophoresis and Qubit (Thermo Fisher, Singapore). After library construction, the qualified libraries were sequenced using the Illumina MiSeq Sequencing platform (Illumina, San Diego).

Taxonomy classification and diversity detection

After filtration, overlapped paired reads were assembled as tags with FLASH (v1.2.11), and clustered to operational taxonomic units (OTUs) through USEARCH (v7.0.1090)[26]. Representative OTUs were mapped against the Greengenes database (v201305)[27] and classified with RDP classifier (v2.2)[28]. The diversity of microbiota was calculated with MOTHUR (v1.31.2)[29].

Principal component analysis and statistical analysis

PCA was performed with R software (v3.2.5). Wilcoxon rank-sum test was used to compare GM in diseased infants and healthy controls (Health group). Comparative analysis between the epileptic infants before (P1 group) and after treatment (P2 group) was conducted by Wilcoxon signed-rank test. Linear discriminant analysis Effect Size (LEfSe) analysis was used to identify microbial species which were apparently enriched in a specific group.

RESULTS

Data output and patients' characteristics

The average number of high-quality sequencing reads produced for each sample was 117196 (range, 31900 to 305190). The number of assembled tags averaged 22800, with a range from 12655 to 27337. Both gender and age had no significant effect on GM (P = 0.069 and 0.234, respectively).

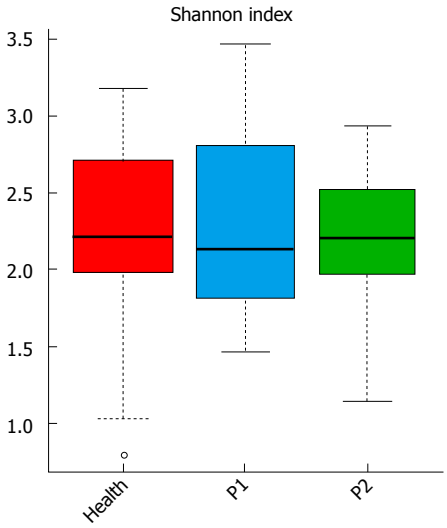


Figure 1 Gut microbial diversity of the three groups. Distribution of Shannon index (evenness) is shown. Red, blue, and green represent the Health, P1 and P2 groups, respectively. The gut microbiota (GM) of the healthy infants was more stable than that of the other two groups.

GM of healthy individuals differs dramatically with that of diseased infants

Shannon index analysis indicated higher GM diversity in healthy infants, in comparison with infants with refractory epilepsy (Figure 1, Supplementary Table 2). PCA of GM profile also identified that healthy infants could be clearly distinguished from patients (Figure 2, Supplementary Table 3). The phylum Firmicutes predominated in patients (45.82%) and was unchanged after KD therapy (47.00%) (Supplementary Table 4). Bacteroidetes accounted for 53.01% of GM in healthy infants, followed by Firmicutes (34.38%). After KD treatment, Bacteroidetes increased from 26.75% to 38.71%. Actinobacteria was enriched in healthy infants (8.49%) and occupied a lower percent in patients (2.38% before treatment and 2.92% after treatment). Proteobacteria was highly accumulated in infants with refractory epilepsy (24.34%) and decreased dramatically after KD therapy (10.77%). At the genus level, *Cronobacter* was dominant in the patients (23.30% *vs* 0.00% in the healthy group). By contrast, healthy subjects harbored more than twice *Bacteroides* (42.68%) than infants with refractory epilepsy (17.93%). *Prevotella* and *Bifidobacterium* also accumulated in the healthy group (7.25% and 7.84%, respectively) (Supplementary Table 5).

KD therapy ameliorates epilepsy and GM of patients started to improve

After a week of KD therapy, 3 (21%) patients were seizure-free and 6 (43%) had a 50% to 90% decrease of seizure frequency (Supplementary Table 1). The

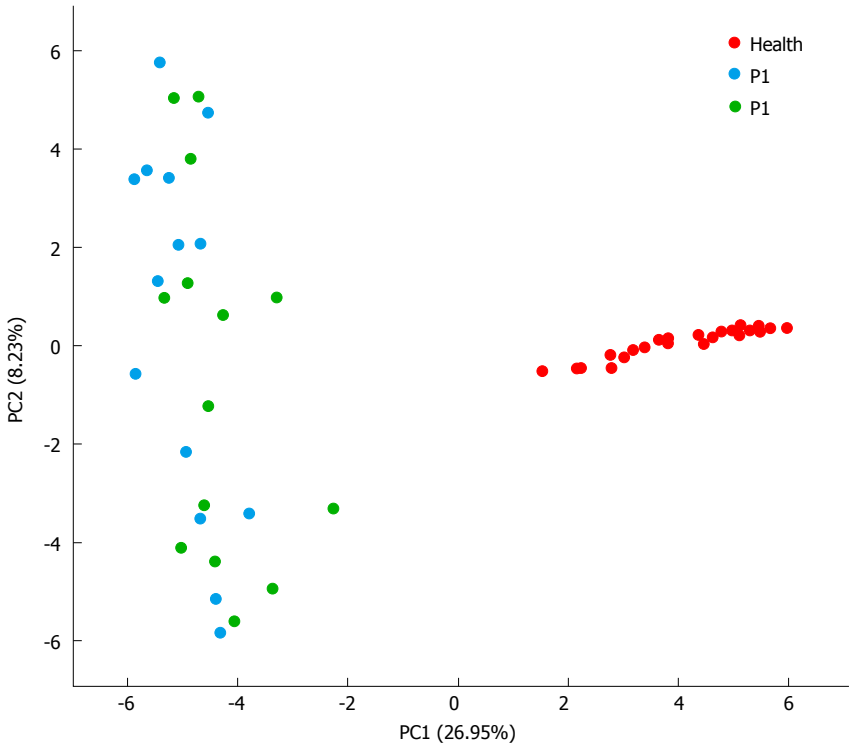


Figure 2 Principal component analysis. Each plot in the principal component analysis (PCA) graph stands for a sample. Red, blue and green colors represent the Health, P1 and P2 groups, respectively.

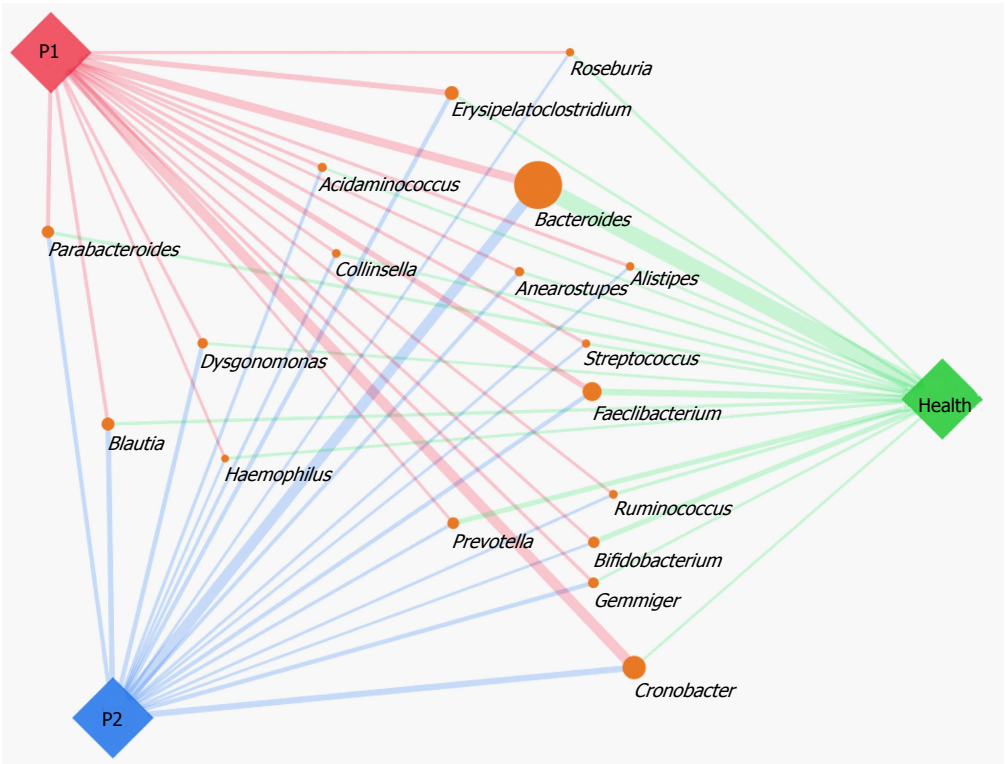


Figure 3 Gut microbiota structures in the Health, P1 and P2 groups at the genus level. SVG package (version 1.1) was used to produce the paragraph. The size of the circle representing each genus was determined by the relative abundance of the three groups, and the width of line linking the P1, P2 and Health groups indicates the relative abundance of each group.

remaining 5 (36%) infants experienced no significant improvement in seizure control (Supplementary Table 1). GM of the P2 group was more similar to that of the Health group, by comparison with P1 group (Figures 3 and 4). After KD treatment, *Bacteroides* increased significantly, by 24.42%. *Prevotella* also

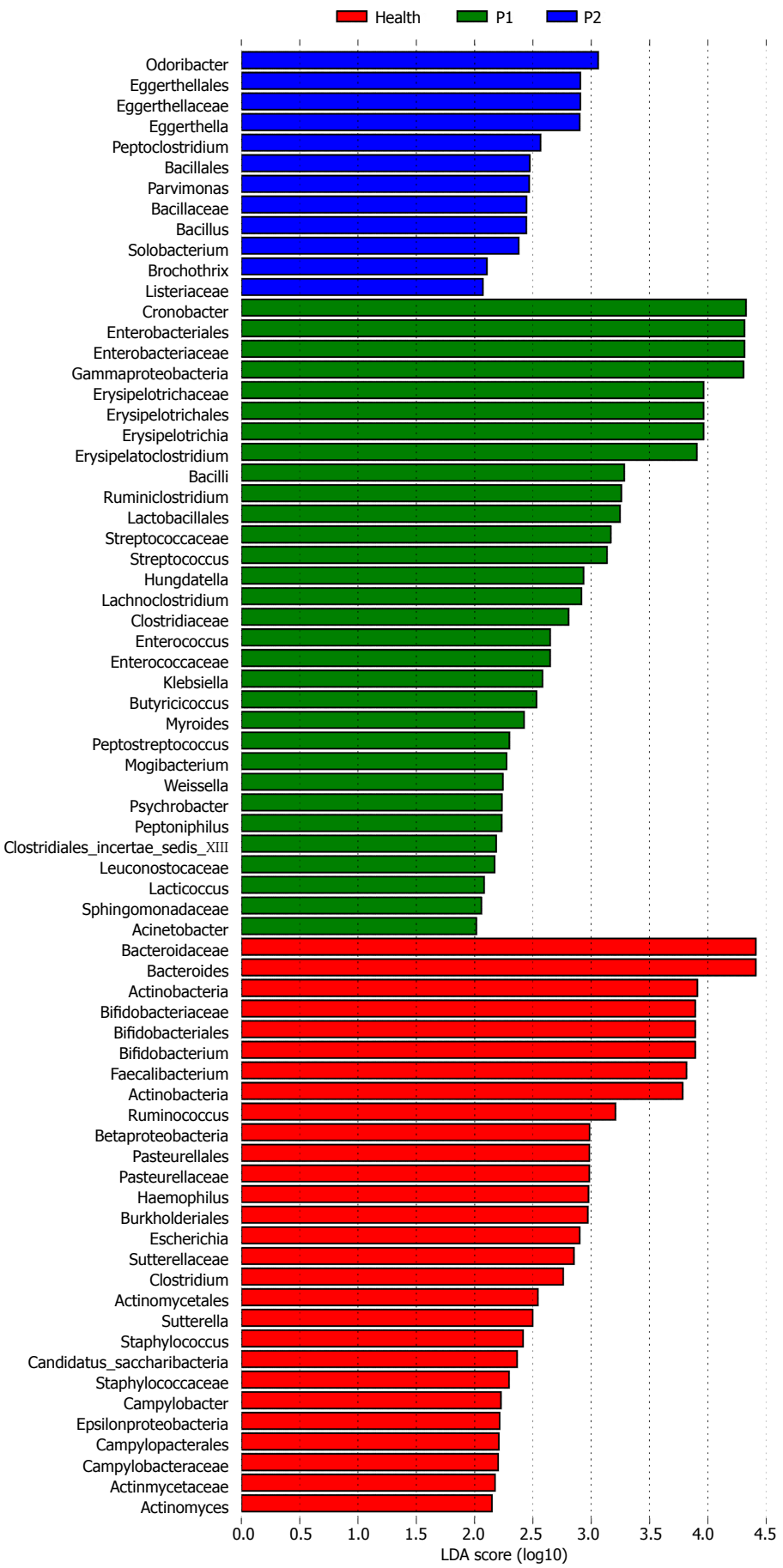


Figure 4 Significantly enriched gut microbiota components in the Health, P1 and P2 groups. LEfSe analysis was applied to detect the gut microbiota (GM) components in the three groups. Red, green, and blue represent the Health, P1 and P2 groups, respectively. The LDA score was set as ≤ 2. The enrichment degree is proportional to the LDA score.

increased in the P1 group from 0.37% to 1.85% after KD treatment (Figure 3 and Supplementary Table 5). *Cronobacter* decreased sharply in after-treatment patients, from 23.3% to 10.44 % (Figures 3 and 4 and Supplementary Table 5). KD exposure also induced a decrease in *Erysipelatoclostridium* (by 8.67% in the P1 group and 4.89% in the P2 group); it represented just 0.64% in healthy infants (Figures 3 and 4 and Supplementary Table 5). *Streptococcus*, *Alistipes*, *Ruminiclostridium*, *Barnesiella* and *Enterococcus* also decreased after KD therapy (Figures 3 and 4 and Supplementary Table 5).

DISCUSSION

KD is increasingly used for the treatment of refractory epilepsy in childhood, but the mechanism remains unclear. Previous reports indicated that GM played an important role in the gut-brain axis[24], and was affected significantly by intake of high-fat food[16]. This study focused on differed GM structures between healthy and epileptic infants, as well as altered GM in patients after one week of KD treatment. The results pointed to an imbalanced GM in patients and a significant improvement after KD therapy.

Proteobacteria comprises a variety of notorious pathogens, such as *Escherichia*, *Salmonella* and *Vibrio*. It accounted for 24.34% in pediatric patients and decreased dramatically after KD treatment. Bacteroidetes was dominant in healthy infants and increased largely in after-treatment patients.

We identified accumulated *Bacteroides* in healthy subjects as well as in patients after treatment. *Bacteroides* was reported to digest and metabolize high-fat food and to regulate the secretion of IL-6 and IL-17 in dendritic cells (DCs), a process strongly associated with seizure severity of epileptic patients[4,16]. However, patients-enriched *Cronobacter* decreased dramatically after KD therapy. Prior reports demonstrated that there were multiple virulence determinants of *Cronobacter*, including *Cronobacter* plasminogen activator and ferric ion transporter protein, which paly a detrimental role in human health[30-32]. *Prevotella* is a robust producer of SCFAs[33], which could protect the intestinal mucosa and function as neurotransmitters. Previous reports also indicated that SCFAs mediated nervous impulse and mitigated Parkinson's disease[33,34]. Similarly, we identified increased *Prevotella* in the Health and P2 group, when compared with the P1 group. Some other genera also offer clues to epilepsy recovery, such as *Erysipelatoclostridium*, *Blautia, Bifidobacterium* and *Streptococcus*. *Bifidobacterium* was well known to be beneficial to health[35], and *Streptococcus,* a common pathogen, played a role especially in respiratory diseases[36]. Although GM imbalance in diseased infants was identified and GM improved after KD treatment, more exploration was needed to elucidate the contribution of a healthy GM to epilepsy onset/recovery.

This study revealed that KD can mitigate the symptoms of epilepsy and correct an imbalanced GM in epileptic infants. However, further analysis is needed to unravel how GM may be involved in epilepsy onset/recovery.

There are some limitations that need to be clarified. First, 16S rDNA analysis identified microbes at the genus level, which makes it difficult to unravel different microbes at the species or function level. Second, it would be more useful to evaluate the efficacy of KD treatment and its effect on the GM if this could be done with a longer period of follow-up. Third, an animal model might be applied to demonstrate whether GM imbalance could induce epilepsy associated symptoms. Considering these limitations, we are planning to perform metagenomic analysis on GM of healthy and epileptic infants. This will provide more insights into distinct metabolic networks in imbalanced GM.

In conclusion, we found that GM of infants with refractory epilepsy differed dramatically from that of healthy infants. Epileptic patients harbored significantly enriched pathogens and decreased beneficial bacteria. Although this study provides new insight into the involvement of GM in pediatric refractory epilepsy, the gap between KD and epilepsy recovery is still huge. To uncover the mechanism and pathogens involved in refractory infantile epilepsy, further research should underscore functional gene networks in GM.

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COMMENTS

Background

Infants with refractory epilepsy could not be cured by several anti-epileptic drugs (AEDs) and ketogenic diet (KD) was increasingly used as an alternative therapy to refractory epilepsy. High-fat diet was reported to pose a significant impact on gut microbiota (GM), which could regulate neural systems.

Research frontiers

Previous reports demonstrated that GM could affect neural systems by secreting metabolites as neurotransmitters. In parallel, the gut-brain axis is a research hot spot in biomedicine, including the study of autism, Parkinson's disease, and depression.

Innovations and breakthroughs

This study showed that the GM pattern of diseased infants differs significantly from that of healthy controls. The decreased number of dominant pathogens and significantly increased number of beneficial bacteria after KD treatment offer new insight into KD therapy for epilepsy.

Applications

This study found several types of bacteria altered in the GM, suggesting that these bacteria could be monitored as biomarkers to provide an important reference for epilepsy treatment.

Terminology

GM, which consists of many kinds of bacteria including pathogens, commensals,

and probiotics, plays an important role in the human body.

Peer-review

The authors have performed important research in pediatric epilepsy. They discovered that the composition of the GM in healthy and diseased infants was significantly different, specifically in healthy infants as opposed to those with refractory epilepsy. Bacterial patterns were dramatically changed after KD therapy, and this was associated with a reduction in the frequency of seizures. These findings should enhance our knowledge of the relationship between epilepsy and GM and provide new insight into the clinical treatment of epilepsy.

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ORIGINAL ARTICLE

Observational Study

Definition of colorectal anastomotic leakage: A consensus survey among Dutch and Chinese colorectal surgeons

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Data sharing statement: The technical appendix and dataset are available from the corresponding author at stefan.van.rooijen@mmc.nl.

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Abstract

AIM

To determine the level of consensus on the definition of colorectal anastomotic leakage (CAL) among Dutch and Chinese colorectal surgeons.

METHODS

Dutch and Chinese colorectal surgeons were asked to partake in an online questionnaire. Consensus in the online questionnaire was defined as > 80% agreement between respondents on various statements regarding a general definition of CAL, and regarding clinical and radiological diagnosis of the complication.

RESULTS

Fifty-nine Dutch and 202 Chinese dedicated colorectal

surgeons participated in the online survey. Consensus was found on only one of the proposed elements of a general definition of CAL in both countries: 'extravasation of contrast medium after rectal enema on a CT scan'. Another two were found relevant according to Dutch surgeons: 'necrosis of the anastomosis found during reoperation', and 'a radiological collection treated with percutaneous drainage'. No consensus was found for all other proposed elements that may be included in a general definition.

CONCLUSION

There is no universally accepted definition of CAL in the Netherlands and China. Diagnosis of CAL based on clinical manifestations remains a point of discussion in both countries. Dutch surgeons are more likely to report 'subclinical' leaks as CAL, which partly explains the higher reported Dutch CAL rates.

Key words: Colorectal anastomotic leakage; Colorectal surgery; Definition; Complication

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Core tip: The present international online survey proves the inconsistent views as to what is considered colorectal anastomotic leakage among surgeons in the Netherlands and China, and shows large differences between the countries. This is in line with the current literature, since there is no uniformly accepted definition worldwide. We therefore propose to perform a systematic literature review to identify the available definitions. The final stage would be to perform a Delphi analysis within a representative panel of colorectal surgeons to develop a widely accepted definition of colorectal anastomotic leakage.

van Rooijen SJ, Jongen ACHM, Wu ZQ, Ji JF, Slooter GD, Roumen RMH, Bouvy ND. Definition of colorectal anastomotic leakage: A consensus survey among Dutch and Chinese colorectal surgeons. *World J Gastroenterol* 2017; 23(33): 6172-6180 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6172.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6172

INTRODUCTION

Colorectal anastomotic leakage (CAL) remains gastrointestinal surgeons' most feared complication, despite important improvements in perioperative care and the development of novel surgical techniques. It is associated with high rates of morbidity and mortality[1,2], poor quality of life[3], and increased healthcare costs[4,5]. Since CAL influences the direct postoperative course and has recently been proven to impact oncological outcome as well[6-8], it is frequently used as an outcome measure in clinical studies. However, the CAL rates vary considerably in the international literature from 1.5% to 23%[9,10]. Large variations in leakage rates have been reported between studies published by Western and Asian research groups, in which the reported incidence of CAL in Asian publications is substantially lower[5,11-14]. Such differences can be partly explained by the variations of operation technique, tumor location, and patient characteristics[15,16]. However, little attention has been paid to potential differences in the CAL definition and the available methods of diagnosis.

Although CAL is sometimes defined as "a defect in the bowel wall at the anastomotic site, leading to communication of intra- and extraluminal compartments"[17], this definition translates rather difficult to the clinical situation. Therefore, many authors formulate new definitions or diagnostic criteria in their studies, which usually include clinical and radiological features[18], and the impact of a leak on the treatment plan. However, since the pathophysiology of anastomotic leakage is multifactorial, the manifestation of a clinical leak can be rather variable[15]. Furthermore, due to the increased use of (routine) diagnostics such as CT or contrast enema, "radiological" leaks that do not eventually influence patient management are diagnosed more often. These factors complicate comparison of study results, and weaken the reliability of further analyses. This in turn hampers the construction of evidence-based guidelines on patient management and surgical technique.

Clearly, there is a need for a generally accepted and practical definition for CAL and its diagnostic criteria to serve as a template for future research on CAL and the clinical decision-making process[19]. Several surveys have been performed to reach consensus regarding the definition of CAL, however, most of them were restricted to a single country[19]. We hypothesized that the aforementioned reported differences in incidence rates between Asian and Western countries can partly be explained by differences in the definitions and diagnostic methods used. The aim of this study was therefore to determine the level of consensus regarding different aspects of a general definition of CAL within and between populations of Chinese and Dutch colorectal surgeons, that can be considered good representatives of the East and West, respectively.

MATERIALS AND METHODS

An online survey was performed among colorectal surgeons from the Netherlands and China. In the Netherlands, the survey was constructed and run through an online database using SurveyMonkey TM (Palo Alto, CA, United States). Colorectal surgeons in the Netherlands were identified from the contacts section of the colorectal subdivision of the Dutch Society of Gastro Intestinal Surgery (NVGIC): the Taskforce Coloproctology (WCP). Within this subdivision, 141 senior and junior colorectal surgeons were identified. Respondents were invited to partake in the online

Table 1 English questionnaire on definition of colorectal anastomotic leakage

		Yes	No
General definition			
Do we have to consider the following findings as anastomotic leakage?			
1	Extravasation of contrast after rectal enema on a CT scan		
2	Radiological collection around the anastomosis and no treatment		
3	Radiological collection around the anastomosis treated with antibiotics		
4	Radiological collection around the anastomosis treated with percutaneous drainage		
5	Abdominal sepsis and reoperation needed		
6	Necrosis of the anastomosis seen at reoperation		
7	Necrosis of the blind loop seen at reoperation		
8	Signs of peritonitis during reoperation		
9	Air bubbles around the anastomosis seen on a CT scan		
10	Free intra-abdominal air seen on a CT scan		
Clinical diagnosis			
In what extent do the following clinical parameters contribute to the suspicion of colorectal anastomotic leakage? Please note the relevance on a numeric scale of 0-10:			
1	Increased C-reactive protein		
2	Increased leukocytes		
3	Tachycardia		
4	Increased respiratory rate		
5	(Sub-) febrile temperature		
6	Postoperative ileus (> 4 d)		
7	Deterioration in clinical condition		
8	Abdominal pain, other than wound pain		
Radiological diagnosis			
Answer the following questions using percentages (0% = never, 100% = always)			
1	In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radiodiagnostics?		
2	In how many percent of patients with clinical suspicion of anastomotic leakage do radiodiagnostics change your treatment policy?		
3	In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage.		
4	In how many percent of cases do you consider a reoperation without previous radiodiagnostics?		
Early anastomotic leakage			
In your opinion, is 'very early (< 3 d) anastomotic leakage the result of technical failure?			
1	Yes		
2	No		

survey by email. Dutch surgeons completed the questionnaire between May and June 2015.

In China, the survey was conducted on the platform provided by DXY (www.dxy.cn), which is the largest medical website in China with more than one million registered medical users. An invitation was sent to all the registered colorectal surgeons to invite them to participate in a five-minute survey. Due to a relatively large number of registered users, the survey was designed to be terminated when 200 replies were received. Surgeons from Hong Kong, Macao, and Taiwan were not invited in this survey, because of the application of different medical systems in those areas. A demographic chart of the regions represented by the respondents can be observed in Figure 1.

The survey was divided into three major categories with questions addressing the general definition, and the clinical and radiological diagnosis of CAL. It was partly adapted from a previous study of Adams *et al*[20] and was initially constructed in English, and then translated to Dutch and Chinese by surgeons fluent in both English and Dutch and English and Chinese for the Dutch and Chinese versions, respectively, and checked for interpretation bias. Details of the English questionnaire are shown in Table 1 (See supplementary data for Dutch and Chinese versions).

Category Ⅰ mainly focused on the agreement of general definitions used in the international literature[20]. Surgeons were asked to state whether ten different clinical situations should or should not be included in a general definition. Category Ⅱ focused on clinical manifestations and their predictive value for CAL. A 10-point grading scale ranging from 1 (not predictive at all) to 10 (very predictive) was used to assess the agreement of the respondents' views on the clinical parameters. The parameters used were partially adapted from the Dutch Leakage Score (DULK)[11]. Category Ⅲ consisted of four questions regarding the use of radiological examination and the influence of this diagnostic method on patient care. This third category was also partially adapted from Adams *et al*[20]. The last general question focused on surgeons' views regarding the cause of very early anastomotic leakage.

Definitions

Very early anastomotic leakage was defined as leakage occurring within the first three days post-surgery.

Postoperative ileus was defined as an interval of more than 4 d from surgery until passage of flatus or stool and the tolerance of an oral diet[21].

Blind loop was defined as a bypassed loop of bowel after the construction of an end-to-end or end-to-side bowel anastomosis.

Statistical analysis

Basic descriptive statistics were used to summarize data for the online survey. Consensus was defined as > 80% agreement between respondents on various statements, as described by Duncan *et al*[22]. If less than 80% of respondents deemed the statements important, it was stated that no consensus was reached. Graphical depictions of information were used where appropriate to facilitate data interpretation. Chi square test or Mann-Whitney test were applied with proper indications. A *P*-value smaller than 0.05 was considered to indicate statistical significance.

RESULTS

Of the 141 colorectal Dutch surgeons who were invited to partake in the online survey, 62 respondents accepted the invitation, and 59 completed the survey, resulting in a 42% response rate and 95% survey completion. In total, 100% of 201 questionnaires



Figure 1 Demographic chart of the Chinese regions this survey covers. The gray scale reflects the number of participants in each region, varying from 21 from Zhejiang to 1 from Hainan. Correlating with the number of colorectal surgeons in each region, more surgeons from the east regions participated in this survey. Tibet and Ningxia had no participants, which also corresponds to the fact that the number of surgeons is very limited compared to the east provinces. Due to the application of different medical systems in these regions, Hong Kong, Macao, and Taiwan were not included in this survey.

received from Chinese surgeons were completed. A demographic chart of the regions represented by the respondents can be observed in Figure 1, as it shows that this survey covers 96.8% (30/31, Hong Kong, Macao, Taiwan not included) of provinces and areas of China.

Consensus was found on only one clinical situation proposed as an element of a general definition in both countries: 'extravasation of contrast on enema' (Figure 2), and in the Netherlands on two additional elements: radiological collection for which percutaneous drainage was needed (50/59 respondents, 85%) and necrosis of the anastomosis visible upon reintervention (51/59 respondents, 86%). For all other items on the available general definitions, clinical and radiological diagnosis of CAL, no consensus was found. Scores were significantly different between China and the Netherlands for the following elements: radiological collection treated conservatively (21% *vs* 39%, respectively, $P = 0.010$), necrosis of the blind loop on reintervention (41% *vs* 69%, respectively, $P \leqslant 0.001$), and air surrounding the anastomosis on a CT scan (65% *vs* 44%, respectively, $P = 0.004$).

Grades given for the clinical parameters are shown in Figure 3 for both China and the Netherlands. Clinical deterioration, increased C-reactive protein (CRP), tachypnea, and tachycardia were seen as being most contributory for the clinical suspicion of CAL in the Netherlands, and were given a weighed score of 7.83, 7.45 7.13 and 7.13, respectively (Table 2). In China, clinical deterioration and abdominal pain other than wound pain were deemed most attributable for the suspicion of anastomotic leakage in the direct postoperative period, with scores of 6.67 and 6.61, respectively. Increased plasma concentration of CRP received the lowest score of all parameters in China (4.35), while in the Netherlands this was deemed

Table 2 Sensitivity scores of clinical parameters for the suspicion of anastomotic leakage in the direct postoperative period in China and The Netherlands

Clinical parameter	China	The Netherlands	*P*-value
	Score ± SD	Score ± SD	
Increased CRP	4.35 ± 2.466	7.45 ± 1.871	< 0.001
Leukocytosis	5.96 ± 2.596	6.53 ± 1.824	0.095
Tachycardia	4.55 ± 2.411	7.13 ± 1.937	< 0.001
Tachypnea	4.46 ± 2.244	7.13 ± 1.937	< 0.001
Febrile temperature	6.23 ± 2.281	5.86 ± 1.963	0.207
Postoperative ileus	4.47 ± 2.363	5.76 ± 1.679	< 0.001
Clinical deterioration	6.67 ± 2.033	7.83 ± 1.205	< 0.001
Abdominal pain	6.61 ± 2.247	6.74 ± 1.835	0.659

CRP: C-reactive protein.

more sensitive (7.45, $P < 0.001$). Upon categorization of the grades for the value of clinical parameters into different categories of the numeric scale: disagree (0-3), neutral (4-6) and agree (7-10), most surgeons from both countries (45%-59% of surgeons for each parameter) remained neutral towards the added value of specific clinical parameters during the postoperative course.

The data on radiodiagnostics are shown in Table 3. The majority of Chinese and Dutch surgeons perform radiodiagnostics upon clinical suspicion of a leak. The distribution of the answers over the different classifications, however, was significantly different between the two nationalities (Chi square test, $P = 0.020$). Expected false-negative rates for CT scans were equal for surgeons in both countries. A significantly larger portion of the Chinese colorectal surgeons (25.4% *vs* 13.6%, $P \leqslant 0.001$) would consider performing a reoperation for the suspicion of CAL without performing radiological diagnostics. The distribution of the scores differed significantly between countries as to in how many cases a reoperation is considered without previous radiodiagnostics (Chi square test, $P = 0.002$).

Concerning the question about early CAL, 90.6% of the Chinese surgeons agreed that the cause of such should be considered a technical failure, whilst only 70.4% of the Dutch colorectal surgeons agreed to this statement ($P \leqslant 0.001$).

DISCUSSION

Despite extensive research in the field of CAL, no international consensus regarding a practical definition exists, which limits the transparency and comparison of study outcomes. Several definitions of CAL have been proposed during the last decade[18,23], but review of the literature shows that newly published papers fail to adopt these definitions[24]. Instead, authors seem to prefer to use their own definitions or no definition at all[24]. It could be postulated that these previously proposed definitions were not yet implemented in clinical practice and (retrospective) research because of limited awareness of the existence of such a

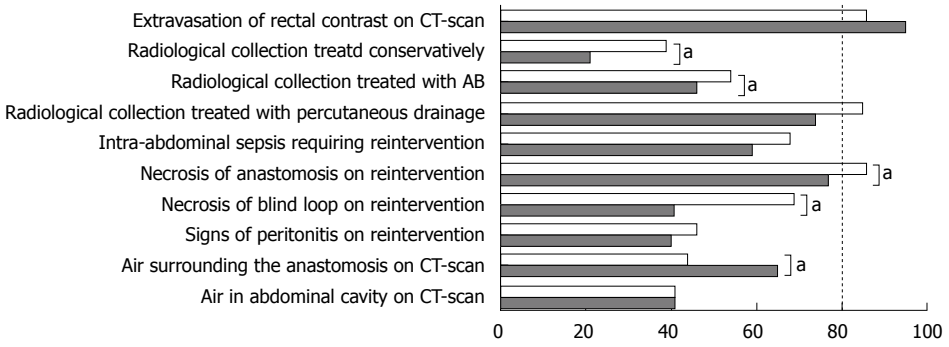


Figure 2 Percentage of respondents in agreement to general definitions of colorectal anastomotic leakage in the Netherlands (white bars) and China (dark grey bars). The dotted line indicates the 80% consensus threshold for the different statements. An a indicates a significant ($^aP < 0.05$) difference between percentages of agreement of Dutch and Chinese surgeons.

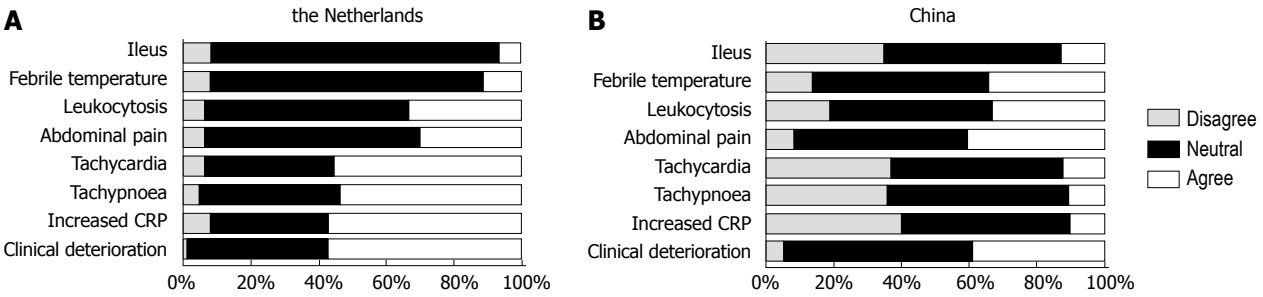


Figure 3 Distribution of categorized scores for the value clinical parameters in the direct postoperative phase. A: Comparison between the Netherlands and China. B: Scores are divided into three categories: numeric scales ranging from 0-3 are depicted in grey (disagree), 4-6 depicted in black (neutral), and numeric scales ranging from 7-10 (agree) in white.

definition and/or lack of support from a large expert group.

Reports from Asian studies show CAL rates that are substantially lower than those reported by Western research groups[25]. This could partially be explained by demographic differences that exist in patient population, availability and use of diagnostic tools, or how perioperative care is structured. Another explanation could be that Chinese surgeons only report a leak as such when reintervention is required.

On the other hand, despite the lower prevalence of obesity in Asian countries, rates of type Ⅱ diabetes mellitus and metabolic syndrome are relatively high due to ethnic and genetic factors. Indeed, Asians account for 60% of diabetes mellitus cases worldwide[26], which is considered an important risk factor for CAL[27,28]. Furthermore, the most common location of colorectal tumors in the Asian population is the left hemicolon[16,29], compared to the Western population, in which the predominant side is the right[29,30]. The literature shows a significantly higher CAL risk for surgeries on colorectal tumors located in the left hemicolon[31]. These regional differences therefore fail to completely explain the variation in reported CAL rates. It is very likely that important regional differences exist as to what is considered an anastomotic leak, *i.e.*, Asian surgeons may report a leak mostly when a reintervention is required, while the Western surgeons may report latent leaks. In order to gain more insight into these differences in views, the present survey was conducted both in China and in the Netherlands, countries that are considered to be representative for their continent.

In the first part of the survey, surgeons were asked whether different statements including clinical and radiological signs and interventions regarding CAL should be considered anastomotic leakage. Of ten statements, only one was deemed as CAL by more than 80% of respondents in both countries: 'Extravasation of contrast after rectal enema visible on a CT scan'. This is generally considered a radiological hallmark sign for anastomotic leakage after left-sided colorectal surgery and should naturally be included in a general definition. Moreover, other important and evident CAL signs including "Radiological collection around the anastomosis treated with percutaneous drainage" and "Necrosis of the anastomosis seen at reoperation" received more than 80% positive responses in the Netherlands, however, not in China, and thus were not considered as consent according to the predetermined criteria. Despite this, the majority of the Chinese surgeons also agreed on these items and their answers did not differ significantly from those of their Dutch colleagues. In conclusion, it seems that for the evident signs of CAL, the majority of surgeons from both countries have quite similar views.

"Radiological collection treated conservatively" was only considered to be CAL in 21% of the Chinese surgeons (versus 39% in the Netherlands), which is almost a consensus of NOT including this statement in a general definition of CAL. On the contrary, it is at

Table 3 Surgeons' opinion regarding the value of radiodiagnostics in the diagnosis of colorectal anastomotic leakage

Responders, *n* (%)	China (%)	The Netherlands (%)	*P*-value
In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radiodiagnostics?			
	202 (100)	55 (93)	
0%-20%	3.0	0	
21%-40%	6.4	0	
41%-60%	6.9	1.8	
61%-80%	24.3	16.4	
81%-100%	59.4	81.8	
Average	83.3	91.5	0.285
In how many percent of patients with clinical suspicion of anastomotic leakage do radiodiagnostics change your treatment policy?			
	202 (100)	54 (91.5)	
0%-20%	10.9	13.0	
21%-40%	9.9	5.6	
41%-60%	27.7	44.4	
61%-80%	30.2	25.9	
81%-100%	26.7	11.1	
Average	63.6	55.9	0.028
In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage?			
	202 (100)	52 (88.1)	
0%-20%	40.6	51.9	
21%-40%	29.2	28.8	
41%-60%	25.2	15.4	
61%-80%	4.0	1.9	
81%-100%	1.0	1.9	
Average	31.8	28.7	0.221
In how many percent of cases do you consider a reoperation without previous radiodiagnostics?			
	202 (100)	53 (89.8)	
0%-20%	58.4	84.9	
21%-40%	18.8	13.2	
41%-60%	17.3	0	
61%-80%	4.5	0	
81%-100%	1.0	1.9	
Average	25.4	13.6	< 0.001

least remarkable that in current grading systems, a radiological collection can be considered anastomotic leakage. As such this is reported as a grade A CAL according to the International Study group of Rectal Cancer (ISREC)[18] and grade Ⅰ-Ⅱ CAL according the Clavien-Dindo Scale.

Despite the fact that only a minority of Dutch surgeons consider conservatively treated radiological collections as CAL, these numbers are higher than those among the Chinese. These differences in views regarding the subclinical signs of CAL may eventually lead to a significantly higher reported CAL rate in the Dutch studies than the Chinese ones. However, considering the fact that more than 30%[32] of the CAL do not require invasive intervention, the treatment provided by surgeons from both countries may eventually be similar, *i.e.*, leading to a similar intervention rate for the complication. To rule out the reporting difference in this regard, one solution is to report complications with a Clavien-Dindo score higher than Ⅲa, which actually is also commonly accepted and applied in recent studies.

The second part of the survey focused mainly on clinical markers and parameters for CAL. Early clinical diagnosis of CAL remains a challenge for surgeons worldwide. Many clinical symptoms and biomarkers have been suggested as early signs of CAL[33-36]. However, previous studies of these parameters have shown that almost none of these parameters yield sufficient diagnostic accuracy to allow for a confirmative diagnosis[37]. This explains our findings that most surgeons do not base their diagnosis of CAL on these parameters, which results in a relatively low score of their contribution to the suspicion of CAL. Surgeons from both countries deemed "deterioration of clinical condition" as an important symptom of CAL, which further accentuates the complexity of CAL diagnosis based on its clinical manifestations. We believe the surgeons' opinions indeed reflect the unsatisfactory status of CAL diagnosis, which stresses the need for further research in this field[38]. However, important differences exist between the two countries. Although surgeons from both countries agreed about the predictive value of higher temperature, abdominal pain other than wound pain, and increased leukocyte count, more than half of the clinical parameters scored significantly lower in China than in the Netherlands. Although these abnormal clinical manifestations are indeed very common after gastrointestinal surgery[39], it seems that they are considered less suggestive by the Chinese surgeons.

The third part of the survey focused on radiological tools used in the diagnosis of CAL. Based on the present data, the majority of the surgeons in both countries would perform radiological examination on patients in whom CAL was suspected (these numbers are slightly higher in the Netherlands), and more than half of the treatment plans would be changed after the imaging. In this regard, although differences have been found in the views of Chinese and Dutch surgeons regarding the definition of CAL, the treatment they provide is similar. However, our data also show that surgeons from both countries do not blindly rely on the results from radiodiagnostics. Instead, they state that in approximately 30% of the cases in which CAL is suspected, CAL is eventually diagnosed in spite of a negative radiological report. This correlates with previously reported false negative rates of CT scans[40]. Experience with inaccurate CT scan reports may be a reason for surgeons to consider reoperation without affirmative CT results, which according to the data, occurs in about 25% of cases.

Further research and education may facilitate the achievement of international consensus. However, definition without considerations of the practical issues in different regions is unlikely to gain sufficient popularity. In 2010, the ISREC proposed a graded system for the diagnosis and treatment of CAL[18]. Grade A CAL refers to anastomotic leakage for which no active therapeutic intervention is required. It seems that this grade correlates with the second statement "Radiological collection surrounding the anastomosis

treated conservatively" that is not classified as CAL according to the majority of both Dutch and Chinese surgeons. This discrepancy between an established definition and the views of colorectal surgeons could partly explain why the ISREC definition has not been adopted in practice and science. In accordance with that, our survey clearly demonstrates how different practices may influence surgeons' opinion.

For example, in the Netherlands the Enhanced Recovery After Surgery (ERAS) program has been widely adopted for years, and recommends no abdominal drainage after surgery. In China, on the contrary, ERAS is less commonly implemented, and an intra-abdominal drain is often left *in situ* for longer periods after surgery. Moreover, CT imaging is less commonly used as radiodiagnostic for CAL, and laboratory analysis by means of CRP is not yet implemented in routine practice in many rural areas. This could explain why increased CRP was deemed least contributory in the diagnostic process in the present survey. These points, though small, significantly influenced the results, and would certainly impact the applicability of a proposed CAL definition.

To successfully embed a definition in clinical practice, research on CAL would greatly benefit from establishing a uniform definition and recording in national databases. We will therefore continue to perform an extensive and systematic literature review. The results from that review and the consensus assessment described in this paper will lead to an international Delphi analysis that will allow us to reach consensus on a new definition proposal that will be supported by a large panel of experts. We sincerely welcome others to participate in this further research, in order to formulate a new definition based on joint experience and opinions.

The most important limitations of the study are the following. The content of questionnaires is always susceptible to researcher imposition and there may be a level of subjectivity in the answers given. Furthermore, the relatively low numbers of respondents from both countries would have a negative influence of the generalizability of study results. Finally, the original questionnaire was constructed in English and translated into Dutch and Chinese, which could introduce bias and weaken the validity of comparisons between the countries. Finally, as some of the clinical parameters used in the questionnaire were derived from the DULK-score, which was constructed and validated in the Netherlands, it is plausible that the Dutch participants scored similarly on these items because they were familiar with the content of the DULK-score, because they have been (in)directly involved in the construction of the scoring system. However, the use of the DULK-score has not remained limited to the Netherlands, and it is unknown whether the subset of Dutch surgeons familiar with the DULK-score is higher than the number of Chinese surgeons who use this score routinely, and whether this difference is large enough to alter the data significantly.

In conclusion, no international consensus of a practical definition of CAL is yet available, which limits the transparency and comparison of published results. The present international online survey proves the inconsistent views as to what is considered CAL among surgeons in the Netherlands and China, and shows large differences between countries. Dutch surgeons are more likely to report 'subclinical' leaks as CAL, which partly explains the higher reported Dutch CAL rates. Surgeons from both countries rely on radiological diagnostics and laboratory parameters in the decision-making process, but are well aware of the limitations of these diagnostic aids. A Delphi analysis within a representative panel of colorectal surgeons is desired to develop a widely accepted definition of CAL.

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COMMENTS

Background

Colorectal anastomotic leakage (CAL) is the most feared complication after colorectal surgery. No international consensus exists regarding a general definition of CAL.

Research frontiers

Over the past decades, thousands of articles on CAL have been published. Unfortunately, a uniform and accepted worldwide definition of CAL is not available. This limits the transparency and comparison of study results and usefulness in clinical practice.

Innovations and breakthroughs

An international survey has been performed to identify the differences in reported definitions of CAL and to evaluate the opinions of expert leaders in both a Western and Eastern country.

Applications

The present international online survey proves the inconsistent views as to what is considered CAL among surgeons in the Netherlands and China, and shows large differences between the countries. This is in line with the current literature, since there is no uniform accepted definition worldwide. We therefore propose to perform a systematic literature review to identify the available definitions. The final stage is to perform a Delphi analysis within a representative panel of colorectal surgeons to develop a widely accepted definition of CAL.

Terminology

CAL is the major complication after colorectal surgery with a stable incidence (1.5%-23%). It is associated with high rates of morbidity and mortality, poor quality of life, and increased health expenditure. Since CAL influences the direct postoperative course and has recently been proven to impact oncological outcome as well, it is frequently used as an outcome measure in clinical studies.

Peer-review

In this study, the authors have presented a thorough and critical analysis of the availability of a definition of CAL and the opinions of both Dutch and Chinese surgeons regarding this definition.

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CASE REPORT

How to treat intestinal obstruction due to malignant recurrence after Whipple's resection for pancreatic head cancer: Description of 2 new endoscopic techniques

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Author contributions: Mouradides C, Taha A, Deprez PH and Moreels TG performed the endoscopic procedures; Borbath I was in charge of the clinical follow-up of the patients; Mouradides C, Taha A and Moreels TG wrote the report; Mouradides C, Taha A, Borbath I, Deprez PH and Moreels TG read and approved the report.

Institutional review board statement: All endoscopic procedures were performed according to good clinical practice standards approved by the Institutional Review Board of the Cliniques universitaires Saint-Luc.

Informed consent statement: Both patients consented to undergo the endoscopic procedures described in the current manuscript.

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Abstract

The prognosis of pancreatic cancer remains poor, even after initial surgical therapy. Local recurrence after Whipple's pancreatico-duodenectomy may lead to intestinal obstruction at the level of the afferent limb or the alimentary limb. Endoscopic insertion of a self-expandable metal stent (SEMS) into the intestinal malignant stricture is the preferred method of choice for palliation. We describe two new endoscopic techniques to treat a malignant intestinal obstruction with the insertion of a SEMS into the afferent limb and the alimentary limb. A case of malignant gastric outlet obstruction after a Whipple's resection was treated by the creation of an endoscopic gastrojejunostomy by the insertion of a lumen apposing HotAxios stent in between the stomach and the alimentary limb under fluoroscopic and endoscopic ultrasound control. Biliary obstruction and jaundice caused by a malignant stricture of the afferent limb after a Roux-en-Y Whipple's resection was treated by the insertion of a SEMS by means of the single-balloon overtube-assisted technique under fluoroscopic control. Feasibility and advantages of both techniques are discussed.

Key words: Intestinal obstruction; Self-expandable metal stent; Whipple's pancreatico-duodenectomy

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Core tip: Malignant recurrence after Whipple's pancreatico-duodenectomy is frequent and may lead to intestinal obstruction. Endoscopic palliation of the intestinal obstruction is challenging. We present 2 endoscopic techniques: Endoscopic ultrasound-guided creation of a gastrojejunostomy using a fully covered lumen apposing metal stent, and insertion of a self-expandable metal stent by means of the single-balloon overtube-assisted technique under fluoroscopic control.

Mouradides C, Taha A, Borbath I, Deprez PH, Moreels TG. How to treat intestinal obstruction due to malignant recurrence after Whipple's resection for pancreatic head cancer : Description of 2 new endoscopic techniques. *World J Gastroenterol* 2017; 23(33): 6181-6186 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6181.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6181

INTRODUCTION

Whipple's pancreatico-duodenectomy was named after Alan Oldfather Whipple who described the technique in 1935[1]. It still is the surgical treatment of choice to cure pancreatic head cancer[2]. Modifications of the technique have emerged, including pylorus-preserving Whipple's resection and Roux-en-Y intestinal reconstruction[3]. After resection of the pancreatic head, both the remnant bile duct and pancreatic duct have to be anastomosed to the afferent limb (hepaticojejunostomy/pancreaticojejunostomy) and food passes through the alimentary limb. Despite the different surgical Whipple's variations, the risk of local tumor recurrence after Whipple's resection for pancreatic head cancer remains more than 50%[4]. Few cases present with malignant intestinal obstruction at the level of the afferent or the alimentary limb. Endoscopic palliative therapy by means of insertion of self-expandable metal stents (SEMS) is the preferred method of choice to treat malignant intestinal obstruction in case of pancreatic cancer[2]. However, endoscopic SEMS placement in the afferent or the alimentary limb remains a challenging procedure[5]. We report on the endoscopic treatment modalities of two cases of malignant intestinal obstruction due to tumor recurrence after Whipple's pancreatico-duodenectomy.

CASE REPORT

Case 1

A 71 year-old woman was referred with symptoms of upper gastrointestinal obstruction because of local tumor recurrence at the level of the gastrojejunal anastomosis with peritoneal carcinomatosis. She underwent Whipple's resection for pancreatic head cancer two years earlier. Initially, an uncovered enteral SEMS (Wallflex 60 x 22 mm, Boston Scientific) was inserted and ended up in the afferent limb, eventually obstructing the alimentary limb worsening the gastric outlet syndrome. CT scan confirmed the dilated afferent limb towards the hepaticojejunostomy, with food remnants and the empty alimentary limb underneath the stomach (Figure 1). Since the uncovered SEMS could not be removed from the afferent limb because of tumor ingrowth obstructing the entrance to the alimentary limb, we decided to create an endoscopic gastrojejunostomy, using the HotAxios device (XLumina Axios 10 mm x 15 mm, Boston Scientific), under endoscopic ultrasound (EUS) guidance (linear-array echoendoscope GF-UCT 180, Olympus) and fluoroscopy. The HotAxios device is a cystotome catheter with a preloaded covered lumen apposing metal stent. It is developed to drain pancreatic pseudocysts with an all-in-one device[6]. Here we used it to create an endoscopic gastrojejunostomy. The empty alimentary limb was identified by transgastric EUS, accessed with a 19 gauche needle (Figure 2), and confirmed fluoroscopically by intraluminal contrast dye injection.

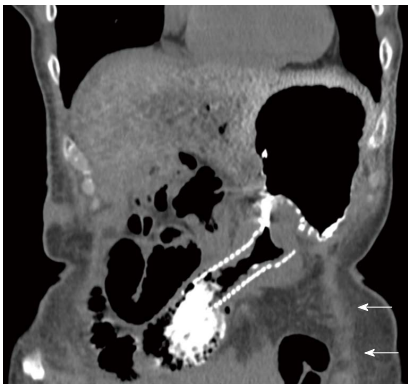


Figure 1 Coronal computed tomography image of a non-covered metallic stent with peroral contrast in the dilated afferent limb in a patient with classical Whipple's duodenopancreatectomy. Note the obstructed empty alimentary limb underneath the stomach (arrows).

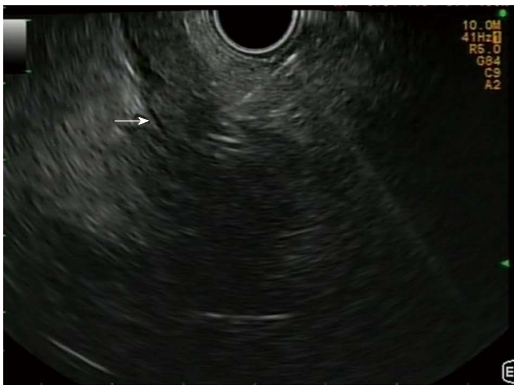


Figure 2 Linear endoscopic ultrasound image of the transgastric puncture of the obstructed alimentary limb (arrow) with a 19 gauche needle.

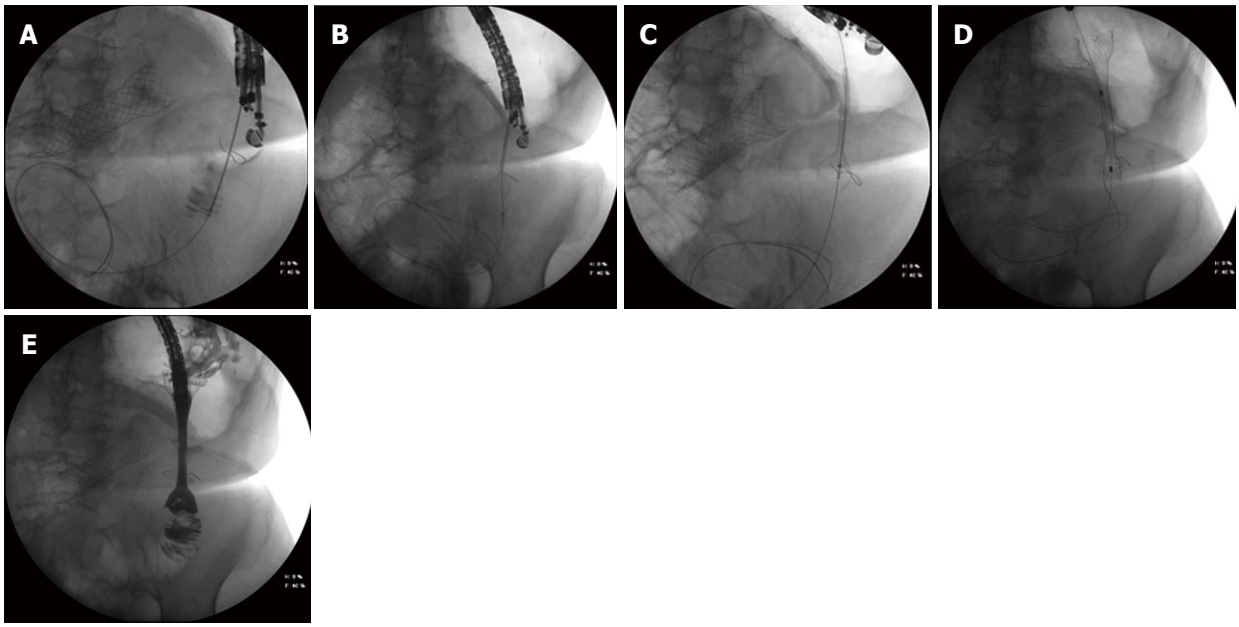


Figure 3 Fluoroscopic image. A: The transgastric access of the alimentary limb using a 19 gauche needle. Correct access of the limb was confirmed after injection of contrast dye. A: 0.035 inch guidewire is introduced into the alimentary limb. Note the SEMS in the afferent limb; B: The HotAxios lumen apposing stent deployment between the alimentary limb and the gastric lumen; C: The HotAxios lumen apposing stent migrating outside to stomach. The distal flange is well positioned inside the lumen of the alimentary limb; D: The fully covered oesophageal stent inside the HotAxios lumen apposing stent, creating a gastrojejunostomy; E: Fluoroscopic image confirming the creation of a gastrojejunostomy between the stomach and the alimentary limb using the combination of a HotAxios lumen opposing stent and a fully covered oesophageal stent. Water soluble contrast dye is injected through the oesophageal stent into the alimentary limb. Note the non-covered SEMS in the afferent limb.

Next, a 0.035 inch guidewire was placed (Figure 3A), allowing smooth introduction of the HotAxios stent deployment system into the alimentary limb using electrocautery. The fully covered 10 mm HotAxios lumen apposing stent was deployed under fluoroscopic and endoscopic guidance, with the distal flange in the intestinal lumen, and the proximal flange in the stomach (Figure 3B). However, upon release, it immediately migrated outside the stomach because of the distance between the alimentary limb and the stomach (Figure 3C). A fully covered metal esophageal stent (Taewoong Medical Niti, 80 mm x 22 mm) was introduced over the guidewire and deployed inside the HotAxios stent, with the proximal part protruding into the gastric lumen (Figures 3D and E). After the procedure, the patient quickly resumed oral feeding.

Three months later, she was readmitted for vomiting due to intragastric migration of the esophageal stent. The HotAxios stent was still in place. A fully covered Nagi stent (Taewoong Medical, 20 mm x 16 mm) was placed inside the HotAxios stent, and the patient resumed oral feeding. Seven months after the initial presentation, she died due to malignant progression of the disease.

This case illustrates the feasibility of the HotAxios system in creating an EUS-guided gastrojejunostomy to treat malignant gastric outlet syndrome. This technique has recently emerged as an alternative to surgical gastrojejunostomy treating benign and malignant gastric outlet syndrome, with technical success rate of 92% and 85% clinical success[7-9]. Serious adverse events of this endoscopic ultrasound technique were reported in 11.5%, and encompass gastrointestinal perforation, peritonitis and bleeding[9]. We encountered another complication. After deployment of the HotAxios lumen apposing stent to create the gastrojejunostomy, the gastric flange migrated outside the stomach without the possibility to reposition it into the stomach. Since the guidewire was still in place, we were able to save the gastrojejunostomy by inserting a second fully covered oesophageal SEMS inside the HotAxios stent.

Case 2

A 76-year-old woman who underwent Roux-en-Y Whipple's resection two years earlier for pancreatic head cancer, was referred because of obstructive jaundice and dilated intrahepatic bile ducts. Percutaneous transhepatic cholangiography and biliary drainage excluded an obstruction at the level of the bilioenteric anastomosis. However, afferent limb syndrome was diagnosed based on the obstruction of the afferent limb 20 cm distally from the hepaticojejunostomy with a dilated intestinal segment between the hepaticojejunostomy and the enteral stricture of the afferent limb (Figure 4A). Next, we performed a single-balloon enteroscopy (SBE) through the Roux-en-Y anastomosis into the afferent limb under fluoroscopic guidance, and reached the jejunal stenosis, with endoscopic characteristics of a malignant stricture (Figure 5). The length of the

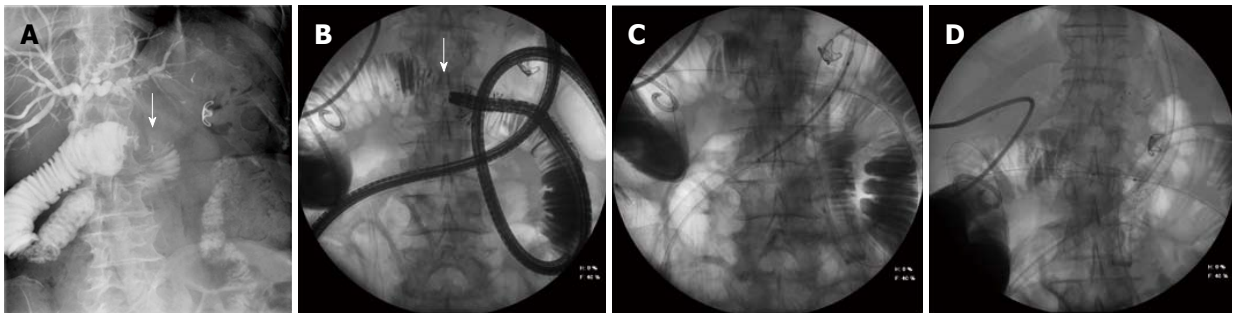


Figure 4 Fluoroscopic image. A: A percutaneous transhepatic cholangiography confirming patency of the biliary anastomosis. The malignant stricture (arrow) is located at the level of the afferent limb (afferent limb syndrome), leading to dilated intrahepatic bile ducts and a dilatation of the afferent limb. Note the presence of an old Ovesco clip in the stomach. B: The single-balloon enteroscopy up to the level of the malignant stricture in the afferent limb. Contrast injection through the working channel of the enteroscope clearly delineates the length and the malignant aspect of the stricture (arrow). C: The peroral insertion technique of the self-expandable metal stent over a stiff guidewire through the overtube with the balloon inflated to protect the intestinal mucosa and to guide the catheter up to the malignant stricture of the afferent limb. D: The self-expandable metal stent in place in the malignant stricture of the afferent limb. Both the stiff guidewire and the overtube of the single-balloon enteroscope were used to insert the SEMS. SEMS: Self-expandable metal stent.

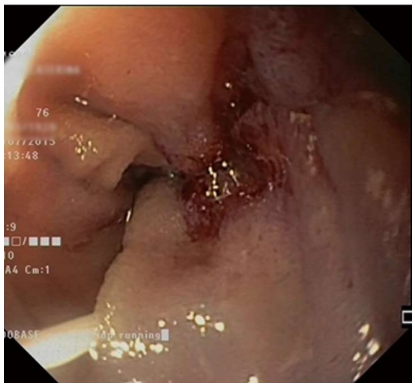


Figure 5 Endoscopic image of the malignant stricture of the afferent limb. Note the guidewire inserted into the stricture.

stricture was estimated by means of the injection of water soluble contrast. A 0.035 inch guidewire was inserted into the stricture under endoscopic and fluoroscopic control, and a CRE dilation balloon (8-10 mm) was inserted over the guide wire to dilate the stricture. Biopsies confirmed the intestinal recurrence of a pancreas adenocarcinoma. After a multidisciplinary oncology discussion, a non-surgical endoscopic approach was suggested. One week later, a second SBE was performed in order to place a SEMS into the malignant stricture. Again, the length of the stricture was estimated by contrast injection under fluoroscopic control (Figure 4B). A 0.035 inch stiff guidewire (VisiGlide 450 cm, Olympus) was inserted into the dilated jejunal segment proximal of the stricture. The overtube of the SBE was anchored as close as possible near the malignant stricture with the balloon inflated. Next, the enteroscope was removed under fluoroscopic guidance, leaving the guidewire and the overtube in place. An uncovered SEMS (Wallflex 60 mm x 22 mm, Boston Scientific) was inserted over the guidewire through the overtube into the stricture, located by means of the previously injected contrast (Figure 4C). The stent was deployed under fluoroscopic guidance (Figure 4D). Afterwards, the enteroscope was reintroduced through the overtube to control for the correct positioning of the SEMS. The percutaneous biliary drain was removed and the patient was discharged two days later with normal oral food intake. Gemcitabine-based chemotherapy was started.

Three months later, she presented again with obstructive jaundice and afferent limb syndrome suggestive of tumor invasion of the uncovered SEMS. A third SBE procedure was performed, confirming the diagnosis of SEMS obstruction. We used the same single-balloon overtube-assisted technique to insert a new partially covered SEMS (ComVi Enteral Colonic Stent 100 mm x 22 mm, Taewoong) inside the uncovered SEMS. No complications were encountered. Ten months after the second SEMS insertion, she died due to malignant progression of the disease.

This case emphasizes the usefulness, feasibility and reproducibility of the single-balloon overtube-assisted technique for SEMS insertion in malignant strictures of the small intestine, especially in patients with altered anatomy such as Roux-en-Y anastomosis where conventional endoscopes cannot reach. Since the currently available enteral SEMS deployment catheters cannot be used through the working channel (2.8 or 3.2 mm) of the 200 cm long enteroscope, the SEMS is introduced over a long stiff guidewire through the balloon-anchored overtube under fluoroscopic control. This balloon-assisted overtube technique enables to relieve intestinal obstruction without surgical intervention by a combined endoscopic and fluoroscopic approach [10,11].

DISCUSSION

Malignant recurrence after (modified) Whipple's pancreatico-duodenectomy for pancreatic head adenocarcinoma remains high[4]. Sometimes, local

recurrence may lead to the afferent limb syndrome (stricture of the afferent limb) or a gastric outlet-like syndrome (stricture of the alimentary limb) because of intestinal tumor infiltration. In the case of malignant intestinal obstruction following Whipple's resection, palliative surgery is generally indicated to obtain symptom relief (resection of an intestinal segment, enterostomy or intestinal bypass)[12]. Case reports have shown that endoscopic enteral SEMS placement has become feasible, either by the creation of an EUS-guided gastrojejunostomy or by insertion of a SEMS by means of the balloon-assisted overtube technique[7-11]. We illustrated both procedures in two cases of malignant intestinal obstruction (afferent and alimentary limb obstruction) after Whipple's resection.

COMMENTS

Case characteristics

Both patients underwent Whipple's pancreatico-duodenectomy as a curative treatment of pancreatic head cancer. However, local tumor recurrence in the alimentary limb resulted in the gastric outlet syndrome (case 1) and local tumor recurrence in the afferent limb resulted in obstructive jaundice (case 2).

Clinical diagnosis

The patient with the gastric outlet syndrome presented with recurrent vomiting and weight loss (case 1). The patient with the afferent limb syndrome presented with fever, abdominal pain and jaundice.

Differential diagnosis

Vomiting may be a side-effect of chemotherapy (gemcitabine) or may be caused by peptic ulcer disease (case 1). Obstructive jaundice may be the clinical sign of a postoperative stricture at the level of the hepaticojejunostomy (case 2).

Laboratory diagnosis

The patient with the gastric outlet syndrome presented a slight increase in CA19.9 tumor marker (case 1). The patient with the afferent limb syndrome presented with elevated CRP and liver function tests (case 2).

Imaging diagnosis

Abdominal CT scan of the patient with the gastric outlet syndrome suspected peritoneal carcinomatosis (case 1). MRI-MRPC of the patient with the afferent limb syndrome suspected a postoperative stenosis at the level of the hepaticojejunostomy (case 2).

Pathological diagnosis

No histological biopsies were taken during the endoscopic ultrasound (EUS)-guided gastrojejunostomy procedure to confirm local tumor recurrence (case 1). During the first single-balloon enteroscopy biopsies were taken at the level of the afferent limb stricture, confirming the presence of a well-differentiated pancreatic adenocarcinoma (case 2).

Treatment

Both patients were treated endoscopically in a palliative setting. The first patient underwent EUS-guided gastrojejunostomy followed by chemotherapy (Oxaliplatine) (case 1). The second patient underwent self-expandable metal stent (SEMS) insertion into the afferent limb followed by chemotherapy (Gemcitabine) (case 2).

Related reports

The two endoscopic techniques presented here have been described previously under the form a case reports.

Term explanation

Whipple's resection is the surgical curative treatment for pancreatic head cancer, with resection of the pancreatic head and the duodenum and the creation of a hepaticojejunostomy and a pancreaticojejunostomy on to the afferent jejunal limb. The afferent limb syndrome is caused by an obstruction at the level of the afferent limb after Whipple's resection. The gastric outlet syndrome is caused by an obstruction at the level of the alimentary limb after Whipple's resection.

Experiences and lessons

Local tumor recurrence remains high after initial curative Whipple's pancreatico-duodenectomy for pancreatic head cancer. The clinical presentation as the gastric outlet syndrome or the afferent limb syndrome is rare. New endoscopic techniques may allow to overcome these clinical syndromes by creating an EUS-guided gastrojejunostomy or by SEMS insertion into the afferent limb.

Peer-review

This is a well-written and illustrated overview of two endoscopic techniques for the palliation of intestinal obstruction due to local tumor recurrence after Whipple's pancreatico-duodenectomy for pancreatic head cancer.

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CASE REPORT

Arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer

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Author contributions: Kim HB were the major contributors in writing the manuscript; Park SG were involved in drafting, writing and editing the manuscript, and reviewed the manuscript as corresponding author; all authors read and approved the final manuscript.

Informed consent statement: Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

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Abstract

Arterioportal shunt (APS) is an organic communication between the hepatic arterial system and the portal venous system. The APS is one of the major causes of transient hepatic attenuation differences on dynamic computed tomography (CT) or magnetic resonance imaging (MRI). This condition is usually associated with trauma, liver cirrhosis, and malignancies of the liver. However, there has been no report about oxaliplatin-induced APS. A 41-year-old male was diagnosed with Stage ⅢB gastric cancer. The patient initially underwent neoadjuvant chemotherapy with capecitabine and oxaliplatin After 3 cycles of therapy, the mass had markedly decreased, and a total gastrectomy with splenectomy was performed. Since the malignancy was locally invasive, the patient was continued on the same regimen of the adjuvant chemotherapy. After 3 more cycles, a computed tomography revealed a 1 cm sized arterial-enhancing nodule in the right lobe of the liver. An MRI revealed an arterial enhancing lesion, and a positron emission tomography CT scan showed a hypermetabolic lesion in the same portion of the liver. We tried to perform a liver biopsy; however, an ultrasonography could not detect any mass. A presumptive diagnosis of an APS due to a recurred cancer was made. We found a similar but slightly different case report of an oxaliplatin-induced liver injury, mimicking a metastatic tumor on an MRI. Based on a prior report, the patient was continued

on treatment with adjuvant chemotherapy following discontinuation of oxaliplatin. After 2 cycles, the arterial enhancing liver mass resolved, supporting the final diagnosis of an APS, related to oxaliplatin-induced sinusoidal injury. The patient has not experienced any a relapse after two years of additional follow up recurrent gastric cancer upon interpretation of multiple imaging modalities.

Key words: Liver; Arterioportal shunt; Recurred cancer; Oxaliplatin; Transient hepatic attenuation differences

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Core tip: Although there have been several recent reports about oxaliplatin-induced sinusoidal injury, this is first case report of a non-tumorous incidental arterioportal shunt following oxaliplatin chemotherapy. We made a presumptive diagnosis of an AP shunt mimicking a recurred gastric cancer, due to an oxaliplatin-induced transsinusoidal injury. This case is the first report of an oxaliplatin-induced incidental arterioportal shunt mimicking recurred gastric cancer on various images.

Kim HB, Park SG. Arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer. *World J Gastroenterol* 2017; 23(33): 6187-6193 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6187.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6187

INTRODUCTION

Transient hepatic attenuation differences (THAD) lesions indicate areas of parenchymal enhancement, visible during the hepatic artery phase on a dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and are thought to be a physiological phenomenon due to the dual hepatic blood supply. The arterioportal shunt (APS) is one of the most important THAD[1,2].

APS is an organic communication between the hepatic arterial system and the portal venous system, and is caused by the redistribution of arterial flow into a focal region of the portal venous flow. This condition is associated with hepatic tumors, trauma, intervention, liver cirrhosis and metastatic tumors[2-5].

CASE REPORT

A 41-year-old Korean man was admitted with a chief complaint of epigastric pain. The patient initially underwent an endoscopy, which revealed a 4 cm sized ulcerofungating mass in the gastric cardia and CLO test (Campylobacter-like organism test; Rapid urease test) was negative. Pathologic findings revealed poorly differentiated adenocarcinoma without intestinal metaplasia and a CT scan showed a bulky ulcerative mass with direct invasion of the pancreas, and the left diaphragm. Furthermore, there were multiple perigastric and upper aortic lymph node enlargements. Based on these findings, the patient was diagnosed with Stage ⅢB gastric cancer (T4N2M0) (Figure 1A and B). He had a far advanced stage of gastric cancer; therefore, the patient underwent firstly a neoadjuvant chemotherapy with capecitabine and oxaliplatin. After 3 cycles of this therapy (#1-#3), the mass had markedly decreased and a total gastrectomy with splenectomy was performed (Figure 1C and D). Since this was a stage ⅢB gastric cancer [T4bN0 (0/48) M0], the patient was continued on the same regimen as adjuvant chemotherapy

After another 3 cycles of therapy (#4-#6), a follow-up CT demonstrated an arterial enhancing mass like lesion in the peripheral portion of the right lobe of the liver (Figure 2).

We presumed initially a new hepatocellular carcinoma or recurred gastric cancer, and we evaluated the arterial enhancing mass like lesion. He had a no history of hepatitis (hepatitis B and C) and he was social alcohol drinking. There was no proof of liver cirrhosis in CT, too. The test results showed HBsAg (-), HBsAb (+), and HCVAb (−) and tumor marker of hepatocellular carcinoma was under the normal range such as Alpha-fetoprotein (α-FP) of 2.2 ng/mL (normal; < 5 ng/mL) and protein induced by vitamin K absence-Ⅱ of 23 mAU (0–39 mAU). CEA (Carcinoembryonic antigen) and CA 19-9 (carbohydrate antigen) were under the normal range, too. Various other image studies were done. An MRI showed the arterial enhancing lesion, and a PET CT scan showed a hypermetabolic lesion in that same portion of the liver (Figures 3 and 4). We tried to perform a pathological confirmation of the lesion through a liver biopsy. However, we could not obtain the tissue because no typical mass was detected on ultrasonography.

In summary, he had a no risk of hepatocellular carcinoma and normal range of tumor markers, and there was no mass in liver. He was initially far advanced stage of gastric cancer, and he was high risk subtype of recurrence. Base on the data, we assumed it might be an arterial-portal shunt in the liver. However, this phenomenon strongly indicated a recurred mass in the liver despite of no obvious proof of recurrence such as typical mass or elevated tumor marker (CEA, CA 19-9). After reviewing literature, we found a few case report of an oxaliplatin-induced liver injury, mimicking a metastatic tumor on an image, although it was a slightly different case (mainly colon cancer and different enhance pattern)[6-10].

Therefore, we continued with the adjuvant che-

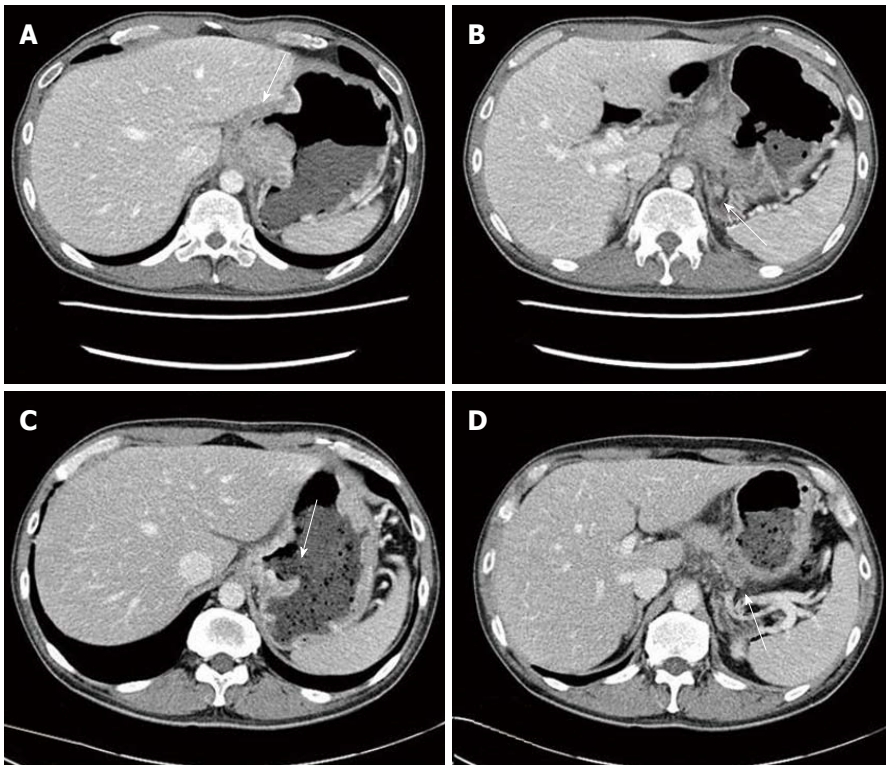


Figure 1 On admission, computed tomography revealed a bulky ulcerative mass with direct invasion of pancreas and left diaphragm (A and B). After 3 cycle of neoadjuvant chemotherapy (capecitabine and oxaliplatin), the mass had markedly decrease (C and D).

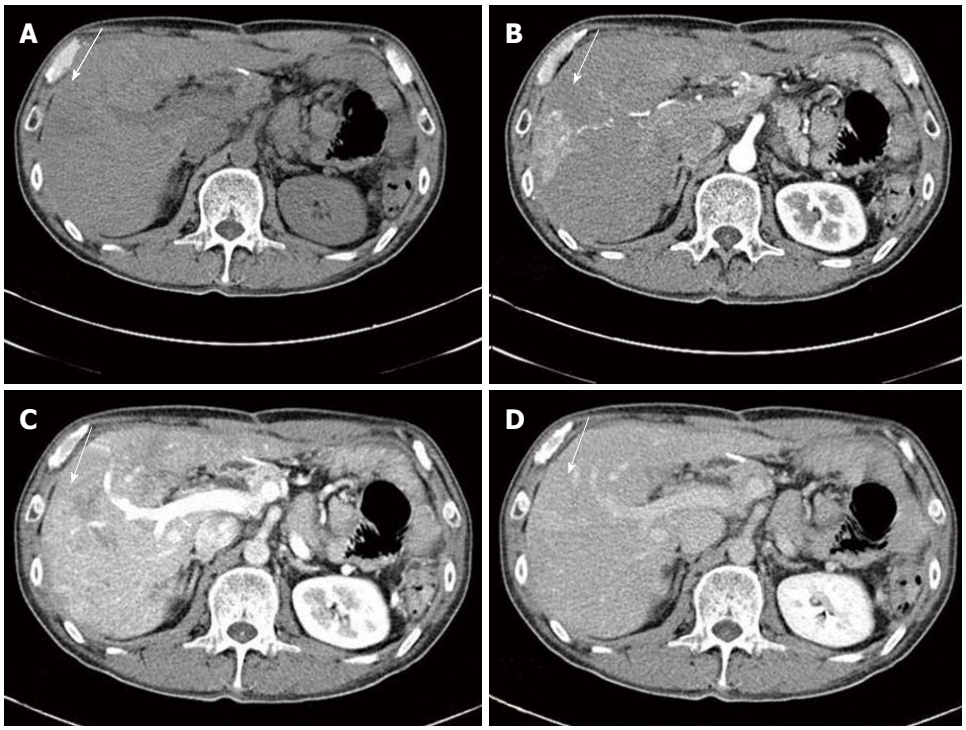


Figure 2 After total six cycle of chemotherapy (capecitabine and oxaliplatin), dynamic computed tomography showed an arterial enhanced mass like lesion. A: Pre-enhanced image; B: Arterial phase image; C: Portal phase image; D: Delayed phase image.

motherapy, this time, eliminating oxaliplatin. After 2 cycles of capecitabine monotherapy, the arterial enhancing mass in the liver disappeared (Figure 5). We were able to confirm the diagnosis of an AV shunt due to oxaliplatin-induced sinusoidal injury, the patient has not relapsed in 2 years.

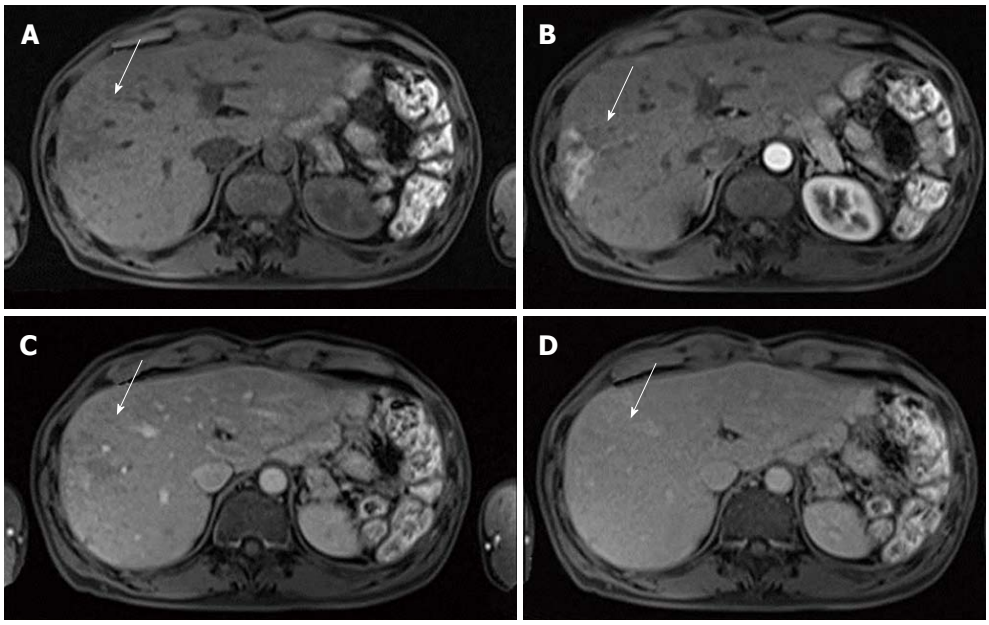


Figure 3 Dynamic magnetic resonance imaging demonstrated an arterial enhance mass like lesion as same as computed tomography scan. A: Pre-enhanced image; B: Arterial phase image; C: Portal phase image; D: delayed phase image.

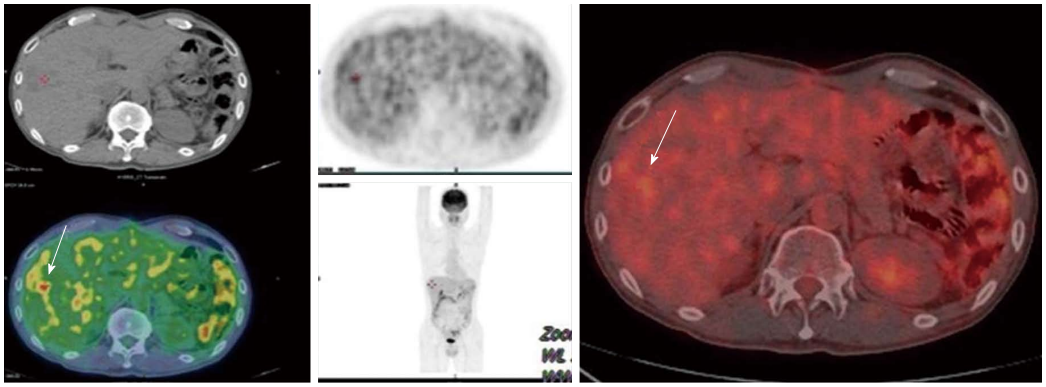


Figure 4 This arterial enhances mass like lesion had a hypermetabolism in positron emission tomography computed tomography.

DISCUSSION

A unique feature of the liver's blood supply system is its dual blood supply, which comes from the hepatic artery (25%) and the portal vein (75%). A perfusion gradient between the artery and portal vein under various conditions such as diminished blood flow through the portal vein can result in an increased attenuation or signal intensity difference during the early phases of a contrast-enhanced dynamic imaging of the liver. In most cases, this finding reflects an increased arterial blood flow, related to an APS. APSs tend to show an amorphous or nodular appearance in the peripheral portion of the liver. There are various causes of APS: hepatic neoplasms, hepatic trauma including interventional procedure, liver cirrhosis, inflammatory diseases, obstruction of the portal or hepatic vein due to various causes, external compression such as hepatic tumor (hepatocellullar carcinoma and cholangiocarcinoma, metastatic tumor, hematoma), or an internal obstruction (tumor thrombus, invasion of cancer). There are various routes, depending on each cause. These routes are transtumoral (through the tumor itself), transversal (due to portal vein tumor thrombosis), transplexal (peribiliary vascular plexus) and transsinusoidal (between microscopic hepatic arterioles and portal venules, distal to portal vein compression or thrombosis), each of which can occur alone or in combination with each other[2-5].

However, our case was an incidental arterioportal shunt, mimicking a recurred gastric cancer, on various images (dynamic CT, MRI and PET). Actually, we are uncertain about why the arterial enhancing nodule (suspected APS) spontaneously disappeared. However, our patient had no history of liver cirrhosis, intervention of liver, or liver trauma (including trauma

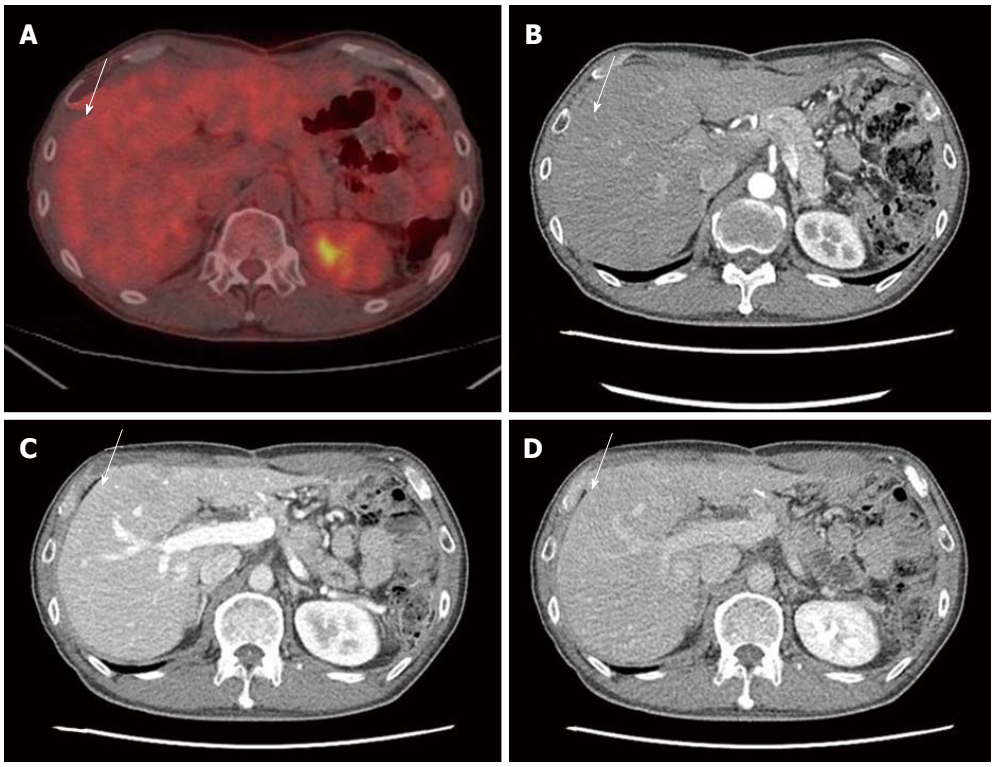


Figure 5 After adjuvant chemotherapy without oxaliplatin, the arterial enhance lesion disappeared. A: PET CT; B: Arterial phase image; C: Portal phase image; D: Delayed phase image.

from surgery of the liver). Moreover, no typical mass was picked up by the sonography.

Oxaliplatin is platinum-based, and a third generation chemotherapeutic agent, administered to patients with gastric cancer, and colorectal cancer. It has comparably less toxic side effects than that of cisplatin. However, there are increasing numbers of reports indicating that oxaliplatin-based chemotherapy could cause damage to a non-tumor-bearing liver. Several reports have confirmed the association between hepatic sinusoidal injury and oxaliplatin. Oxaliplatin-induced hepatic sinusoidal injury can range from a mild sinusoidal dilatation to a hepatic sinusoidal obstruction syndrome. Clinical manifestations vary from asymptomatic to hepatomegaly, jaundice, and ascites. The oxaliplatin sinusoidal injury lesions appeared after the administration of oxaliplatin; however, most lesions disappeared after the cessation of oxaliplatin treatment[6-10].

The incidence of hepatic sinusoidal injury ranges between 19% and 54%. A retrospective study demonstrated that approximately half the patients who received preoperative chemotherapy with oxaliplatin developed some degree of hepatic sinusoidal dilation and microscopic hemorrhage related to damage to the hepatic sinusoidal endothelial cell barrier[11,12].

Oxaliplatin-induced liver damage is characterized histologically by sinusoidal dilatation and congestion outlined by atrophic hepatocyte and/or fibrosis and venular obstruction. An immunohistochemical study of cluster of differentiation 34 revealed a decrease in sinusoidal endothelial cells in this lesion. Such an injury often increases the porosity of the sinusoidal endothelium, and increases cellular fenestrations. This led to the obstruction of the sinusoids and interruption of the portal circulation, resulting in hepatic congestion and eventually elevated portal pressures. Finally, some advanced cases of oxaliplatin-induced sinusoidal injury, have induced veno-occlusive disease, sinusoidal obstruction and portal hypertension[13-15]. Among the pathogenesis of oxaliplatin-induced sinusoidal injury, hepatic congestion due to an obstruction of the sinusoids and an interruption of portal circulation is similar to APS, through a transsinusoidal route. It is thought that oxaliplatin-induced sinusoidal obliteration of peripheral hepatic venules could lead to a retrograde filling of small portal vein branches, by way of a transsinusoidal route[2-5].

Our patient underwent splenectomy, and there might be a question that it may affect to oxaliplatin induced sinusoidal injury. However, we couldn't find the relationship between splenectomy and oxaliplatin-induced sinusoidal injury or APS, and splenectomy was rather as option of treatment of hypersplenism from oxaliplatin-induced sinusoidal injury[16].

In conclusion, although we had no established

evidence of a relationship between oxaliplatin and incidental APS, we concluded on the diagnosis because of the following reasons: Firstly, there was no reason lead to be APS such as trauma, intervention and liver cirrhosis. Secondly, the arterial enhancing lesion appeared after the administration of oxaliplatin and disappeared after the cessation of oxaliplatin. Thirdly, although no definitive mechanism exists for our case, oxaliplatin-induced sinusoidal injury has been identified, and this is enough evidence from literature, to support our case. To the best of our knowledge, this is the first case report of arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer upon interpretation of multiple imaging modalities.

COMMENTS

Case characteristics

After adjuvant chemotherapy with capecitabine and oxaliplatin in stage IIIb gastric cancer, a computed tomography revealed a 1 cm sized arterial-enhancing nodule in the liver.

Clinical diagnosis

The authors presumed initially a new hepatocellular carcinoma or recurred gastric cancer.

Differential diagnosis

Hepatocellular carcinoma, recurred gastric cancer, arterioportal shunt.

Laboratory diagnosis

Various tumor marker were under the normal range.

Imaging diagnosis

Various imaging including dynamic computed tomography (CT), magnetic resonance imaging, and Positron emission tomography-CT showed an arterioportal shunt.

Pathological diagnosis

The authors could not obtain the tissue because no typical mass was detected on ultrasonography.

Treatment

The patient was continued on treatment with adjuvant chemotherapy following discontinuation of oxaliplatin.

Related reports

There was no report of non-tumorous incidental Arterioportal shunt (APS) related to treatment with oxaliplatin.

Term explanation

APS is an organic communication between the hepatic arterial system and the portal venous system.

Experiences and lessons

The authors want to share this case as rare condition of arterioportal shunt due to oxaliplatin induced sinusoidal injury.

Peer-review

This is the first case report of arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer upon interpretation of multiple imaging modalities

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LETTERS TO THE EDITOR

Comment on "Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics"

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Author contributions: Kazakos EI, Polyzos SA, Deretzi G and Kountouras J wrote the letter; Dorrell N and Kountouras J revised the manuscript.

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Abstract

Attaran *et al*[1] have recently shown that decreased susceptibility of established *Helicobacter pylori (H. pylori)* biofilms to specific antibiotics, was associated with the overtly enhanced transcription of two efflux pump genes, *hp1165* and *hefA*, involved in specific resistance to tetracycline and multiple antibiotics, respectively. Apart from antibiotic exposure, secretion of multiple antimicrobial peptides, such as human β-defensins (hβDs), by the gastric epithelium upon *Hp* challenge, may act as early triggering events that positively impact biofilm formation and thus, antibiotic resistance. In this regard, we undertook genomic transcriptional studies using *Hp* 26695 strain following exposure to sublethal, similar to those present in the gastric niche, concentrations of hβDs in an attempt to provide preliminary data regarding possible mechanisms of immune evasion and selective sensitivity of *Hp*. Our preliminary results indicate that hβD exposure ignites a rapid response that is largely due to the activation of several, possibly interconnected transcriptional regulatory networks – origons - that ultimately coordinate cellular processes needed to maintain homeostasis and successful adaptation of the bacterium in the gastric environment. In addition, we have shown that both antibiotic and hβD resistance are mediated by dedicated periplasmic transporters, including the aforementioned efflux pump genes *hp1165* and *hefA*, involved in active export of antibiotics from the cell membrane and/or, as recently suggested, substrate sensing and signalling. Furthermore, it

appears that sublethal doses of hβDs may enhance biofilm formation by the sustained expression of, mainly, quorum sensing-related genes. In conclusion, we provide additional data regarding the role of specific innate immune molecules in antibiotic cross-resistance mechanisms that may deepen our understanding in the context of the development of novel eradication regimens.

Key words: *Helicobacter pylori*; Human β-defensins; Biofilm; Antimicrobial resistance

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Core tip: In the course of *Helicobacter pylori* infection, epithelium-derived human β-defensins may act as early triggering signals that induce biofilm formation and enhanced expression of antibiotic resistance genes, regardless of prior antibiotic exposure.

Kazakos EI, Dorrell N, Polyzos SA, Deretzi G, Kountouras J. Comment on "Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics". *World J Gastroenterol* 2017; 23(33): 6194-6196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i33/6194.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i33.6194

TO THE EDITOR

Attaran *et al*[1] concluded that, in biofilm-forming populations, overexpression of two efflux pump genes, *hp1165* and *hefA,* conferring resistance to tetracycline and multiple antibiotics respectively, may favor reduced antibiotic susceptibility of *Helicobacter pylori* (*H. pylori) in vivo*.

Further to antibiotic exposure, additional, epithelial-derived molecules may function as triggering signals during the dynamic *H. pylori* interaction with the gastric mucosa, provoking overexpression of efflux pumps that in turn, regulate the bacterium's biofilm-producing capacity and promote its virulence. Several studies have unraveled the role of constitutive and/or induced expression of human β-defensins (hβDs)1 - 4 in the bacterium's adaptation in the human stomach and *H. pylori* -related pathologies [2,3].

In this respect, we performed whole genome transcriptome analyses (competitive genomic RNA/RNA hybridisations) using *H. pylori* -specific microarrays based on the *Hp* 26695 and J99 genome sequences and annotation available at the time. Briefly, *H. pylori* 26695 strain was exposed to sublethal, similar to those encountered at the gastric epithelium concentrations of hβDs, in an attempt to identify possible mechanisms of *H. pylori* immune escape and clarify their role in biofilm development *in vitro*. Our preliminary results have identified profound changes in the transcriptional profile

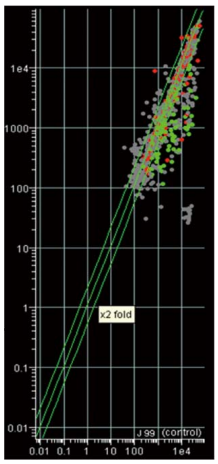


Figure 1 Representative scatter analysis of the general patterns of *H. pylori* genomic response to human β-defensin 3 (hβD3) revealed by transcriptional profiling. Scatter plots (log2 ratio) of average normalized intensities representing Cy5-red channel versus Cy3-green channel are shown for experiments in the presence of sublethal concentrations of hβD3 compared with "control" conditions (no hβD3). Differential expression of a given gene is reflected by deviation from the central diagonal line. The upper diagonal defines ⩾ 2-fold up-regulation and the lower one defines ⩾ 2-fold down-regulation.

of *H. pylori* 26695 demonstrated by the induction or suppression of multiple gene components of distinct regulatory and signaling cascades activated as a result of environmental stress (Figure 1, unpublished data). Overall, the vast majority of genes affected, encoded components of the cell wall stimulon, possibly as means to prevent hβD-specific binding and proper immune recognition, or could be further assigned to certain origons, essential for colonisation of the gastric niche and long-term adaptation, intracellular metal homeostasis and urease activation that largely determine *H. pylori* pathogenicity. Apart from the marked induction of *hp1165* and *hefA*, also reported by the authors[1], several other genes coding for transmembrane ABC transporters (*glnP, dppF, hp1458, hp1486*), efflux proteins (*hp0656, hp0946*), multidrug and toxic extrusion proteins were found to be significantly up-regulated, thereby indicating their prominent role in the cellular response to hβDs challenge, membrane detoxification and maintenance of osmotic balance.

Interestingly, enhanced biofilm production by *Hp* 26695, observed in our studies upon exposure to sublethal concentrations of hβD1 and hβD3, was primarily attributed to the down-regulation of *metK* and *luxS* genes, involved in synthesis of quorum-sensing autoinducer-2, in accordance to previously published data[4,5].

Collectively, our results indicate that sublethal doses of epithelial-secreted antimicrobial peptides such as hβDs, may select co-resistance to antibiotics commonly used in *Hp* eradication therapies and *vice versa*, considering that they provoke the activation of shared, contact-dependent signaling networks, including efflux pumps. Furthermore, it appears that hβDs may independently act as triggering stimuli

promoting biofilm formation *in vivo* which in turn, accounts, at least partly, for the observed failure of eradication regimens and the establishment of *H. pylori* -related chronic inflammation.

Given the complexity of *H. pylori* -host epithelial crosstalk aforementioned data warrant further investigation to achieve the development of successful anti-biofilm strategies that will ultimately re-enforce our therapeutic options mainly towards eradication of *H. pylori* -related resistance. Furthermore, future research focus on the polymorphic variability of the human genome that directly affects epithelial dynamics of hβDs expression may reveal important correlation patterns between *H. pylori* pathogenesis, including biofilm formation, and individual disease susceptibility.

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EDITORIAL

Defining and predicting deep remission in patients with perianal fistulizing Crohn's disease on anti-tumor necrosis factor therapy

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Abstract

Perianal fistulas can occur to up to one-third of patients with Crohn's disease (CD) leading to significant disabling disease and morbidity. Fistulising perianal CD treatment often necessitates a combined pharmacological and surgical approach. Anti-tumor necrosis factor (anti-TNF) therapy, particularly infliximab, has been shown to be very effective for both perianal and internal fistulising CD. Nevertheless, current data suggest that sustained remission and long-term complete fistula healing can be achieved in only 30% to 50% of patients. Moreover, these percentages refer mostly to clinical rather than deep remission, defined as endoscopic and radiologic remission, which is quickly emerging as the preferred goal of therapy. Unfortunately, the therapeutic options for perianal fistulising CD are still limited. As such, it would be of great value to be able to predict, and more importantly, prevent treatment failure in these patients by early and continued optimization of anti-TNF therapy. Similar to ulcerative colitis and luminal CD, recent data demonstrate that higher infliximab concentrations are associated with better clinical outcomes in patients with perianal fistulising CD. This suggests that therapeutic drug monitoring and a treat-to-trough therapeutic approach may emerge as the new standard of care for optimizing anti-TNF therapy in patients with perianal fistulising CD.

Key words: Inflammatory bowel disease; Infliximab; Adalimumab; Magnetic resonance imaging; Drug monitoring; Fistula healing

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Core tip: Defining and predicting deep remission is important to guide the management of patients with perianal fistulizing Crohn's disease (CD). Deep remission, defined as complete fistula healing based on objective endoscopic and radiologic findings, should be the goal of care in the treatment of patients with perianal CD. Currently, anti-tumor necrosis factor (anti-TNF) are the standard of care for perianal CD, but long-term outcomes are disappointing. Data suggests that higher infliximab concentrations are associated with better clinical outcomes in patients with perianal fistulising CD and thus therapeutic drug monitoring may be a valid therapeutic strategy for optimizing anti-TNF therapy towards improved objective outcomes and deep remission.

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INTRODUCTION

Perianal fistulas can develop to up to one-third of patients with Crohn's disease (CD) leading to disabling disease, morbidity, and a significant impairment in quality of life[1]. The treatment of fistulising perianal CD is not simple and often requires a multidisciplinary approach of both pharmacological and surgical therapy especially for complex perianal fistulae[2]. Anti-tumor necrosis factor (anti-TNF) therapy has revolutionized the treatment of both perianal and internal fistulising CD[3-18]. Nevertheless, therapeutic outcomes from randomised controlled trials (RCTs), post-hoc analyses of RCTs and real-life prospective or retrospective studies show that long-term remission can be achieved in only 30%-50% of patients (Table 1). Moreover, these percentages refer mostly to clinical remission, based on symptoms and physician global assessment (PGA), and not to objective endoscopic and/or radiological healing. At this time, the preferred goal of treatment should be deep remission, or the combination of clinical and the more objective measures, including radiologic and endoscopic healing. As therapeutic options for perianal fistulising CD are still limited it is very important to attempt to predict and subsequently prevent treatment failure in these patients. Preliminary data demonstrate that higher infliximab concentrations are associated with improved clinical outcomes in patients with perianal fistulising CD, suggesting that therapeutic drug monitoring (TDM) and a treat-to-trough approach is likely a valid therapeutic strategy for optimizing anti-TNF therapy in these patients[19,20].

Defining deep remission

Most studies typically use clinical remission, defined as absence of any draining fistulas based on PGA and patients' reports, as a therapeutic endpoint for perianal fistulising CD[3-18]. Nevertheless, deep remission, defined as mucosal and/or radiological healing of fistulas, is likely a more appropriate goal of therapy for perianal fistulising CD. T2-weighted magnetic resonance imaging (MRI) with fat-suppression is considered the gold-standard for fistula imaging and an MRI-based score is currently available for defining disease activity, although it is still not widely used in clinical practice[1]. Thomassin *et al*[11] have recently showed that deep remission, defined as a composite clinical (absence of any draining fistulas and self-reported drainage episodes by the patient at two successive evaluations), endoscopic (absence of ulcers in the anal canal) and radiological (absence of T2 hyperintensity and contrast enhancement on MRI) remission, was achieved in approximately one-third of patients with perianal fistulizing CD[11].

Predicting deep remission

As new drugs for the treatment of perianal fistulising CD are still awaited, it is important to be able to predict who will achieve deep remission and who will not respond adequately to typical anti-TNF dosing and will require early (and continued) optimization[1,2]. Although several variables have been associated with improved outcomes (Table 2), prediction of deep remission remains a challenge. Thomassin *et al*[11] have recently identified absence of rectal involvement on MRI (OR = 4.6; 95%CI: 1.03-20.5) as the only variable associated with deep remission in patients with perianal fistulizing CD[11]. Similar to ulcerative colitis and luminal CD[19-25], recent data demonstrate that higher infliximab concentrations are associated with better clinical outcomes in patients with perianal fistulising CD[26,27]. Regarding maintenance therapy Yarur *et al*[26] recently showed that infliximab trough concentrations $\geqslant$ 10.1 μg/mL are associated with fistula healing and based on quartile analyses proposed that physicians should aim for even higher concentrations (> 20.2 μg/mL) before giving up and moving on to alternative therapies with a different mechanism of action.

CONCLUSION

Deep remission defined as a composite clinical, endoscopic and radiological remission should really be considered the goal of therapy in patients with perianal fistulizing CD. TDM may be a valid therapeutic strategy for optimising anti-TNF therapy, improving therapeutic outcomes, and moving towards more personalized medical care.

Table 1 Long-term outcomes of patients with perianal fistulizing Crohn's disease on anti-tumor necrosis factor maintenance therapy

Type of anti-TNF therapy	*n*	Complex fistulas, %	Follow up, wk	Therapeutic outcome of interest	Therapeutic outcome, %	Ref.
IFX	68	75	52	Complete fistula closure & CDAI < 150	34	[4]
IFX	59	85	> 56	Complete fistula closure (PGA)	41	[5]
IFX	13	ND	95[1]	Reduction of fistulas number (MRI)	15	[5]
IFX	156	82	250[1]	At least 1 fistula closure	69	[6]
IFX	12	ND	156	Clinical remission (PGA)	33	[7]
IFX	12	ND	156	Radiological healing (MRI)	42	[7]
IFX	19	ND	52	Absence of draining fistulas (PGA)	53	[8]
IFX	26	69	255[2]	Complete fistula closure	42	[9]
IFX (RCT)	96	ND	54	Complete fistula closure	36	[10]
IFX/ADM	49	ND	160[2]	Deep remission (PGA, MRI, endoscopy)	33	[11]
IFX/ADM	49	ND	160[2]	Absence of draining fistulas (PGA)	53	[11]
IFX/ADM	20	ND	52	Absence of draining fistulas (PGA)	35	[12]
IFX/ADM	78	67	192[1]	Absence of drainage with seton removal	53	[13]
IFX/ADM	20	ND	78	Radiological healing (MRI)	30	[8]
ADM	7	ND	156	Absence of draining fistulas (PGA)	0	[7]
ADM	7	ND	156	Radiological healing (MRI)	14	[7]
ADM	7	ND	52	Absence of draining fistulas (PGA)	29	[8]
ADM	39	ND	52	Clinical remission (FDAI)	41	[14]
ADM	14	ND	52	Radiological healing (MRI)	43	[14]
ADM	53	ND	40	Complete fistula closure	41	[15]
ADM (RCT)	70	ND	56	Absence of draining fistulas (PGA)	33	[16]
ADM (post hoc)	70	ND	116	Absence of draining fistulas (PGA)	31	[17]
CZP (RCT)	28	ND	26	Complete fistula closure	36	[18]

[1]Median; [2]Mean. CDAI: Crohn's disease activity index; TNF: Tumor necrosis factor; ADM: Adalimumab; IFX: Infliximab; CZP: Certolizumab pegol; RCT: Randomized controlled trial; PGA: Physician global assessment; ND: Not defined; FDAI: Fistula drainage assessment index; MRI: Magnetic resonance imaging.

Table 2 Variables associated with improved therapeutic outcomes of anti-tumor necrosis factor maintenance therapy in patients with perianal fistulizing Crohn's disease

Variables	Ref.
Clinical or phenotypic	
Ileocolonic disease	[6]
Concomitant immunosuppressants	[6]
Duration of seton drainage (< 34 wk)	[6]
Duration of infliximab treatment (> 118 wk)	[6]
Number of infliximab infusions (> 19)	[6]
Absence of complex fistulas	[14]
Male gender	[26]
Absence of switch of anti-TNF therapy	[11]
Imaging	
Absence of persisting fistulas on MRI	[5]
Absence of collections at baseline on MRI	[5]
Absence of rectal wall involvement on MRI	[5]
Absence of single-branched fistulas on MRI	[5]
Absence of rectal involvement on MRI	[11]
Serologic	
Infliximab (maintenance) trough concentrations ⩾ 10.1 μg/mL	[26]
Endoscopic	
Absence of active proctitis	[11]

TNF: Tumor necrosis factor; MRI: Magnetic resonance imaging.

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MINIREVIEWS

Evidences supporting the vascular etiology of post-double balloon enteroscopy pancreatitis: Study in porcine model

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Abstract

Double balloon enteroscopy (DBE) is an endoscopic technique broadly used to diagnose and treat small bowel diseases. Among the associated complications of the oral DBE, post-procedure pancreatitis has taken the most attention due to its gravity and the thought that it might be associated to the technique itself and anatomical features of the pancreas. However, as the etiology has not been clarified yet, this paper aims to review the published literature and adds new results from a porcine animal model. Biochemical markers, histological sections and the vascular perfusion of the pancreas were monitored in the pig during DBE practice. A reduced perfusion of the pancreas and bowel, the presence of defined hypoxic areas and disseminated necrotic zones were found in the pancreatic tissue of pigs. All these evidences contribute to support a vascular distress as the most likely etiology of the post-DBE pancreatitis.

Key words: Double balloon enteroscopy; Pancreas; Animal model; Pancreatitis; Pig

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Core tip: Double balloon enteroscopy (DBE) has been occasionally associated with pancreatitis. As

the etiology has not been clarified yet, this paper reviews the published literature and adds new results from the porcine animal model. Based on a growing number of indirect clinical evidences and supported by experimental studies in animal model, a vascular distress is suggested as the most likely etiology of post-DBE pancreatitis.

Latorre R, López-Albors O, Soria F, Morcillo E, Esteban P, Pérez-Cuadrado-Robles E, Pérez-Cuadrado-Martínez E. Evidences supporting the vascular etiology of post-double balloon enteroscopy pancreatitis: Study in porcine model. *World J Gastroenterol* 2017; 23(34): 6201-6211 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6201.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6201

INTRODUCTION

Double balloon enteroscopy (DBE) is an endoscopic technique for diagnosis and treatment of small bowel diseases[1]. This variety of deep enteroscopy allows a complete exploration of the small bowel by alternatively combining the inflation and deflation of two balloons placed at the tip of the tube and the overtube following a precise sequence of push and pull manoeuvres, which allow the endoscope to progress deep into the small bowel[2]. DBE is not only exploratory but also a therapeutic technique which after more than fifteen years has even been considered as the "gold standard" technique for the endoscopic exploration of the small bowel. Furthermore, the combination of the endoscopic capsule and DBE has been highlighted as the most effective tool for diagnosis and treatment of diseases affecting the small bowel[3]. This was openly stated in the "guideline" published by the ESGE in 2008[4,5] and has also been largely reported in the scientific literature. However, as in any other endoscopic technique complications can appear, and among them post-procedure pancreatitis (post-DBE) is within the most severe.

There is a large series of studies describing direct and indirect complications associated to DBE[6]. In a multicentric study involving 10 endoscopy units from four different continents[7], a total of 40 complications from a total of 2362 DBE were reported (1.7%). This number is within the range of complication currently associated to the therapeutic conventional endoscopy techniques. Concerning the post-DBE pancreatitis several case-series published in Europe[8,9], Asia[7,10,11] and United States[12] refer figures as low as 0.3%, and in the opposite, as high as 3%[13-15] or even 12.5%[16]. Overall, in a recent paper, Kopacova reported a historic accumulation of more than 100 post-DBE pancreatitis already published[17].

According to the international consensus of classification of acute pancreatitis[18,19], the diagnosis requires two of the following three items: (1) abdominal pain consistent with acute pancreatitis; (2) serum lipase activity or amylase activity at least three times greater than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on contrast-enhanced computed tomography (CT) and less commonly magnetic resonance imaging (MRI) or transabdominal ultrasonography. In clinical practice suspicion of post-DBE pancreatitis must always be ruled out before patients are delivered home. Severe pancreatitis is not difficult to recognize, however the mild form may be a problem. Mild acute pancreatitis is characterized by the absence of organ failure and the absence of local or systemic complications. Patients with subclinical mild acute pancreatitis are usually discharged during the early phase and usually do not require pancreatic imaging, and mortality is very rare. Besides many of mild pancreatitis could be missed in patients with DBE performed on an outpatient basis[17]. It is unknown how many patients with self-controlled abdominal pain have been able to have mild acute pancreatitis post-DBE[17]. This circumstance of lack of patient follow-up suggests to some authors a generalized underestimation of the number of post-DBE pancreatitis[13].

Increased levels of one or more of the pancreatic serum markers is very common after oral DBE[16,20,21]. Thus, amylase concentration below the double of the basal levels has been found in 39%[15], 46%[21] or even 58%[20] of oral DBE at 24 h of procedure. Hyperlipasemia has been described from as soon as 4 h[16,17,20,22], to up 12 h[16] or 24 h[16,17,20] after DBE. Also, in patients where pancreatic disease was diagnosed lipase levels remained increased even after amylase returned to normal figures[23]. The C-reactive protein (CRP) can also be related to either non-specific inflammation of the bowel mucosa or pancreatic inflammation, thus its diagnostic significance very much depends on the concomitant appearance of increased levels of amylase and/or lipase[14,16]. Regarding the evolution of these three serum markers, in a recent study, we determined pre- and post-procedure (4 h) levels of amylase, lipase and CRP in a series of 55 randomly selected patients for oral DBE (non-published data). Results in demonstrate a significant increase of the three markers after 4h of DBE. Contrastingly, neither the age, sex, geographical origin, insertion depth and duration of the procedure, nor the type of procedure (diagnostic *vs* therapeutic) showed any significant correlation with the levels of the pancreatic markers.

Up to here, the most common evidences for suspicion and diagnosis of the oral post-DBE pancreatitis have been summarized. However, despite more than 15 years of worldwide DBE practice the ethiopathogenesis of the post-DBE pancreatitis is not yet completely clarified. A shared belief in the literature is that the cause of this grave complication is associated to the technique itself[14,20,21,24,25]. Thus, a combination of different circumstances such as mechanical stress by the instruments (balloons, mainly) or the push

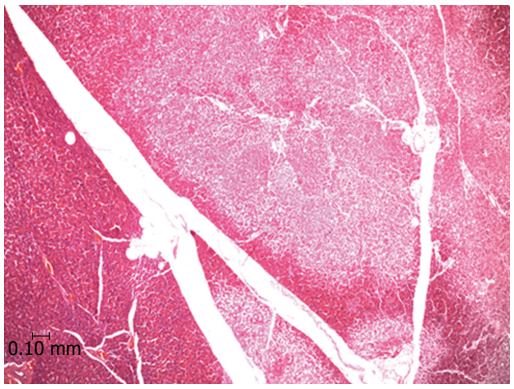


Figure 1 Light microscopy image of the porcine pancreas after double-balloon enteroscopy showing located ischemic necrosis in pancreatic interlobular tissue.

and pull manoeuvres, anatomical features of the pancreas and its vascularization, and undetermined conditions of the patient could be implicated in each case[2,10,20,24-26]. Nevertheless, up to three different hypotheses have been formulated. One refers to a reduced blood perfusion of the pancreas and the bowel (vascular etiology)[14,24,27], another to obstruction of the duodenal papilla by mechanical pressure or damage (papilla injury etiology)[13,15,16,21,25,28-30], and the third one to potential pancreatic reflux due to increased intraluminal pressure within the small bowel (reflux etiology)[24,31]. To better understanding the problem in the last decade we have carried out several of experiments in porcine model[32-39] where oral DBE were systematically monitored. Although the porcine model was previously used both *ex vivo* and *in vivo* for DBE training and to increase the security conditions in translational studies[2,35,36], no previous *in vivo* studies were directly aimed at finding out the etiology of the post-DBE pancreatitis. In the present work, we are summarizing our findings[33,34,39], with addition of some unpublished results, and reviewing the related literature in order to gather substantial evidences supporting the vascular etiology as the most likely explanation for the potential appearance of pancreatitis after oral DBE.

VASCULAR ETIOLOGY: REDUCED PANCREATIC AND INTESTINAL PERFUSION DURING DBE BY ORAL APPROACH

A broad number of scientific studies have suggested this cause[14,24,27]. This hypothesis establishes that during DBE the continuous repetition of push and pull manoeuvres progressively recoils the root of the mesentery such that the vascularization supplying the pancreas and the first portion of the small bowel is significantly reduced[14,24,27]. The increasing ischemia causes different degrees of cellular damage and a reactive condition leading to subclinical or occasionally clinical pancreatitis after the procedure. Also, the potential compression of the pancreas and its vascularization against the vertebral spine during the exploration may intensify the damage to this organ and increase the risk of inflammation[26]. The tail and body of the pancreas are the portions less mobile and more susceptible to such an ischemic and mechanical stress, which may explain why these portions are the most frequently affected in many cases of clinically diagnosed post-DBE pancreatitis[20,21,25,40].

Evidences of structural disorders and hypoxia in the pancreas

In an experiment set up already published oral DBE were performed in Large White pigs[33]. Serum levels of pancreatic markers (amylase, lipase and CRP) were monitored before, during and after (24 h and 7 d) the procedure, which lasted for either 90 (n = 10) or 140 min (n = 10). In order to avoid any pressure over the duodenal papilla the balloons were never inflated until the Treitz ligament was reached. Amylase, lipase and CRP levels at 24 h post-DBE were significantly increased, however none of the pigs showed any clinical symptom of pancreatitis after the procedure. Besides, no association between the serum markers and the duration of the procedure or the total explored distance was found. Seven days post-DBE the pigs were euthanized and the pancreases removed and processed for histopathological study. A remarkable result was that 47.4% of the animals showed pancreatic lesions which consisted in disseminated focal areas of ischemic type tissue necrosis with inflammatory cell infiltration around the necrotic foci. There was a lack of dilation of the pancreatic ducts. The lesions mainly affected the exocrine component and always affected the tail of the pancreas (Figure 1). The pancreatic tail (left lobe in pigs) was preferentially damaged because it is the portion with the most limited blood supply. In pigs, it is supplied by a single artery, which is a branch of the splenic artery, and a similar situation is found in humans where the main artery of the tail of the pancreas is the major pancreatic artery[41]. Such anatomical particularity predisposes the left pancreatic lobe to suffer from hypoxia or even ischemia if blood supply is mechanically restricted. It is interesting to highlight that CT has revealed that human pancreatitis is predominantly located in the tail of pancreas[14,40,42]. This preferential location of the histopathological lesions and the lack of clinical symptoms in spite of substantial elevations of serological markers is a clue to assume a similar etiopathogeny for the appearance of the lesions in the pigs and humans[15,26].

To further investigate whether a pancreatic ischemia during DBE was in the origin of the histopathological findings immunohistochemistry of hypoxia markers was carried out. The vascular endothelial growth factor

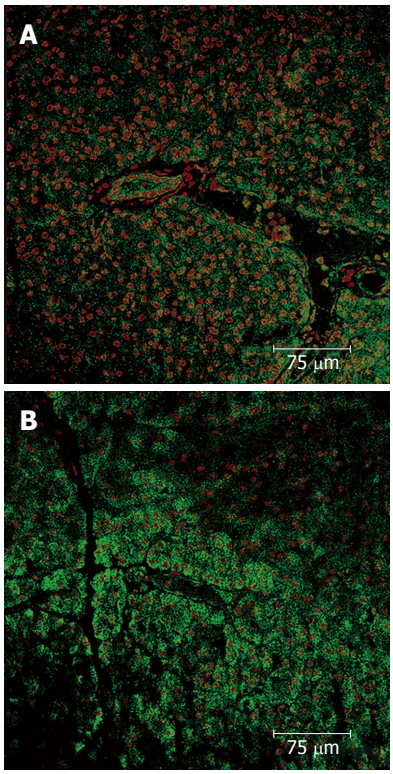


Figure 2 Pancreas immunohistochemistry. A: Expression of the VEGF in a normal parenchyma; B: Pancreatic acini with normal structure and nuclei, which express VEGF. In upper right corner a ischemic zone where less VEGF expression is shown, the pancreatic acini structure has been lost and nuclei are pyknotic. VEGF: Vascular endothelial growth factor.

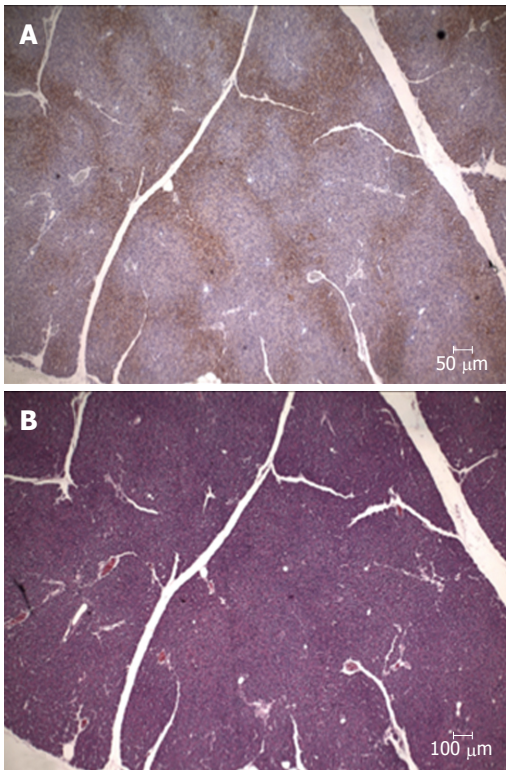


Figure 3 Pancreas immunohistochemistry. A: Expression of the pimonidazole hydrochloride (Hypoxyprobe®) presence of focal areas positively stained; B: Serial section stained with hematoxyline-eosine.

(VEGF) is an endogenous factor involved in vascular permeation and angiogenesis which may be over expressed when blood supply is reduced[43]. In the endocrine component of the pancreas an increased expression of VEGF has been observed in experimental situations reproducing a hypoxic scenario[44-46]. Likewise, in the pancreases of our pigs (same animals of the previous experiment) a positive immunostaining for VEGF was observed in both the endocrine and exocrine components. No VEGF expression was found in the control group while in the experimental group there were areas where VEGF was not expressed alongside others where it was highly expressed. In the necrotic areas low or nule VEGF expression was observed, as corresponds to areas with damaged cells. Around these necrotic areas however high VEGF expression was found, which correspond to a reactive expression due to ischemia. Overall the highest expression of VEGF was in the tail and body of the pancreas (Figure 2).

Complementarily, the exogenous marker pimonidazole hydrochloride (Hypoxyprobe®) was used in another set of pigs (non-published results). This compound is a derivative of the 2-nitroimidazole, which forms irreversible adducts with thiol groups in proteins, peptides and amino acids in tissues with oxygen tension of below 10 mmHg[47]. This exogenous marker has been largely tested in animal models and humans for immunodetection of hypoxic tissues harvested from both normal organs and malignant tumors[48,49]. In our experiment Hypoxyprobe® was administered endovenously to 5 anaesthetized Large White pigs (70-90 kg). Then, an oral DBE was performed for 60 min, and after euthanasia, the presence of hypoxic areas in the pancreas assessed by immunostaining. Results demonstrated the presence of focal areas positively stained for pimonidazole adducts (Figure 3), which was an unequivocal demonstration of pancreatic hypoxia (proof of concept). Like in the previous experiment the hypoxic areas were preferentially located (more abundant and wider) in the tail of the pancreas, and affected the exocrine and endocrine components. Although pancreatic hypoxia associated to oral DBE was conceptually demonstrated in this study, it could not be concluded that the direct cause of the hypoxia suffered by the pancreas was DBE. In theory, the presence of pimonidazole adducts could either be a consequence of reduced blood flow or increased oxygen demands by the pancreatic tissue[49], thus further studies were needed to directly assess if a reduced perfusion of the pancreas do really exist during oral DBE.

Evidences for reduced perfusion of the pancreas in the oral DBE

Blood flow to the human and porcine pancreas is primarily supplied by the celiac and superior mesenteric

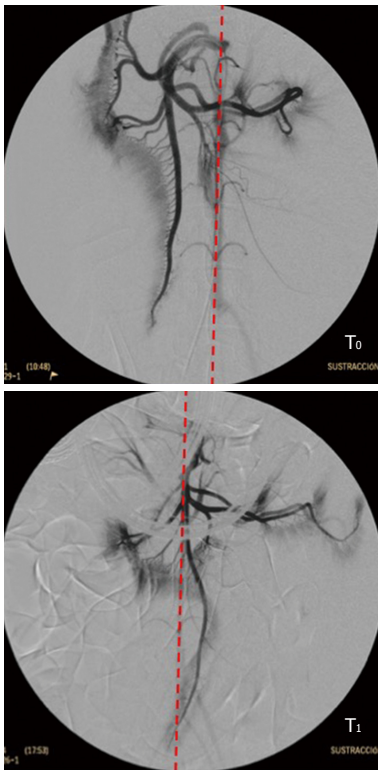


Figure 4 Selective angiogram of the cranial mesenteric artery. T_0: Before the DBE; T_1: With the endoscope inserted at maximum during DBE. Red line shows the aorta topography. DBE: Double balloon enteroscopy.

arteries. These two main vessels supply the head and body of the pancreas by a vascular arcade resulting from anastomosis of the cranial and caudal pancreaticoduodenal arteries. The left pancreatic lobe, as mentioned above, is supplied by a single, terminal, and constant major pancreatic artery which arises from the first portion of the splenic artery, a branch of the celiac artery[41,50-52].

To assess whether a reduced perfusion of the pancreas is associated with the oral DBE two experiments were designed in porcine model. In a former study[39] an endovascular approach under fluoroscopic guidance was carried out in 10 Large White pigs of 45-50 kg. The femoral artery was approached and after selective catheterization angiographies of the superior mesenteric and celiac arteries obtained (time T_0). Then, conventional oral DBE was performed for 90 min, and at the end of procedure, with the endoscope inserted at maximum depth the angiographic study was repeated (time T_1). During the procedure continuous estimation of the explored distance was done[35], but to avoid potential interference of the vascular catheterization neither serum markers of pancreatitis nor histopathological study were done. At T_0, the cranial mesenteric vascular axis was located mainly to the right of the spine, in its usual anatomical position, but at T_1 it was completely to the left (Figure 4), which was related with the intestinal folding and the pull-and-push manoeuvres. Besides, at T_1 a lack of vascular repletion was seen in the caudal pancreaticoduodenal artery. There was a reduction in the level of opacification of the branches of the cranial mesenteric artery addressed to the pancreas and bowel. As revealed by the injected contrast the perfusion of the jejunal arteries, the network of straight arteries, and the capillary network in the antimesenteric border of the small intestine was significantly reduced. With regards to the selective angiography of the celiac trunk, differences between T_0 and T_1 were also relevant. As it was common in all the experiments, none of the animals presented signs of post-DBE pancreatitis. The study described was most surely the only angiographic evaluation of the pancreas and small bowel carried out simultaneously to DBE. The results give radiological evidences of a reduced perfusion to these organs and agree with the recommendation of handling the overtube gently during the push-and-pull manoeuvres to avoid excessive restrain of the mesentery and the traction force on the pancreas[27].

A step further in the evaluation of the vascularization of the pancreas during DBE was achieved in a second experiment (non-published results). The similar and genuine vascular pattern of the tail of the pancreas in pigs and humans supplied by a major pancreatic artery from the splenic artery, lead us to design an endovascular super-selective approach to this vessel at the end of an oral DBE. 5 pigs (40-50 kg) were anaesthetised and, first of all, a CT angiogram (Philips Brillance CT-6) with vascular 3D reconstruction obtained from each animal so as to monitor his individual vascular anatomy before angiographic procedure (Figure 5). The images displayed the whole pattern of ramifications of the celiac artery, the pancreatic branch for the tail of pancreas included. Then, an oral DBE was currently done for 60 min, and by the end of procedure, when the endoscope was inserted at the maximum depth, a super-selective angiography of the major pancreatic artery achieved. In brief, this was the followed protocol. Under fluoroscopic guidance (BV-300; Philips, Best, the Nertherlands), a 0.035- inch hydrophilic guide wire (Radifocus guidewire, Terumo, Tokyo, Japan) and a 5-French cobra catheter (Radiofocus Cobra Small; Terumo, Tokyo, Japan) were selectively addressed towards the celiac artery. Celiac arteriograms clearly depicted the vascular anatomy of the hepatic and splenic arteries and their branches. By road-map guidance, the cobra catheter was selectively positioned and wedged into the major pancreatic artery. The super-selective angiography of this artery was conducted by manual injection of 3-5 mL contrast medium to ensure that the tip of the catheter was at the desired site[53]. The nuclear dye Hoechst (Bizbenzimida H 33258 fluorochrome) was injected through that vessel to allow its contact with the cells in the tail of the pancreas (Figure 5). Hoechst is a membrane-permeable, adenine-thymine specific fluorescent dye useful for *in vivo* staining of DNA. It

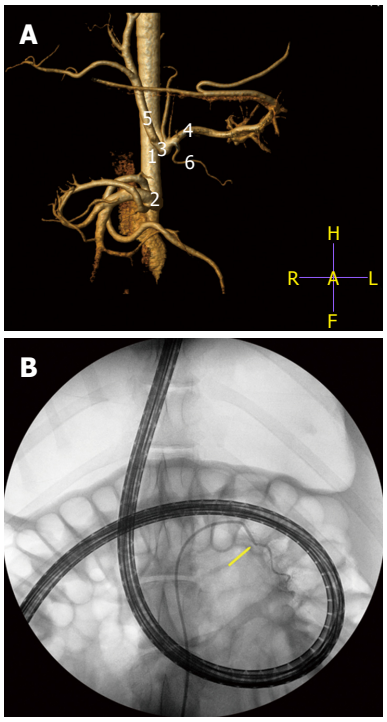


Figure 5 Computed tomography angiogram (A) with vascular 3D reconstruction to monitor his individual vascular anatomy (1) abdominal aorta; (2) cranial mesenteric artery; (3) celiac artery; (4) splenic artery; (5) hepatic artery; and (6) major pancreatic artery. B: Super-selective angiography of the major pancreatic artery (arrow) with the endoscope inserted at maximum during DBE. The nuclear dye Hoechst (Bizbenzimida H 33258 fluorochrome) is injected through that vessel to allow its contact with the cells in the tail of the pancreas. DBE: Double balloon enteroscopy.

has been proved very useful as a marker of tumour cells and to assess the vascular supply to tumours[54-56] as well as vascular perfusion[57] and hypoxia[58]. After euthanasia the pancreases were collected, the tail trimmed in 1 cm × 1 cm × 1 cm blocks, which were identified by zone and frozen in liquid nitrogen. Criosections (10 μm thick) were obtained and stained with the nuclear marker TO-PRO®3 iodide (Molecular Probes) at a dilution of 1:100 in PBS. To-Pro3 was used as a control dye of the whole cellular population in the tissue sections. Fluorescence microscopy was used to evaluate the nuclear DNA staining with the two dyes.

The quantification of staining for both dyes in a digital analyser allowed to directly asses potential changes in the perfusion of the tail of pancreas during DBE (Figure 6). In average, from the total number of nuclei (To-Pro staining) only 35% of them were Hoechst positive. Moreover, the sequenced study in proximo-distal and latero-medial direction of the tail of the pancreas allowed to identify the proximo-lateral portion as the one with the lowest level of Hoechst staining (25%) and the disto-medial portion the one with the highest level (42%). The other two portions, proximo-medial and disto-lateral, showed a 35% and 38% of positive Hoechst cells, respectively. According to these results, a positive gradient in proximo-distal direction was observed in terms of potential blood supply to the tail of the pancreas during oral DBE.

These conclusive results, which directly associated the oral DBE with a reduced vascular supply to the tail of the pancreas, together with all the previous evidences give consistent clues to support the vascular etiology as a likely cause of post-DBE pancreatic inflammation in the porcine model. The anatomical similarities between the human and porcine pancreas supports a translation of results which may help to better understand the pathogeny and clinics of post-DBE pancreatic disorders.

OCCLUSIVE ETIOLOGY: MANIPULATION AND OCCLUSION OF THE DUODENAL PAPILLA

The potential occlusion of the duodenal papilla by the balloons during oral DBE is a risk largely mentioned in the literature[13,15,16,21,25,28-30]. Evidences of pancreatic disorders during Endoscopic Retrograde Cholangio-pancreatography (ERCP) practice are conclusive. For this reason, there is a recommendation of avoiding the inflation of the balloons in the pancreato-biliar area until the Treitz ligament has been reached[16,31,36]. However, awareness of this anatomical landmark by the endoscopist is not always guaranteed[14], especially during the learning curve of DBE. Some studies have related the increase of amylase and lipase levels after DBE with the distance of the first balloon inflation from the Treitz ligament[14,16,59,60]. The longer the distance the lower the figures of the serum markers. Nevertheless, a correspondence between this trend and the incidence of persistent hyperamylasemia or pancreatitis has not been concluded[9].

In the experimental field, we used a porcine model to demonstrate if the direct contact of the inflated balloons with the duodenal papilla was responsible for significant and persistent changes in the serum markers as well as the appearance of structural disorders in the pancreatic tissue[34]. The results were conclusive. The longer the contact of the inflated overtube's balloon with the papilla the higher the levels of amylase (up to 22.7% increase), lipase (up to 7.5 times higher) and CRP (up to 6.3 times higher). Contrastingly, in the DBE control group, where the balloons were inflated after the ligament of Treitz, the serum markers did not increase more than two times the baseline.

Moreover, in those pigs where the overtube's balloon was inflated and kept around the duodenal papilla the histopathological study showed moderate to severe acinar vacuolization, oedema and even necrosis. The histopathological pattern resembled the structural disorders currently found in experimental studies of pancreatitis caused by forced obstruction of the pancreatic duct[61,62]. On the other hand, it has been described that slight vacuolization and/or oedema are reversible if the cause of obstruction disappears, which is compatible with a complete functional and

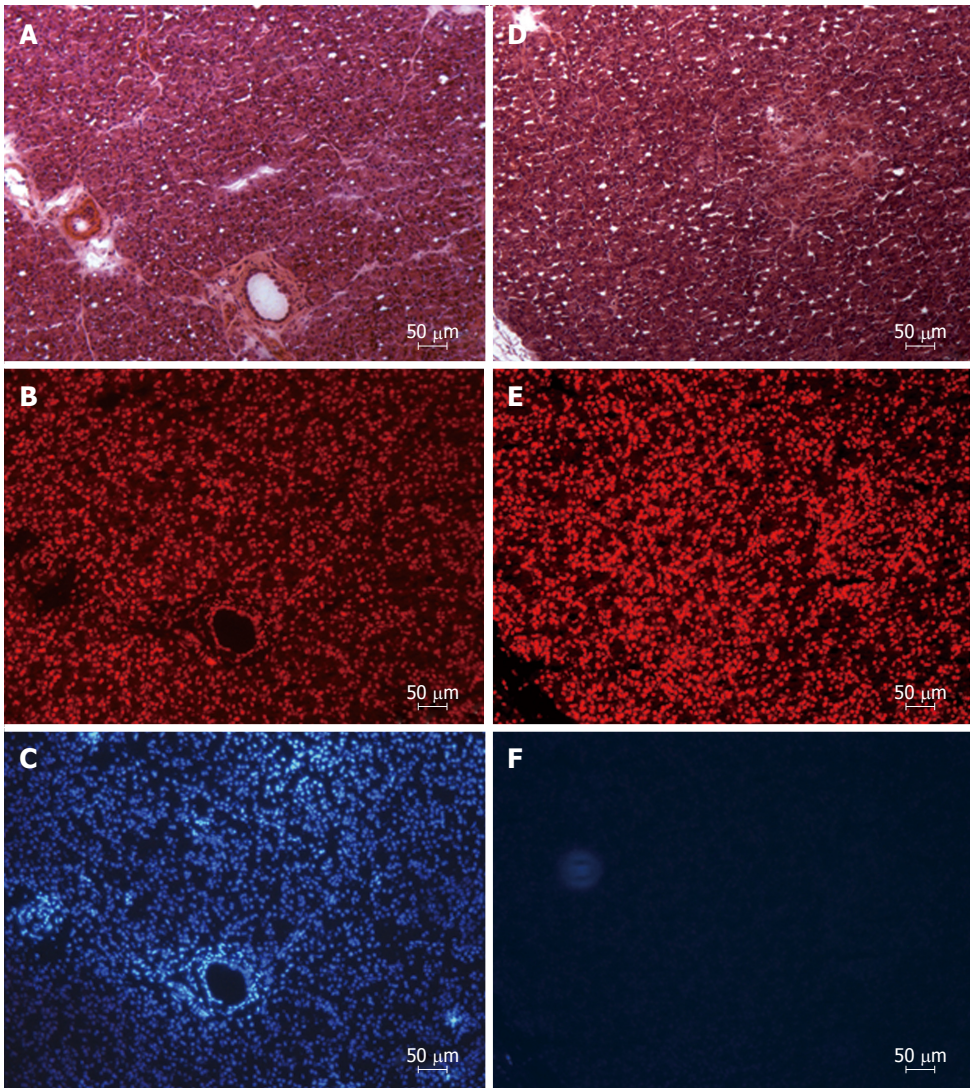


Figure 6 Fluorescence microscopy image of cryosections from left lobe of the pancreas showing distribution of endocrine and exocrine cells with nuclei stained in disto-medial (A-C) and proximo-lateral (D-F) portions. A and D: Serial sections stained with hematoxyline-eosine; B-E: Serial sections stained with the nuclear marker TO-PRO®3 iodide; C and F: Serial sections showing the location the nuclear dye Hoechst (Bizbenzimida H 33258 fluorochrome).

structural recovery of the pancreas[63]. This could explain the absence of symptoms of pancreatitis in any of the pigs despite the persistent contact of the balloon with the duodenal papilla.

These results in animal model clearly support the theory that avoiding contact with the duodenal papilla during DBE insertion decreases the risk of iatrogenic lesions in the pancreas[14,16,59,60]. However, even when the pancreato-biliar area is avoided hyperamylasemia and symptoms of pancreatitis can still appear after DBE. Therefore, avoiding the obstructive picture does not seems to be the only solution, although it reduces the risk of post-DBE pancreatitis[2,33].

THE REFLUX ETIOLOGY

This hypothesis is based on the fact that the endoluminal pressure within the small bowel and particularly, around the pancreato-biliar area rises while DBE progresses. The increased pressure within the duodenal lumen leads to reduced drainage, retrograde circulation or even reflux of the pancreatic secretion[25,31], which cause a severe pancreatic lesion[24]. Accordingly, the pancreatic structural disorders and correspondent inflammation are expected to affect the whole organ in a diffuse pattern. However, that is not the case of the post-DBE pancreatitis, as revealed by imaging diagnosis and histopathology, where the pancreatic affection is preferentially located in the tail of the pancreas in both human[7,13,14,40,42] and porcine models[33]. In addition, no significant dilatation of pancreatic ducts is currently found. Thus, rather than a primary cause of post-DBE pancreatitis this etiology should be considered as secondary or occasional. So, it seems difficult that the etiopathogeny of post-EDB pancreatitis is mainly due to a duodenal reflux mechanism[2].

ADDITIONAL RISK FACTORS FOR POST-DBE PANCREATITIS

In addition to reviewing the etiologies of the post-DBE pancreatitis, a discussion about potential risks involved in this complication is pertinent. The time length of the procedure, the final depth of endoscope insertion and the purpose of the DBE (diagnostic *vs* therapeutic) have been mentioned[14,16,17,20,31,40]. Again, the use of a validated animal model for DBE allows a more accurate approach to this topic[32-35].

The question about how long can DBE take to avoid complications such as pancreatitis has not received yet a unanimous answer. A large variety of figures are found in the literature. Explorations as short as 30 min or as long as 240 min[64] have been referred. Between those limits, DBE of 148 min[14,20], 115 min[16] and 95 min[25] are mentioned. Some authors agree a time window between 70 and 100 min as the criteria to avoid DBE complications such as perforation[2,65,66], while in relation with the learning curve other authors highlight the important reduction in time after approximately 10 DBEs, which in average was quantified from 109 min to 92 min[67]. From another perspective, some works define the maximum effective time of exploration as the period from the beginning of procedure till the moment when no more than 1-2 cm are progressed after several push-and-pull cycles. Such a time of effective exploration, which in humans and pigs takes around 60 min, has been referred as "the gold hour" of DBE practice. However, the relevance of this reference is uncertain. While it is noticeable that oral DBE longer than one hour have been associated with increases of pancreatic lesion or pancreatitis[14,16,17,20,31,40], other studies do not find any consistent reason to consider the time as a direct risk factor[14,21]. This was also the overall conclusion of an experimental study in porcine model[33]. The time and length of each push-and-pull manoeuvre was carefully monitored and no significant association between DBE duration (90 min *vs* 140 min) and the risk of pancreatitis (amylase and lipase levels) or the grade of histopathological disorders was found.

The final insertion depth of the endoscope is another controversial risk associated to DBE, especially because the estimation of the explored distance is always subjective. Several methods to assess insertion depth have been proposed[2,68], but the most frequently used was presented by May in 2005[2]. This method was later validated for experimental purposes in a porcine model[35]. On the other hand, evaluating how insertion depth may be a risk of pancreatitis is neither easy nor accurate in DBE practice. The influence of this variable cannot be isolated from other factors associated to the patient and/or the pathology, which strongly condition how deep the endoscope is addressed. Even under experimental conditions the total insertion depth of the endoscope was not correlated with the level of pancreatic markers or the appearance of structural disorders in the pancreas[33]. Nevertheless, when both the time length DBE interval and the depth of insertion are combined potential complications are more likely.

Several other risks for post-DBE pancreatitis have been suggested[17]. The indication (purpose) of DBE, whether diagnostic or therapeutic is one of them. Diagnostic DBE are less invasive and have a lower risk of major complications (around 1%), such as pancreatitis, haemorrhage or perforation, than therapeutic (up to 5%)[17,69]. The learning curve has also been suggested as a risk for major complications[14]. Up to a minimum of 50 DBE are needed to finish the learning curve[69] but this was never related with higher amylase or lipase levels, or post-DBE pancreatitis[20,59,70]. On the other hand, the use of CO_2 insufflation during DBE is highly recommended as it prevents over-inflation of the small bowel. CO_2 is helpful for an easier and deeper insertion of the endoscope, and has a possible preventive effect to post-DBE pancreatitis[14,17].

CONCLUSION

Based on a growing number of indirect clinical evidences and supported by experimental studies in porcine model, a vascular distress is suggested as the most likely etiology of post-DBE pancreatitis. During oral DBE the push-and-pull manoeuvres cause mechanical strength on the small bowel, pancreas and mesentery, which reduce the normal blood supply. The resulting situation of pancreatic ischemia commonly determines transient cellular hypoxia, with a lack of clinical signs of pancreatitis, however it may occasionally drive severe hypoxia, necrosis and clinical pancreatitis.

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ORIGINAL ARTICLE

Basic Study

Circulating inflammatory factors associated with worse long-term prognosis in colorectal cancer

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Abstract

AIM

To investigate association of circulating inflammatory factors at the time of colorectal cancer (CRC) surgery with survival.

METHODS

Plasma levels from 174 CRC patients (69 females and 105 men), with median age 70 years (range 29-90), localized in the colon (n = 105) or rectum (n = 69), with stage Ⅰ (n = 24), stage Ⅱ (n = 54), stage Ⅲ (n = 67) and stage Ⅳ (n = 29) were measured using commercially available Bio-Plex Pro™ Human Chemokine

Panel 40-Plex, including 40 different chemokines, cytokines and interleukins. The prognostic association of each inflammatory factor was analysed as CRC-specific and total mortality.

RESULTS

Out of 174 patients, 66 died during the follow-up, 40 because of CRC specific mortality. High tertile levels of 8 factors were significantly associated with increased CRC-specific mortality, of which CCL1, CCL20, CCL24, CX3CL1, IL-4 and TNF-α remained significant in a multivariate Cox regression analysis. High tertile levels of 14 factors were associated with increased total mortality, of which CCL1, CCL15, CCL20, CX3CL1, CXCL13, IFN-γ, IL-2, IL-4 and IL-10 remained significant after adjustment for clinical covariates. For most of the inflammatory factors the association between higher tertile levels and an increased mortality in general appeared two years after surgery. High tertile levels of TNF-α and CCL24 were exclusively associated with CRC-specific mortality. The distribution of these factors were not associated with TNM stage with exception for CCL20.

CONCLUSION

High plasma levels of inflammatory factors are associated with increased risk of mortality among CRC patients and could be potential biomarkers for revealing prognosis.

Key words: Colorectal cancer; Inflammation; Cytokines; Plasma; Prognosis; Mortality

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Core tip: Plasma levels of 40 different cytokines, chemokines and interleukins were analyzed in colorectal cancer (CRC) patients of which high tertile levels of nine factors were associated with total mortality and six factors with CRC-specific mortality. For most of the inflammatory factors the association between higher tertile levels and an increased mortality in general appeared two years after surgery.

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INTRODUCTION

Inflammation is of importance in cancer development, and many tumours develop due to prolonged or chronic inflammation throughout their progression[1]. Carcinogenesis in colorectal cancer (CRC) is a multistep process maintained by accumulation of genetic and epigenetic aberrations in several pathways[2,3]. Also, local immunoregulation mediated by inflammatory cells, such as white blood cells, of the tumour microenvironment are involved in the release of inflammatory factors that are able to activate local immune networks to promote both the development and growth of malignant CRC cells by increasing their proliferation, survival and angiogenesis[1,4]. Inflammatory factors such as cytokines together with angiogenic factors are able to trigger the development of invasive abilities as they increase the migration and motility of tumour cells, resulting in the occurrence of metastasis[1]. Cytokines, which include chemokines and interleukins, are a broad and loose category of small proteins produced by white blood cells, stromal cells and cancer cells[1,5]. They are able to regulate the intensity and duration of the immune response by either stimulating or inhibiting the activation, proliferation and/or differentiation of various cells and are also able to regulate the secretion of antibodies and other cytokines[5]. By targeting selected cytokine networks or pathways one may be able to restrain CRC tumorigenesis or even improve the response rate in CRC tumours to chemotherapies, and there are several clinical trials that have focused on evaluating the blockage of different cytokines[6-8].

Increased levels of inflammatory factors have been associated with increased mortality in CRC patients, also in stage I which has a good oncological prognosis, but also in asymptomatic assumed healthy individuals[9-12].

The aim of this paper was to study the association of plasma levels of cytokines, chemokines and interleukins in CRC patients at the time of surgery with survival. The hypothesis is that strong inflammation at the time of surgery is associated with worse prognosis.

MATERIALS AND METHODS

Study population

This study involved analysis of plasma samples from 174 CRC patients from southeastern Sweden who had undergone surgical resections for primary colorectal adenocarcinoma between 2006-2013 at the Department of Surgery, County Hospital Ryhov, Region of Jönköping County, Jönköping, Sweden. The clinicopathological characteristics of the patients were obtained from surgical and pathological records. Follow-up was performed by consulting the medical records from all hospital departments and the primary care up to January 31, 2016. The date of an eventual cancer recurrence and the date and cause of death as related to CRC-specific mortality or not were determined from a review of the patient's files. The study was approved by the Regional Ethical Review Board in Linköping, Linköping, Sweden, and written informed consent was obtained from each patient.

Plasma samples

Venous blood samples were collected at the time of surgery and centrifuged to separate plasma and blood cells. Plasma was stored at -80 ℃ until analysis. Plasma samples were available from 174 patients (69 females and 105 men), and their median age was 70 years (range 29-90). The patients' tumours were localized in the colon (n = 105) or rectum (n = 69) and were classified as stage Ⅰ (n = 24), stage Ⅱ (n = 54), stage Ⅲ (n = 67) and stage Ⅳ (n = 29).

Human cytokine assay

Diluted plasma (1:4) from 174 CRC patients and an eight-point standard curve were analysed for each of the 40 factors using a commercial Bio-Plex Pro™ Human Chemokine Panel 40-Plex (Bio-Rad Laboratories, Inc., CA, United States) including chemokines, cytokines and interleukins according to the manufacturer's recommendations. Magnetic separation was performed using the Bio-Plex Pro Wash Station (Bio-Rad Laboratories). Bead fluorescence readings were taken using the Bio-Plex Manager version 6.1.0.727 (Bio-Rad Laboratories) with Low PMT (Low RP1) setting on the Bio-Plex 200 System (Bio-Rad Laboratories). The results are presented as pg/mL and grouped into tertiles defined as low, middle or high tertile.

Statistical analysis

A Shapiro-Wilk test was used to determine the normal distribution. The Pearson χ^2 test was used to determine differences in distribution of covariates; age, gender, localization, cancer recurrence, radical surgery, TNM stage, preoperative treatment, and adjuvant treatment between patients with CRC-specific mortality compared to survivors or deceased by other causes. An association of the inflammatory variables with TNM stage was analysed by comparing the distribution of inflammatory factors using Jonkheere Terpstra test. The association of age, sex, tumour localization, TNM stage, local radical resection, pre- and postoperative adjuvant treatment and tertiles of the examined inflammatory variables with CRC specific and total mortality were performed with Kaplan-Meier curves, log-rank test and Cox's regression analysis. Both univariate and multivariate Cox regression analysis were performed. The proportional hazard assumption was verified by visual inspection of log-log plots. The statistical analyses were performed using the SPSS for Windows computer package (IBM® SPSS® Statistics, 2012, version 21, SPSS Inc., Chicago, IL, United States).

RESULTS

Clinical baseline characteristics

The clinical baseline characteristics of the total study population are presented in Table 1. Out of 174 patients, 40 died because of CRC. Thirty five of these were stage Ⅲ and Ⅳ patients. Twenty-six of the 174 patients died from other causes and 108 were still alive at the end of follow-up. Frequency of radical surgery, TNM stage and adjuvant treatment differed significantly between patients with CRC-specific mortality compared with survivors or deceased by other causes. On the other hand, age, gender, localization of the cancer and preoperative treatment did not differ between the groups. A threshold of $P < 0.20$ was set for the covariates used in the adjustment of statistical analyses. Age was included in the adjusted model since mortality is highly associated with increased age in general.

Associations between TNM stage and levels of inflammatory factors

Each of the inflammatory factors were tested for any eventual association with TNM stage by the Jonkheere Terpstra test.Only CCL20, CCL27, IL-8 and MIF were associated with TNM stage (Table 2).

Total mortality in relation to high tertile levels of inflammatory factors

In a univariate Cox regression analysis, the highest tertile levels of 14 factors, CCL1, CCL3, CCL15, CCL20, CX3CL1, CXCL1, CXCL10, CXCL13, IFN-γ, IL-1β, IL-2, IL-4, IL-8/CXCL8 and IL-10, were significantly associated with total mortality (Table 3). CX3CL1 had the highest hazard ratio (HR) of 3.3 with a 95%CI of 1.8-6.1, $P < 0.001$, for the highest tertile. Nine of the factors in the univariate analysis, CCL1, CCL15, CCL20, CX3CL1, CXCL13, IFN-γ, IL-2, IL-4 and IL-10, remained significant after adjustment of clinical covariates with $P < 0.20$, such as TNM stage, radical surgery, preoperative- and adjuvant treatment and age. Figure 1 shows an example of a Kaplan-Meier analysis of plasma levels of IFN-γ and an increased risk of total mortality. The Kaplan-Meier curves illustrate mortality as a function of follow-up time in relation to tertile levels.

CRC-specific mortality in relation to high levels of inflammatory factors

When investigating the CRC specific mortality among the inflammatory factors, the univariate Cox regression analysis revealed that the highest tertile levels of 8 factors, CCL1, CCL3, CCL15, CCL20, CX3CL1, CXCL16, IL-4 and IL-8/CXCL8, were significantly associated with CRC specific mortality (Table 4). CCL20 showed the highest HR of 4, CI of 1.6-10.1, $P < 0.01$. After adjustment for clinical covariates with $P < 0.20$, only 4 factors remained significant, CCL1, CCL20, CX3CL1 and IL-4. In addition, TNF-α and CCL24 became significant after this adjustment. Kaplan-Meier analysis of CRC specific mortality and tertile levels of CCL1 is shown in Figure 2.

In summary, higher tertile levels of the inflammatory factors CCL1, CCL20, CX3CL1 and IL-4 were all associated with increased risk of both total and CRC-

Table 1 Clinical baseline characteristics in frequencies *n* (%)

	CRC patients	Others (survivors or deceased by other causes)	CRC specific mortality	*P* value
	n = 174	*n* = 134 (77)	*n* = 40 (23)	
Age	70 (range 29-90)	70 (29-90)	72 (36-90)	0.264[1]
Gender	105 men (60)	83 men (62)	22 men (55)	0.431
Localization	105 colon (60)	78 colon (58)	27 colon (68)	0.292
Cancer recurrence	34 (20)	16 (12)	18 (45)	< 0.001
Radical surgery	165 (95)	130 (97)	35 (88)	< 0.05[1]
TNM stage Ⅰ / Ⅱ / Ⅲ / Ⅳ	24/54/67/29 (14/31/38/17)	22/51/54/7 (17/38/40/5)	2/3/13/22 (5/8/32/55)	< 0.001[1]
Preoperative treatment	41 (24)	36 (27)	5 (13)	0.060[1]
Adjuvant treatment	71 (41)	47 (35)	24 (60)	0.005[1]
Total mortality	66 (38)	26 (19)	40 (100)	< 0.001

[1]Covariates with $P < 0.20$ together with age were selected for adjustment in Tables 3 and 4. CRC: Colorectal cancer.

Table 2 Levels of inflammatory factors significantly associated to TNM stage

Factor	TNM stage Ⅰ (*n* = 24), Median[1] (range)	TNM stage Ⅱ (*n* = 54), Median[1] (range)	TNM stage Ⅲ (*n* = 67), Median[1] (range)	TNM stage Ⅳ (*n* = 29), Median[1] (range)	*P* value
CCL20	100 (77-242)	119.5 (80-272)	124 (89-183)	149 (111-378)	0.034
CCL27	11956 (9975-15131)	11293 (8799-14133)	10308 (8173-13923)	9461 (7619-12283)	0.035
IL-8	99 (81-439)	113 (97-146)	104 (85-153)	166 (113-243)	0.009
MIF	19565 (13546-33372)	23720 (14452-43786)	21900 (14146-33330)	47235 (20723-89267)	0.031

[1]Median in pg/mL with 1st-3rd quartile range. Association was determined using Jonckheere Terpstra test.

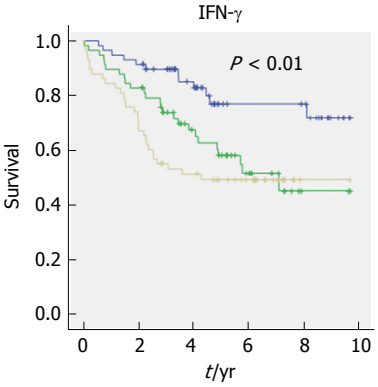


Figure 1 Kaplan-Meier curve of IFN-γ as an example of an inflammatory factor significant for total mortality. Upper blue line: lowest tertile levels in pg/mL; middle green line: middle tertile levels in pg/mL; lower yellow line: highest tertile levels in pg/mL.

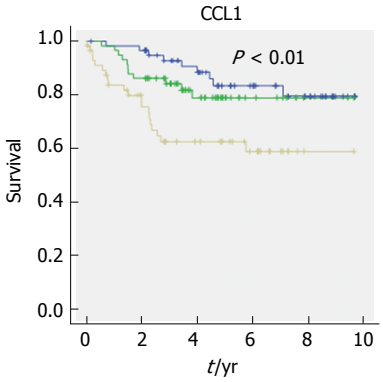


Figure 2 Kaplan-Meier curve of CCL1 as an example of an inflammatory factor significant for colorectal cancer-specific mortality in a multivariate analysis. Upper blue line: lowest tertile levels in pg/mL; middle green line: middle tertile levels in pg/mL; lower yellow line: highest tertile levels in pg/mL.

specific mortality after Cox regression analysis when adjusted for clinical covariates. Higher tertile levels of TNF-α and CCL24 were exclusively associated with CRC-specific mortality. For most of the inflammatory factors the association between higher tertile levels and an increased mortality in general appeared two years after surgery. Twenty three inflammatory factors did not have any association with CRC-specific or total mortality when studying the follow-up time in relation to the tertile levels of these factors (Table 5).

DISCUSSION

In this study, we screened plasma samples from 174 CRC patients for 40 different inflammatory factors using a commercial multiplex kit, which allow simultaneous quantification of several factors of interest, to determine whether the plasma levels of these factors were associated with CRC prognosis.

We found that high tertile levels of CCL1, CCL20, CX3CL1 and IL-4 were associated with increased CRC-specific mortality in a multivariate Cox regression analysis. We also found an increased total mortality in association of high tertile levels of CCL1, CCL15, CCL20, CX3CL1, CXCL13, IFN-γ, IL-2, IL-4 and IL-10.

Several studies have focused on higher or lower levels of some of the inflammatory factors included in our study such as CCL15, CCL20, CX3CL1, CXCL13, CXCL16, IL-4, IL-8/CXCL8, IL-10 and TNF-α by either comparing expression levels in tissue, in serum or

Table 3 Total mortality and association with highest tertile level of inflammatory factors

Factor	Total mortality		Total mortality adjusted[1]	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.0 (1.0-1.1)	0.006	1.1 (1.0-1.1)	< 0.001
TNM stage Ⅱ	1.5 (0.5-4.0)	0.464	1.2 (0.4-3.2)	0.777
TNM stage Ⅲ	1.8 (0.7-4.8)	0.228	2.2 (0.8-6.1)	0.141
TNM stage Ⅳ	6.8 (2.6-17.9)	< 0.001	14.6 (6.0-42.6)	< 0.001
Radical surgery	0.2 (0.1-0.4)	< 0.001	0.3 (0.1-0.6)	0.002
Preoperative treatment	0.8 (0.4-1.5)	0.454	0.7 (0.4-1.4)	0.338
Adjuvant treatment	1.1 (0.6-1.7)	0.842	0.7 (0.4-1.3)	0.226
CCL1	3.2 (1.7-5.9)	< 0.001	2.7 (1.4-5.4)	0.004
CCL3	2.0 (1.1-3.6)	0.030	1.2 (0.6-2.2)	0.605
CCL15	2.3 (1.2-4.3)	0.010	1.9 (1.0-3.7)	0.046
CCL20	3.1 (1.6-6.0)	0.001	2.2 (1.1-4.3)	0.021
CCL26	1.7 (1.0-3.0)	0.055	1.7 (0.9-3.0)	0.078
CX3CL1	3.3 (1.8-6.1)	< 0.001	2.3 (1.2-4.5)	0.014
CXCL1	1.9 (1.0-3.4)	0.038	1.4 (0.8-2.6)	0.295
CXCL10	2.1 (1.1-4.0)	0.017	1.3 (0.7-2.6)	0.387
CXCL13	1.8 (1.1-3.2)	0.033	2.0 (1.0-3.7)	0.039
CXCL16	1.6 (0.9-2.8)	0.139	1.7 (0.9-3.1)	0.113
IFN-γ	3.1 (1.6-6.1)	0.001	3.5 (1.6-7.5)	0.001
IL-1β	2.1 (1.2-3.8)	0.012	1.7 (0.9-3.1)	0.081
IL-2	2.2 (1.2-4.2)	0.011	2.7 (1.4-5.4)	0.005
IL-4	2.4 (1.2-4.6)	0.014	2.3 (1.1-4.5)	0.018
IL-8/CXCL8	2.6 (1.4-4.6)	0.002	1.6 (0.9-3.0)	0.115
IL-10	2.2 (1.2-4.1)	0.014	2.3 (1.2-4.6)	0.014
TNF-α	1.6 (0.9-2.8)	0.104	1.5 (0.8-2.6)	0.202

[1]When adjusted for age, TNM stage, radical surgery, preoperative- and adjuvant treatment.

Table 4 Colorectal cancer specific mortality and the association with highest tertile level of inflammatory factors

Factor	CRC specific mortality		CRC specific mortality adjusted[1]	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.0 (1.0-1.0)	0.532	1.1 (1.0-1.1)	0.006
TNM stage Ⅱ	0.7 (0.1-4.1)	0.674	0.5 (0.1-3.0)	0.444
TNM stage Ⅲ	2.6 (0.6-11.7)	0.201	2.7 (0.6-12.9)	0.209
TNM stage Ⅳ	15.5 (3.6-66.3)	< 0.001	27.6 (5.8-131.1)	< 0.001
Radical surgery	0.2 (0.1-0.5)	0.001	0.19 (0.1-0.5)	0.001
Preoperative treatment	0.5 (0.2-1.2)	0.106	0.4 (0.1-0.9)	0.359
Adjuvant treatment	2.2 (1.2-4.2)	0.013	0.9 (0.5-2.0)	0.947
CCL1	3.2 (1.4-7.0)	0.004	2.8 (1.1-7.1)	0.025
CCL3	2.4 (1.1-5.3)	0.036	1.2 (0.5-2.8)	0.653
CCL15	2.8 (1.3-6.1)	0.011	2.0 (0.9-4.9)	0.107
CCL20	4.0 (1.6-10.1)	0.003	2.7 (1.0-7.0)	0.046
CCL24	2.2 (1.0-4.8)	0.061	2.5 (1.1-5.7)	0.037
CX3CL1	3.7 (1.6-8.3)	0.002	2.6 (1.1-6.4)	0.036
CXCL16	2.5 (1.1-5.4)	0.022	2.0 (0.8-4.8)	0.138
IFN-γ	1.8 (0.8-3.9)	0.138	1.7 (0.7-4.1)	0.253
IL-1β	1.9 (0.9-3.8)	0.090	1.7 (0.8-3.6)	0.193
IL-4	2.5 (1.1-5.7)	0.033	2.4 (1.0-5.5)	0.048
IL-8/CXCL8	3.3 (1.5-7.3)	0.003	1.5 (0.6-3.5)	0.344
TNF-α	2.0 (0.9-4.2)	0.078	2.3 (1.0-5.4)	0.047

[1]When adjusted for age, TNM stage, radical surgery, preoperative- and adjuvant treatment. CRC: Colorectal cancer.

in plasma from CRC patients and controls or among patients only[13-22]. Also, expression levels of some of these factors have been studied in relation to survival[13-15,17,18,20-22].

More recent studies have found an association of increased levels of inflammatory markers and increased mortality in CRC patients, but also in CRC patients stage Ⅰ and in asymptomatic healthy individuals[9-12]. Our findings of a worse prognosis in association with increased level of inflammation may therefore be a more general phenomenon and not directly related to the cancer disease. As mentioned, the local immunoregulation mediated by inflammatory cells, such as white blood cells, of the tumour microenvironment are important for the release of inflammatory factors such as cytokines, which are able to activate local immune networks to promote both the development and growth of malignant CRC cells[1,4]. White blood cells

Table 5 Inflammatory factors not associated with colorectal cancer specific or total mortality

Factor	Factor
CCL2	CCL27
CCL7	CXCL2
CCL8	CXCL5
CCL11	CXCL6
CCL13	CXCL9
CCL17	CXCL11
CCL19	CXCL12
CCL21	GM-CSF
CCL22	IL-6
CCL23	IL-16
CCL25	MIF
CCL26	

play an important part in immunoregulation but play dual roles. They are supposed to defeat the cancer development by producing cytokines and activating inflammatory signalling pathways enabling necrosis or apoptosis of cancer cells. But the cancer itself may also induce an immunomodulation of the white blood cells, making them unable to produce cytokines and other inflammatory factors. In this way, the cancer cells avoid inflammatory recognition and are then able to continue their development[1,4].

The CX3CL1 chemokine is expressed by epithelial cells in both CRC and normal colorectal mucosa. Higher levels of this chemokine have been associated with better prognosis and higher survival rate in CRC patients, depending on anti-tumour immunity through a higher number of attracted lymphocytes[16]. This is contradictory to our findings in the univariate analysis, which show an association between higher tertile levels of CX3CL1 and a > 3.5-fold increased risk for CRC-specific mortality among our CRC patients.

The CCL15 chemokine has a strong chemotactic activity for myeloid cells such as dendritic cells, monocytes, neutrophils and some T-lymphocytes[23]. In a study by Inamoto *et al*[13] a trend between higher levels of CCL15 and poor survival among CRC patients was observed. In this study we confirm that a higher inflammatory tertile level of CCL15 is related to CRC-specific mortality when we do not include age as a covariate in the statistical analysis.

Expression of the CCL20 chemokine has been demonstrated in dendritic cells, macrophages, eosinophilic granulocytes and in B- and T-cell lymphocytes, as reviewed by Schutyser *et al*[24]. In CRC, higher serum levels of CCL20 may serve as a potential biomarker for prognosis. It may also be useful for identification of patients with increased risk of disease recurrence in stage Ⅱ CRC[14]. In the present investigation, higher tertile levels of CCL20 in plasma is associated with a 4-fold increased risk for CRC-specific mortality in the univariate analysis, which could be explained by a relation to TNM stage.

IL-4 is produced by basophils, activated T-lymphocytes and mast cells and seems to be upregulated in CRC[25,26]. It has been suggested that IL-4 might be involved in the process of supporting the tumour-initiating cells, enabling them to escape immune surveillance and in turn promote CRC progression[19]. Our data show that the highest tertile level of IL-4 is associated with an increased risk of CRC specific mortality as a result of ongoing CRC progression over time.

The CCL1 chemokine is secreted from fibroblasts[27] and Th2 cells[28]. CCL1 has been implicated in other types of cancer but little is known about its effects on CRC[27]. The Th2-cells express the CCR8 receptor[29,30], which is activated by CCL1[31], and it mediates Th2 cell recruitment to sites of inflammation[32,33]. In cancer, the CCL1-CCR8 autocrine loop has been shown to have a protective function by enabling lymphoma and T cell leukaemia cells to avoid apoptosis *in vitro*[34,35] and to play a role in T cell transformation[36]. In this study, higher tertile levels of this chemokine were associated with a 2.8 fold increased risk for CRC-specific mortality and one might speculate that the CCL1-CCR8 autocrine loop may help CRC cells to progress their development and spread. Due to our results CCL1 might be a new important factor to consider in further studies regarding its implications on CRC.

There are several limitations identified in our study that need to be taken into consideration. First, a control group was not included in the present work since our focus was on survival among CRC patients, making us unable to study differences and/or associations in levels of the inflammatory factors among patients and healthy individuals. However, there are already several studies that have investigated differences in levels of cytokines among both patients and healthy control subjects[37,38]. Second, our cohort was relatively small influencing the statistical evaluation and especially associations with stage, which was weak due to low power. This also makes it difficult to stratify the patients into respect to more variables such as type of surgery, with inflammatory complicated CRC such as peritumorous abscess, perforation or peritonitis, or inflammatory bowel disease. Third, it is also important to realize that the level of inflammatory factors in the circulation might reflect the release of factors during cancer carcinogenesis, due to other underlying diseases or by systemic inflammation in general. In this study we did not have the possibility to investigate this.

Future aspects should focus on studying these inflammatory factors in a larger CRC patient cohort to see if they might have the potential as biomarkers that can be measured through a rapid, simple, non-invasive and less costly plasma analysis enabling the identification of CRC patients with worse prognosis. Functional studies are needed to elucidate weather these are causative factors for tumour progression or a biomarker for CRC prognosis or a marker for a general fragility.

In summary, high tertile levels of a range of chemokines, cytokines and interleukins are associated with a worse prognosis in patients operated for CRC, both as expressed as cancer specific mortality (CCL1, CCL20, CCL24, CX3CL1, IL-4 and TNF-α) and total mortality (CCL1, CCL15, CCL20, CX3CL1, CXCL13, IFN-γ, IL-2, IL-4 and IL-10). This suggests that the observed association of a worse prognosis in CRC patients with an increased level of inflammation may not only be associated to the cancer disease but expression of a fragile host.

COMMENTS

Background

Inflammation is of importance in cancer development, and many tumours develop due to prolonged or chronic inflammation throughout their progression.

Research frontiers

Several studies have previously investigated expression of some inflammatory factors in circulation of patients with colorectal cancer (CRC).

Innovations and breakthroughs

This study analyzed 40 different inflammatory factors at the same time in plasma from CRC patients which was investigated in multivariate Cox regression analysis taken into account clinical co-variates.

Applications

The identification of high levels of several factors associated with CRC specific mortality makes them interesting targets for further studies and as potential targets for immune therapy. The results further support that CRC progression is an inflammatory active condition.

Terminology

Chemokines are a family of small cytokines that are secreted by a variety of cells and can attract cells expressing the corresponding receptor, normally leukocytes.

Peer-review

The authors in this study investigated whether the circulating inflammatory factors were associated with worse long-term prognosis in CRC. The results showed that high plasma levels of inflammatory factors were associated with an increased risk of total and CRC specific mortality among CRC patients. The whole manuscript is well designed and used a fluency style.

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ORIGINAL ARTICLE

Basic Study

Moxibustion eases chronic inflammatory visceral pain through regulating MEK, ERK and CREB in rats

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Author contributions: Li ZY, Huang Y, Yang YT and Zhang D contributed equally to this work; Huang Y, Zhang D and Ma XP conceived the study; Li ZY, Huang Y, Yang YT, Zhao Y, Wu LJ and Zhang J established the animal model and participated in the treatment; Yang YT, Hong J, Liu J and Zhang CH collected the data; Li ZY and Huang Y analyzed the data and wrote the manuscript; Li ZY, Huang Y, Yang YT and Ma XP approved the final version of the manuscript; Zhang D, Wu HG and Ma XP coordinated the research.

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Abstract

AIM

To investigate the effects of herb-partitioned moxibustion (HPM) on phosphorylation of mitogen-activated extracellular signal-regulated kinase (MEK)1, extracellular signal-regulated kinase (ERK)1/2 and cAMP response element binding protein (CREB) in spinal cord of rats with chronic inflammatory visceral pain (CIVP), and to explore the central mechanism of HPM in treating CIVP.

METHODS

Male Sprague-Dawley rats were randomized into normal, model, HPM, sham-HPM, MEK-inhibitor and dimethyl sulfoxide (DMSO) groups. The CIVP model was established using an enema mixture of trinitrobenzene sulfonic acid and ethanol. HPM was applied at bilateral Tianshu (ST25) and Qihai (CV6) acupoints in the HPM group, while in the sham-HPM group, moxa cones and herb cakes were only placed on the same points but not ignited. The MEK-inhibitor and DMSO groups received L5-L6 intrathecal injection of U0126 and 30% DMSO, respectively. Abdominal withdrawal reflex (AWR), mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were applied for the assessment of pain behavior. The colonic tissue was observed under an optical microscope after hematoxylin-eosin staining. Expression of phosphor (p)MEK1, pERK1/2 and pCREB in rat spinal cord was detected using Western blotting. The levels of MEK, ERK and CREB mRNA in rat spinal cord were detected using real-time polymerase chain reaction.

RESULTS

Compared with the normal group, the AWR scores were increased significantly ($P < 0.01$) and the MWT and TWL scores were decreased significantly ($P < 0.05$) in the model, sham-HPM and DMSO groups. Compared with the model group, the AWR scores were decreased significantly ($P < 0.01$) and the MWT and TWL scores were increased significantly in the HPM and MEK-inhibitor groups ($P < 0.05$). Compared with the sham-HPM and DMSO groups, the AWR scores were decreased significantly ($P < 0.01$) and the MWT and TWL scores were increased significantly ($P < 0.05$) in the HPM and MEK-inhibitor groups. Compared with the normal group, the expression of pMEK1, pERK1/2 and pCREB proteins and the levels of MEK, ERK and CREB mRNA in rat spinal cord were increased significantly in the model, sham-HPM and DMSO groups ($P < 0.01$ or < 0.05). Compared with the model group, the expression of pMEK1, pERK1/2 and pCREB proteins and the levels of MEK, ERK and CREB mRNA in rat spinal cord were reduced significantly in the HPM and MEK-inhibitor groups ($P < 0.01$ or < 0.05). Compared with the sham-HPM and DMSO groups, expression of pMEK1, pERK1/2 and pCREB proteins and the levels of MEK, ERK and CREB mRNA in rat spinal cord were reduced significantly in the HPM and MEK-inhibitor groups ($P < 0.01$ or < 0.05).

CONCLUSION

HPM down-regulates protein phosphorylation of MEK1, ERK1/2 and CREB, and mRNA expression of MEK, ERK and CREB, inhibiting activation of the MEK/ERK/CREB signaling pathway in the spinal cord of CIVP rats, which is possibly a critical central mechanism of the analgesic effect of HPM.

Key words: Herb-partitioned moxibustion; Chronic inflammatory visceral pain; Pain behavior; Analgesia; MEK; Extracellular signal-regulated kinase; cAMP response element binding protein; Signaling pathway

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Core tip: Chronic inflammatory visceral pain (CIVP) (abdominal pain) is one of the major symptoms of inflammatory bowel disease. Herb-partitioned moxibustion (HPM) at bilateral Tianshu (ST25) and Qihai (CV6) acupoints was effective in enhancing the pain threshold and easing pain in CIVP rats. Levels of phosphorylated protein and mRNA expression of mitogen-activated extracellular signal-regulated kinase 1, extracellular signal-regulated kinase and cAMP response element binding protein were abnormally increased in the spinal cord of CIVP rats. HPM effectively down-regulated the abnormally increased expression, which is possibly one of the analgesic mechanisms of HPM.

Li ZY, Huang Y, Yang YT, Zhang D, Zhao Y, Hong J, Liu J, Wu LJ, Zhang CH, Wu HG, Zhang J, Ma XP. Moxibustion eases chronic inflammatory visceral pain through regulating MEK, ERK and CREB in rats. *World J Gastroenterol* 2017; 23(34): 6220-6230 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6220.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6220

INTRODUCTION

Chronic inflammatory visceral pain (CIVP) is a complex process involving a variety of neurotransmitters, cytokines, and signaling pathways. The extracellular signal-regulated kinase (ERK) signaling pathway is a major branch of the mitogen-activated protein kinase (MAPK) pathway and has been thoroughly explored. The ERK pathway is responsible for the regulation of pain and inflammation[1]. It has been shown that electroacupuncture (EA) can enhance the pain threshold of rats with inflammatory pain in the hind paw and that this effect is mediated *via* regulation of the ERK signaling pathway[2].

Mitogen-activated extracellular signal-regulated kinase (MEK)/ERK/CREB is part of the ERK pathway. MEK resides in the upstream region of the ERK pathway, and induces ERK1/2 through phosphorylating its threonine and tyrosine. Activated ERK1/2 regulates phosphorylation of various proteins such as cAMP-response element binding protein (CREB) and transcription factors (TFs), subsequently influencing multiple biological functions[3]. The MEK/ERK/CREB signaling pathway plays an important role in modulating the transmission and maintenance of pain signals[4,5].

Our previous studies have demonstrated that herb-partitioned moxibustion (HPM) reduces chronic inflammatory pain in rat models of trinitrobenzene sulfonic (TNBS)/ethanol-induced ulcerative colitis (UC)[6,7]. However, the questions remain whether

the MEK/ERK/CREB signaling pathway is involved in CIVP and whether HPM exerts its analgesic effect *via* regulation of this pathway. We used Western blotting and real-time polymerase chain reaction (PCR) to observe the effects of HPM on protein phosphorylation and mRNA expression of MEK, ERK and CREB in the spinal cord of rats with TNBS/ethanol-induced CIVP, to discover the analgesic mechanism of HPM from the perspective of the MEK/ERK/CREB signaling pathway.

MATERIALS AND METHODS

Animals

Fifty-four healthy adult male Sprague-Dawley rats, weighing 150 ± 20 g, were provided by Shanghai Sippr-BK Laboratory Animal Co. Ltd. [license no: SCXK(Hu)2013-0016]. The rats were housed in a room with a 12/12-h light/dark cycle (08:00-20:00 light; 20:00-08:00 dark). The feeding room and behavior detection room were both at 20 ± 1 ℃ with a relative humidity of 50%. After 1 wk of adaptive feeding, the rats all presented with normal behavior for ingestion and drinking and were included in the investigation. The experiment was conducted following the Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Committee on Use of Human and Animal Subjects in Teaching and Research, Shanghai University of Traditional Chinese Medicine.

Using a completely randomized design, the 54 rats were randomized into normal, model, HPM, sham-HPM, MEK-inhibitor and DMSO groups, with 9 rats each. A TNBS/ethanol-induced CIVP rat model was replicated in the six groups, except for the normal group[8,9]. Prior to modeling, the rats were fasted for 24 h but allowed free access to water. CIVP was induced with 5% (w/v) TNBS and 50% ethanol in a 2:1 ratio. The rats were weighed and then anesthetized with 2% pentobarbital sodium intraperitoneally at 30 mg/kg. The TNBS enema solution was administered at 3 mL/kg with the needle inserted into anus by 6-8 cm. After enema administration, the tails of the rats were lifted to keep the rats upside down for 1 min. The enema was performed once every 7 d, for four times in total.

After modeling, the rats were subjected to pain behavior tests and one rat from each group was killed to collect colon tissue for hematoxylin-eosin (HE) staining to verify successful induction of the model; after which, the intervention began.

Main reagents and instruments

The following reagents and instruments were used: 5% TNBS (Sigma, St Louis, MO, United States); phospho (p)MEK antibody (Cell Signaling Technology, Danvers, MA, United States); pERK antibody (Cell Signaling Technology); pCREB antibody (Cell Signaling Technology); MEK inhibitor U0126 (Calbiochem, Darmstadt, Germany); DMSO (Santa Cruz Biotechnology, Dallas, TX, United States); horseradish peroxidase goat anti-rabbit IgG (Beyotime, Shanghai, China); Von Frey filaments (Stoelting Co, Wood Dale, IL, United States); BME2410A thermal stimulator (Institute of Medical Biology, Chinese Academy of Medical Sciences, Beijing, China); electrophoresis system: Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA, United States); imaging system: ChemiDoc™ (Bio-Rad, Hercules, CA, United States); PCR primers ABI 7300 (Thermo Fisher Scientific, Waltham, MA, United States).

Pain behavior tests

Abdominal withdrawal reflex (AWR) was measured based on Al-Chaer's method[10] by offering a series of colorectal distending (CRD) stimuli: 20, 40, 60 and 80 mmHg. Each rat was measured three times; each stimulus lasted 20 s, with an interval of 5 min, and the average value of the three measurements was considered as the final record.

Mechanical withdrawal threshold (MWT) was measured using a Von Frey filament (gradient folding force: 2.0, 4.0, 6.0, 8.0 and 15.0 g) to poke perpendicularly the center of the hind paw, lasting ≤ 4 s[11]. It was considered positive when the rat lifted or licked the hind paw. The test started with 2 g; each force was applied five times, with an interval of 30 s between measurements. The maximum force was 15 g, as was the maximum MWT value. The 50% positive response threshold was calculated using an up-down method.

According to Hargreaves' method[12], we examined the thermal withdrawal latency (TWL) by radiating the hind paw with a thermal stimulator. The duration from the beginning of radiation to the withdrawal reaction was designated the TWL. The stimulation was controlled at the same intensity and automatically cut off at 20.1 s to prevent burns. Each rat was examined five times, with an interval of 3 min. The maximum and minimum values were excluded and the mean of the middle three values was taken as the final record.

Intervention

Rats in the normal and model groups were only taken out and fixed at the same time as those in the HPM group but did not receive any treatment. Rats in the HPM group received HPM treatment at bilateral Tianshu (ST25) and Qihai (CV6) points[13-15], with two moxa cones for each point at each session (about 10 min); one session was performed every day, for a total of seven sessions. The herb cakes were mainly made of *Aconiti praeparata* powder and yellow wine. The moxa cones were made using a specific mold, and each moxa cone weighed 90 mg. HPM treatment was performed as shown in Figure 1.

In the sham-HPM group[16,17], when the rats were fixed in position, the moxa cones were placed on the same acupoints for 10 min but not ignited. The control procedure was also conducted once every day, for

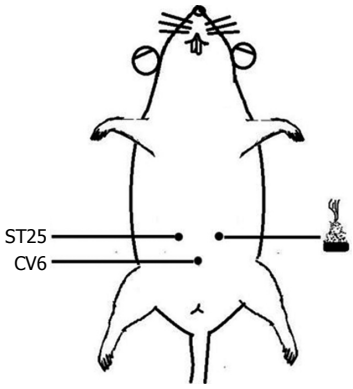


Figure 1 Herb-partitioned moxibustion treatment.

seven times in total. Rats in the MEK-inhibitor group received intrathecal injection at L5-L6 of U0126 which was dissolved in 30% DMSO solution[18] (1 μg/μL), 10 μL each time, once every day, for 7 consecutive days. Rats in the DMSO group received intrathecal injection of 30% DMSO at the same location, 10 μL each time, once per day, for 7 consecutive days.

Sample collection and processing

Pain behavior was retested on the second day of the last intervention session. The rats were killed for collection of colon and spinal cord tissue. After weighing and anesthetic overdose, the abdomen was dissected along the median line and 5 cm of colon was rapidly removed. After cutting longitudinally and rinsing, the colonic tissues were fixed in 4% formaldehyde solution. The samples were then dehydrated in an alcohol series, made transparent, and embedded for subsequent use. The spinal cord from L6 to S2 was collected and kept at -80 ℃.

Pathological observation of colonic tissue

The colonic samples were stained with HE to observe the pathological alterations under an optical microscope.

Western blotting of pMEK1, pERK1/2 and pCREB proteins in spinal cord

The spinal cord tissues were removed from the -80 ℃ freezer, weighed and mixed with lysate solution in a proportion of 100 mg:1 mL. The sample was centrifuged at 4 ℃ and 12000 r/min for 15 min, and the supernatant obtained contained total protein. The bicinchoninic acid method was used for the determination of total protein content. Electrophoresis was performed with each well containing 50 μg total protein.

The resolved protein sample was transferred to a nitrocellulose membrane using a wet transfer printing method. Primary antibody (1:1000) was incubated overnight at 4 ℃, followed by secondary antibody (1:1000) for 2 h at room temperature. After 2-min incubation with electrochemiluminescence, the samples were analyzed using a Bio-Rad imaging system. The gray values of target protein and internal reference protein bands were read, and the ratio between the gray value of the target protein band and that of the internal reference protein band was taken as the relative expression level.

Detection of MEK, ERK and CREB mRNAs by real-time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to acquire total RNA from the tissues. A total RNA equivalent was used to reverse transcribe cDNA using a Thermo Scientific PCR kit (United States). ABI Prism7300 SDS software was used to analyze the mRNA expression level of the target gene. The relative mRNA expression of a target gene was calculated as $2^{-\Delta\Delta CT} \times 100\%$; ΔCT = CT value of the target gene - CT value of the internal reference (GAPDH). The sequences of primers were as follows: GAPDH forward 5′-GGAGAAACCTGCCAAGTATG-3′, reverse 5′-GACAACCTGGTCCTCAGTGT-3′; MEK forward 5′-AGAGCGTGCAGACCTCAAGCT-3′, reverse 5′-AGCCGCAGGGTTCTGCATA-3′; ERK forward 5′-ATCAACACCACCTGCGACCTT-3′, reverse 5′-TGGCCA CATACTCGGTCAGAA-3′; CREB forward 5′-CCACTC AGCCGGGTCTACCAT-3′, reverse 5′-CCAGAGGCAGCTT GAACAACA-3′.

Statistical analysis

SPSS version 19.0 software was used for data analysis. Measurement data in normal distribution were expressed as mean ± SD; measurement data in non-normal distribution were expressed as median (P25, P75). One-way analysis of variance was used to analyze the inter-group differences if the data followed a normal distribution, least significant difference (LSD) for those with homogeneous variances, and Games-Howell for those with heterogeneous variances. A rank-sum test was adopted if the data did not follow a normal distribution. $P < 0.05$ indicated statistical significance.

RESULTS

Comparison of AWR scores

Compared with the normal group, the AWR scores were increased significantly in the model, sham-HPM and DMSO groups under 20, 40, 60 and 80 mmHg distension ($P < 0.01$). Compared with the model group, the AWR scores were decreased significantly in the HPM and MEK-inhibitor groups after intervention under the four levels of distension ($P < 0.01$). Compared with the sham-HPM group, the AWR scores were decreased significantly in the HPM and MEK-inhibitor groups after intervention under the four levels of distension ($P < 0.01$). Compared with the DMSO group, the AWR scores decreased significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.01$). The results indicated that HPM and U0126

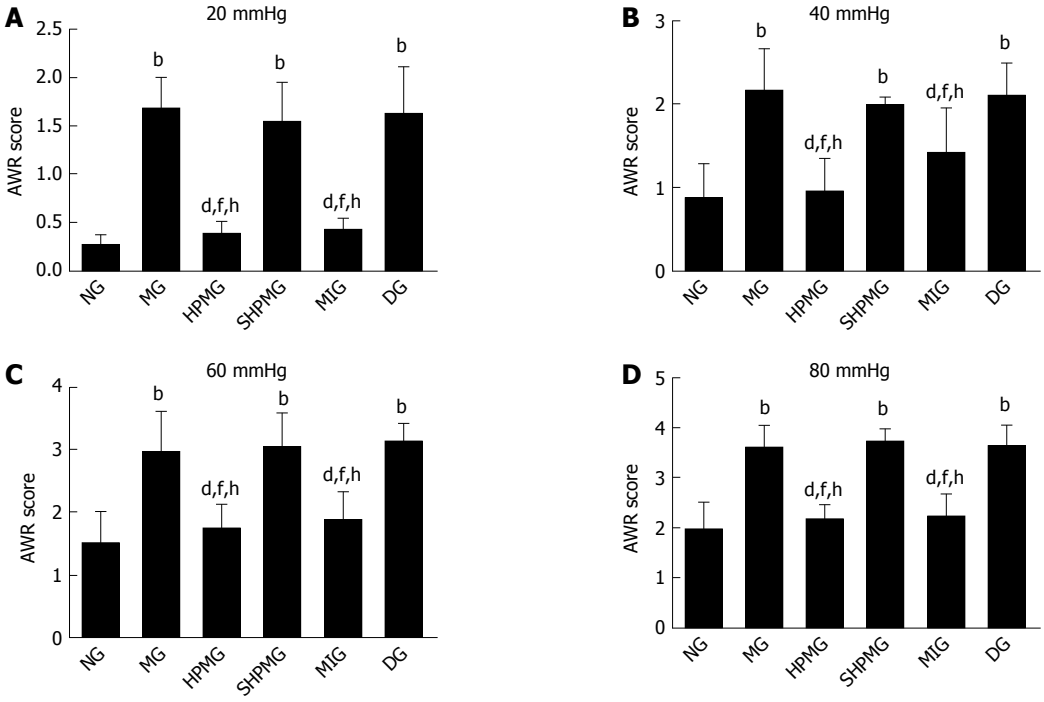


Figure 2 Comparison of the abdominal withdrawal reflex scores after treatment. A: AWR scores under 20 mmHg CRD stimulation; B: AWR scores under 40 mmHg CRD stimulation; C: AWR scores under 60 mmHg CRD stimulation; D: AWR scores under 80 mmHg CRD stimulation. $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.01$ *vs* model group; $^{f}P < 0.01$ *vs* sham-HPM group; $^{h}P < 0.01$ *vs* DMSO group. AWR: Abdominal withdrawal reflex; CRD: Colorectal distending; NG: Normal group; MG: Model group; SHPMG: Sham-HPM group; MIG: MEK-inhibitor group; DG: Dimethyl sulfoxide group; HPM: Herb-partitioned moxibustion.

reduced the AWR scores and enhanced the pain threshold in the CIVP rats (Figure 2).

Comparison of MWT and TWL scores

Compared with the normal group, the MWT and TWL scores were decreased significantly in the model, sham-HPM and DMSO groups ($P < 0.05$). Compared with the model group, the MWT and TWL scores were increased significantly in the HPM and MEK-inhibitor after intervention ($P < 0.05$). Compared with the sham-HPM group, the MWT and TWL scores were increased significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.05$). Compared with the DMSO group, the MWT and TWL scores were increased significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.05$). This indicated that HPM and MEK inhibitor enhanced the MWT and TWL scores, and the pain threshold of the CIVP rats (Figure 3).

Histomorphological observation of colonic tissue

In the normal group, colonic epithelium had the following: a clear structure; mucosa that was complete; arrangement of glands that was regular; and an occasional minor infiltration of inflammatory cells in the lamina propria, without hyperemia, edema or ulceration. In the model group, colonic tissue had an indistinct structure, substantially impaired mucosa, ulcerations, a large amount of inflammatory cell infiltration in the mucosa and submucosa and proliferation of lymph cells, accompanied by hyperemia, edema, damaged glands and indistinct muscular layer. In the HPM group, the mucosal epithelium was generally complete; the arrangement of glands was substantially regular; there was also a large amount of inflammatory cell infiltration in the mucosa and submucosa, accompanied by edema. In the sham-HPM group, the colonic tissue had an indistinct structure, impaired mucosa, a large amount of inflammatory cell infiltration and deficient glands, accompanied by severe hyperemia and edema, but the muscular layer was generally complete. In the MEK-inhibitor group, the colonic tissue had generally complete mucosal epithelium, regular arrangement of glands, obvious infiltration of inflammatory cells (mainly eosinophils and lymph cells) and mild edema. In the DMSO group, the colonic tissue had an indistinct structure, partially impaired mucosal epithelium, ulcerations, obvious hyperemia, edema, inflammatory cell infiltration in the submucosa, damaged glands and indistinct muscular layer (Figure 4).

Comparison of pMEK1, pERK1/2 and pCREB expression in rat spinal cord

Compared with the normal group, expression of pMEK1, pERK1/2 and pCREB was increased significantly in the model, sham-HPM and DMSO groups ($P < 0.01$ or < 0.05). Compared with the model group, expression of pMEK1, pERK1/2 and pCREB was reduced significantly after intervention in HPM and MEK-inhibitor groups ($P < 0.01$ or < 0.05). Compared with the sham-HPM group, expression of pMEK1, pERK1/2 and pCREB was decreased significantly after intervention in the HPM and

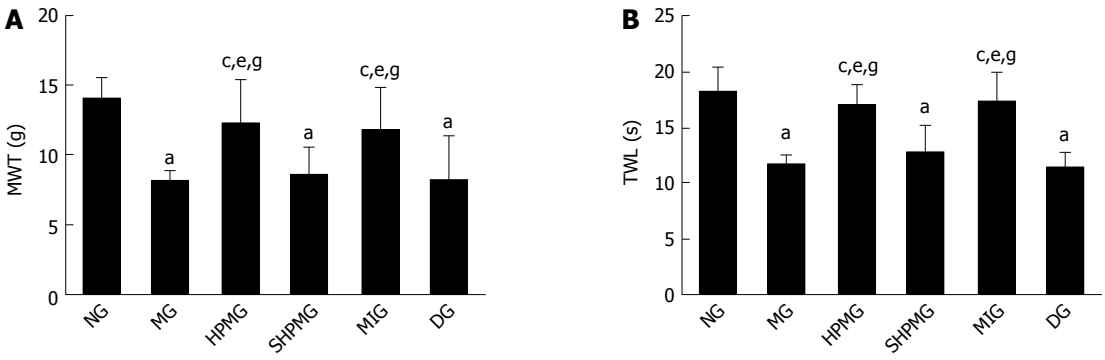


Figure 3 Comparison of the mechanical withdrawal threshold and thermal withdrawal latency scores. A: MWT test used the Von Frey filament; B: TWL test followed Hargreaves. [a]*P* < 0.05 *vs* normal group; [c]*P* < 0.05 *vs* model group; [e]*P* < 0.05 *vs* sham-HPM group; [g]*P* < 0.05 *vs* DMSO group. MWT: Mechanical withdrawal threshold; TWL: Thermal withdrawal latency; NG: Normal group; MG: Model group; SHPMG: Sham-HPM group; MIG: MEK-inhibitor group; DG: Dimethyl sulfoxide group; HPM: Herb-partitioned moxibustion.

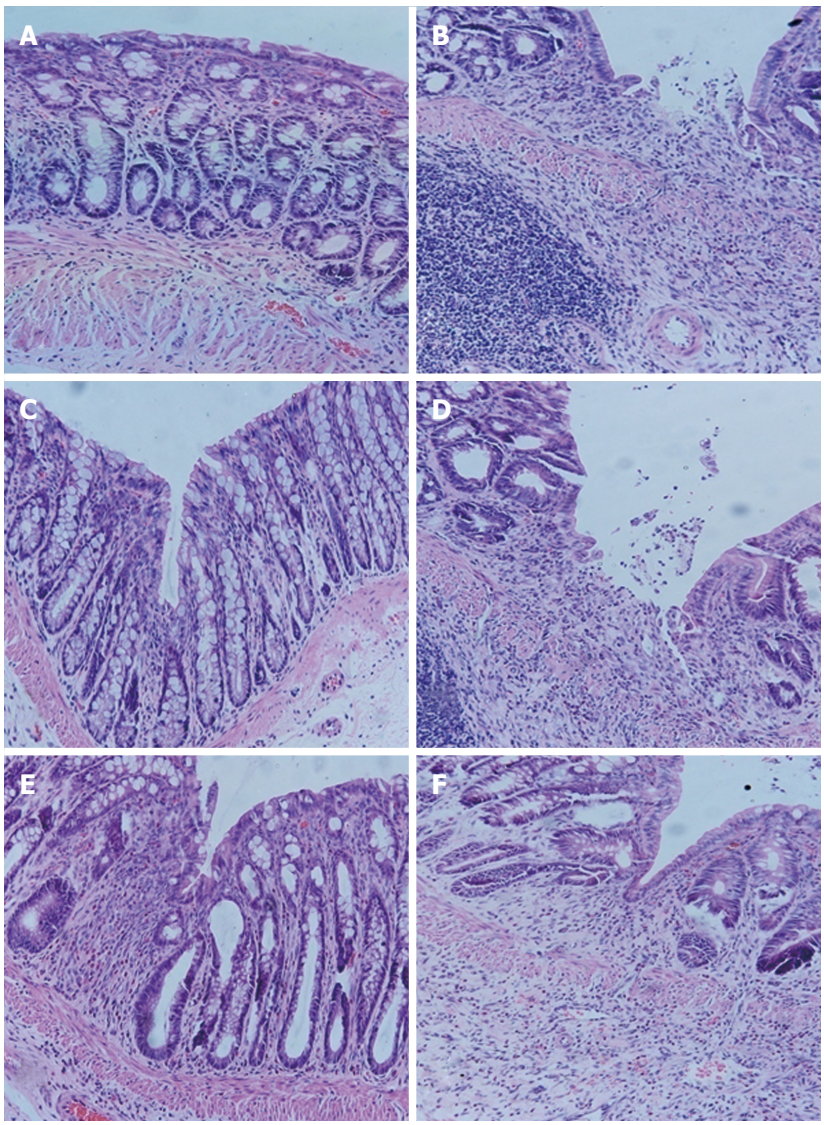


Figure 4 Hematoxylin-eosin staining of colonic mucosa. A: Normal group; B: Model group; C: HPM group; D: Sham-HPM group; E: MEK-inhibitor group; F: DMSO group (HE staining, × 200 magnification). HE: Hematoxylin-eosin; HPM: Herb-partitioned moxibustion; MEK: Mitogen-activated extracellular signal-regulated kinase; NG: Normal group; MG: Model group; SHPMG: Sham-HPM group; MIG: MEK-inhibitor group; DG: Dimethyl sulfoxide group.

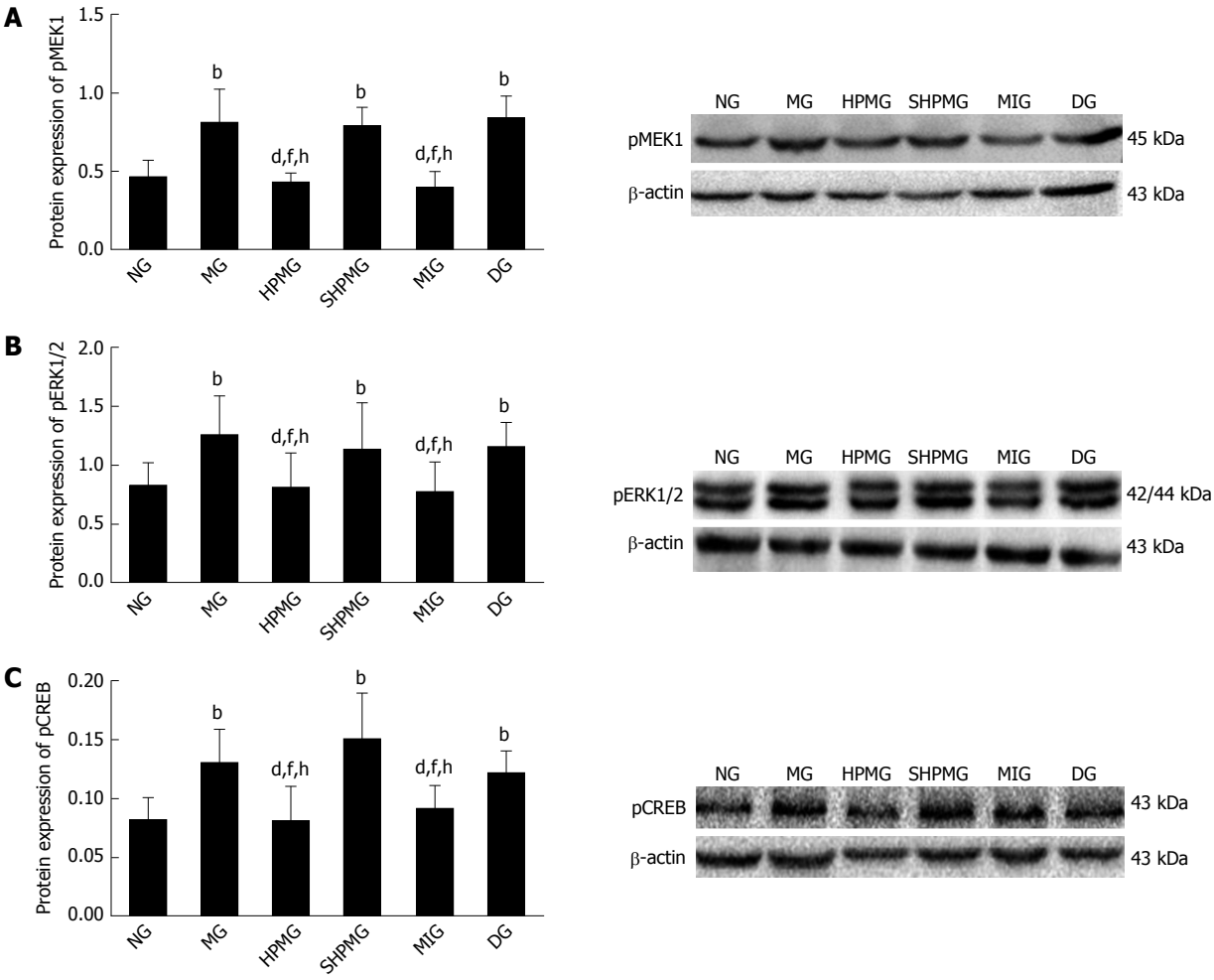


Figure 5 Comparison of expression of phospo-mitogen-activated extracellular signal-regulated kinase 1, phosphor-extracellular signal-regulated kinase 1/2 and cAMP response element binding protein in rat spinal cord. A: Protein expression of pMEK1; B: Protein expression of pERK1/2; C: Protein expression of pCREB. $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.01$ *vs* model group; $^{f}P < 0.01$ *vs* sham-HPM group; $^{h}P < 0.01$ *vs* DMSO group. pCREB: cAMP response element binding protein; DMSO: Dimethyl sulfoxide; pERK: Phosphor-extracellular signal-regulated kinase; HPM: Herb-partitioned moxibustion; pMEK1: Phospo-mitogen-activated extracellular signal-regulated kinase 1; NG: Normal group; MG: Model group; SHPMG: Sham-HPM group; MIG: MEK-inhibitor group; DG: Dimethyl sulfoxide group.

MEK-inhibitor groups ($P < 0.01$ or < 0.05). Compared with the DMSO group, expression of pMEK1, pERK1/2 and pCREB was reduced significantly after intervention in the HPM and MEK-inhibitor groups ($P < 0.01$ or < 0.05) (Figure 5). The results suggested that HPM and U0126 down-regulated expression of pMEK1, pERK1/2 and pCREB, and inhibited activation of the MEK/ERK/CREB signaling pathway.

Comparison of mRNA expression of MEK, ERK and CREB in rat spinal cord

Compared with the normal group, expression of MEK, ERK and CREB mRNA was increased significantly in the model, sham-HPM and DMSO groups ($P < 0.01$). Compared with the model group, expression of MEK, ERK and CREB mRNA was decreased significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.01$). Compared with the sham-HPM group, expression of MEK, ERK and CREB mRNA was decreased significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.01$). Compared with the DMSO group, expression of MEK, ERK and CREB mRNA was reduced significantly in the HPM and MEK-inhibitor groups after intervention ($P < 0.01$) (Figure 6). This indicated that HPM and U0126 down-regulated expression of MEK, ERK and CREB mRNA, and inhibited activation of the MEK/ERK/CREB signaling pathway.

DISCUSSION

CIVP is a common manifestation of inflammatory bowel diseases. Its chronic expansion, unpredictable location and unclear pathogenesis all make it harder to evaluate compared to somatic pain. So far, the measurement of CIVP is generally based on behavioral response, such as visceral pain index, AWR, abdominal contraction record and application of analgesics[19], which may only partially reflect the situation of CIVP. It has been shown by clinical and experimental studies that CIVP involves both somatic (mainly mechanical and thermal hyperalgesia, as shown by

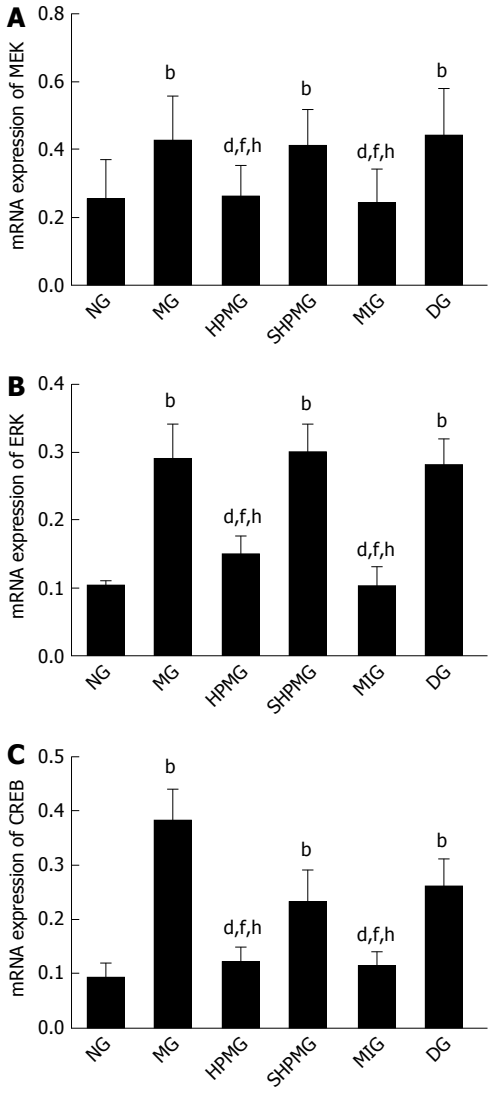


Figure 6 Comparison of expression of mitogen-activated extracellular signal-regulated kinase, extracellular signal-regulated kinase and cAMP response element binding protein mRNAs in rat spinal cord. A: mRNA expression of MEK; B: mRNA expression of ERK; C: mRNA expression of CREB. $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.01$ *vs* model group; $^{f}P < 0.01$ *vs* sham-HPM group; $^{h}P < 0.01$ *vs* DMSO group. CREB: cAMP response element binding protein; DMSO: Dimethyl sulfoxide; ERK: Extracellular signal-regulated kinase; HPM: Herb-partitioned moxibustion; MEK: Mitogen-activated extracellular signal-regulated kinase; NG: Normal group; MG: Model group; SHPMG: Sham-HPM group; MIG: MEK-inhibitor group; DG: Dimethyl sulfoxide group.

animal experiments)[6,20] and visceral pain. This is possibly caused by central sensitization triggered by transmission of peripheral nociceptive stimulation into the spinal cord, or by joint action of visceral afferent and limb efferent fibers in the center[8]. Our previous experiment on a rat model of UC showed that HPM at Tianshu (ST25) and Qihai (CV6) was effective in easing CIVP[6], which has made it a significant project to reveal the analgesic mechanism of HPM.

Currently, neural plasticity is considered one of the key factors in the development of CIVP[21]. Peripheral injury or inflammation induces secretion of large amounts of inflammatory mediators, leading to sensitization of peripheral receptors, central nervous system plasticity, and reorganization and reconstruction of the central neurons involved in pain transmission. This long-term neural plasticity can occur simultaneously in the spinal cord and brain, presenting as allodynia (decreased pain threshold, pain induced by painless stimulators) and hyperalgesia (enlarged pain receptive field, enhanced response to nociceptive stimulations, and prolonged pain duration). The MAPK signaling pathway is widely distributed in various types of cells and transmits extracellular signals from the cell surface to the nucleus through cascade activation and activation of numerous proteinases, nucleoproteins and TFs. This produces a critical regulatory effect on pain, inflammation and tumors that is associated with cell proliferation and differentiation[22].

The ERK signaling pathway is a classic MAPK signaling pathway[23]. By following Ras-Raf-MEK-ERK, ERK plays a key role in signal transduction from cell membrane receptors to the nucleus. This shows that activation of ERK in the spinal cord dorsal horn neurons is significant in producing and maintaining central sensitization[24]. This indicates that the ERK pathway is involved in regulation of nociceptive signals in the spinal cord, and early intervention at the spinal cord level should be effective in blocking pain transmission[25]. The application of specific ERK inhibitor further highlights the signal transduction mechanism in chronic visceral pain.

U0126 effectively and exclusively blocks activation of the ERK pathway and enhances the pain threshold[26]. When the ERK pathway is blocked by U0126, the inflammatory mediators produced are also inhibited[27], which means that U0126 has an anti-inflammatory function. Hence, we selected U0126 for injection into the UC CIVP rat model as a positive control for the HPM group, to observe how HPM intervenes in the ERK pathway in CIVP, by detecting the critical proteins upstream and downstream of the ERK pathway.

When receiving MEK upstream signals, pERK migrates to the nucleus, inducing activation of a series of TFs such as CREB, Elk, activator protein-1, c-myc and c-fos[28]. Therefore, activation of MEK and precise transmission of signals to the ERK pathway are critical to the biological function of cells. When receiving nociceptive stimulation, the phosphorylation of ME34921R and ERK in spinal cord dorsal horn neurons is increased[29], suggesting that nociceptive stimulation can provoke the central ERK signaling pathway. U0126 inhibits activation of MEK and ERK, as well as the downstream functional factors, and thus can regulate inflammation and pain[30].

The downstream factors of the ERK pathway include multiple protein kinases and TFs, and CREB is one of the most important. As a type of endonuclear TF, when phosphorylated, CREB can modulate various pain-related target genes, for example, c-fos, neurokinin-1 and brain-derived neurotrophic factor, through which,

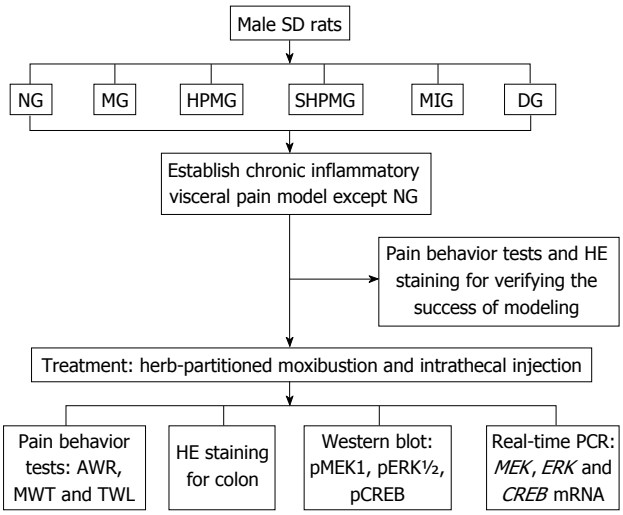


Figure 7 Flow chart of the study design and treatment. AWR: Abdominal withdrawal reflex: CREB: cAMP response element binding protein; ERK: Extracellular signal-regulated kinase; DG: DMSO group; HE: Hematoxylin-eosin; HPM: Herb-partitioned moxibustion; MEK: Mitogen-activated extracellular signal-regulated kinase; MIG: MEK-inhibitor group; MG: Model group; MWT: Mechanical withdrawal threshold; NG: Normal group; SD: Sprague-Dawley; SHPMG: Sham-HPM group; TWL: Thermal withdrawal latency.

it can influence the integration and regulation of pain signals. The whole process is of major significance in adjusting pain signal transduction and central sensitization[31]. ERK and CREB are also closely related in the chronic pain depression model[4,32]. Since CREB is a downstream factor of the ERK pathway, inhibition of pERK leads to decreased expression of pCREB, suggesting that pain can be controlled by inhibiting ERK and expression of its downstream functional factor CREB.

Phosphorylation is the basis of the transcription function of CREB, and pERK can migrate from the cytoplasm to the nucleus and phosphorylate CREB to play its role in modulating the target genes. Peripheral nociception triggers the expression of pCREB in the spinal cord dorsal horn of rat models of chronic pain, while intrathecal injection of CREB inhibitor significantly inhibits pain induced by nociception[33]. This indicates that CREB plays an important role in central sensitization and long-term neural plasticity, which is a potential mechanism by which activated ERK participates in central sensitization and development of chronic pain[34].

By adopting the rat model of Crohn's disease CIVP induced by TNBS/ethanol enema, we observed the effect of HPM on the phosphorylated protein and mRNA expression of MEK1, ERK1/2 and CREB in the spinal cord. CIVP rats had both visceral and somatic pain, manifested by increased AWR, decreased pain threshold, and reduced MWT and TWL. The expression of phosphorylated protein and mRNA of MEK, ERK and CREB was increased significantly in the models, showing that the MEK/ERK/CREB signaling pathway in the spinal cord is activated in CIVP rat models. After application of MEK inhibitor, expression of phosphorylated protein and mRNA of MEK1, ERK1/2 and CREB was decreased significantly, and the pain behavior also showed significant improvement, suggesting a close relationship between the MEK/ERK/CREB pathway in the spinal cord and CIVP. After HPM at Tianshu (ST25) and Qihai (CV6), expression of phosphorylated protein and mRNA of MEK1, ERK1/2 and CREB was also decreased significantly, indicating that HPM can inhibit activation of the MEK/ERK/CREB pathway in the spinal cord of CIVP rats and improve pain behavior. In summary, the MEK/ERK/CREB signaling pathway is closely associated with the development of CIVP. HPM can alleviate the pain in CIVP rats, and this action is related to inhibition of the MEK/ERK/CREB pathway in the spinal cord (Figure 7).

We suppose that HPM exerts its analgesic effect through down-regulating phosphorylation and mRNA expression of MEK1, ERK1/2 and CREB in the spinal cord of CIVP rats, and subsequently inhibiting activation of the MEK/ERK/CREB signaling pathway. However, this study only focused on the MEK/ERK/CREB pathway in the spinal cord to discover the analgesic mechanism of HPM, and it is unclear whether other MAPK pathways, such as p38 MAPK and C-Jun N-terminal kinase, are involved in the signal transmission in CIVP. Pain has a complex pathogenesis, and a variety of non-neural cells, for example, glial cells and stem cells, also play an important role in the development of inflammation and pain[35]. The relationship between these cells and the MEK/ERK/CREB signaling pathway, and the questions in which cells this signaling pathway is activated, and how they bind, require further studies.

COMMENTS

Background

Recent studies suggest that central sensitization is one of the critical factors causing chronic inflammatory visceral pain (CIVP), and the extracellular signal-regulated kinase (ERK) signaling pathway is involved in transduction of pain signals. Moxibustion can effectively mitigate CIVP, but its potential mechanism is still unknown.

Research frontiers

The pathogenesis of CIVP is complicated, involving multiple neurotransmitters, cytokines and signaling pathways. ERK signaling pathway is closely related to the development of pain and inflammation. Moxibustion can successfully mitigate CIVP, and studies on the ERK signaling pathway will help reveal its mechanism of action.

Innovations and breakthroughs

This study discovered that the spinal cord MEK/ERK/CREB signaling pathway was activated in CIVP rats. Herb-partitioned moxibustion (HPM) exerted its analgesic effect by down-regulating protein phosphorylation and mRNA expression of mitogen-activated extracellular signal-regulated kinase MEK, ERK1/2 and cAMP response element binding protein (CREB), and inhibiting activation of the MEK/ERK/CREB signaling pathway in the spinal cord of CIVP rats.

Applications

To provide experimental evidence for HPM in treating CIVP, and the results may

reveal one of the mechanisms of the analgesic effects of HPM in treating CIVP.

Terminology

HPM is an external therapy in traditional Chinese medicine that reduces chronic inflammatory pain.

Peer-review

The authors aimed to investigate the effect of HPM on the phosphorylation of MEK1, ERK1/2 and CREB in spinal cord of rats with CIVP, and to explore the central mechanism of HPM in treating CIVP. This is a great, exciting and very tough study relying on establishing a rat model.

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ORIGINAL ARTICLE

Basic Study

Changes of Ghrelin/GOAT axis and mTOR pathway in the hypothalamus after sleeve gastrectomy in obese type-2 diabetes rats

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Conflict-of-interest statement: We declare that there are no conflicts of interest related to this study.

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Abstract

AIM

To examine the changes of the ghrelin/ghrelin O-acyltransferase (GOAT) axis and the mammalian target of rapamycin (mTOR) pathway in the hypothalamus after sleeve gastrectomy.

METHODS

A total of 30 obese type-2 diabetes Sprague-Dawley (SD) rats, 6 wk of age, fed with high-sugar and high-fat fodder for 2 mo plus intraperitoneal injection of streptozotocin were randomly divided into three groups: non-operation group (S0 group, n = 10), sham operation group (Sh group, n = 10) and sleeve gastrectomy group (SG group, n = 10). Data of body mass, food intake, oral glucose tolerance test (OGTT), acylated ghrelin (AG) and total ghrelin (TG) were collected and measured at the first day (when the rats were 6 wk old), preoperative day 3 and postoperative

week 8. The mRNA expression of preproghrelin, GOAT and neuropeptide Y (NPY), and protein expression of ghrelin, GOAT, GHSR and the mTOR pathway (p-Akt, p-mTOR and p-S6) were measured in the hypothalamus.

RESULTS

SG can significantly improve metabolic symptoms by reducing body mass and food intake. The obese rats showed lower serum TG levels and no change in AG, but the ratio of AG/TG was increased. When compared with the S0 and Sh groups, the SG group showed decreased TG (1482.03 ± 26.55, 1481.49 ± 23.30 and 1206.63 ± 52.02 ng/L, respectively, $P < 0.05$), but unchanged AG (153.06 ± 13.74, 155.37 ± 19.30 and 144.44 ± 16.689 ng/L, respectively, $P > 0.05$). As a result, the ratio of AG/TG further increased in the SG group (0.103 ± 0.009, 0.105 ± 0.013 and 0.12 ± 0.016, respectively, $P < 0.05$). When compared with the S0 group, SG suppressed mRNA and protein levels of preproghrelin (0.63 ± 0.12 *vs* 0.5 ± 0.11, $P < 0.05$) and GOAT (0.96 ± 0.09 *vs* 0.87 ± 0.08, $P < 0.05$), but did not change NPY mRNA expression (0.61 ± 0.04 *vs* 0.65 ± 0.07, $P > 0.05$) in the hypothalamus. The protein levels of p-Akt, p-mTOR and p-S6 were higher in the SG group, which indicated that the hypothalamic mTOR pathway was activated after SG at the postoperative week 8.

CONCLUSION

The reduction of ghrelin expression and activation of the mTOR pathway might have opposite effects on food intake, as SG improves obesity and T2DM.

Key words: Ghrelin; Ghrelin O-acyltransferase; Type-2 Diabetes; Hypothalamus; Obesity; Sleeve gastrectomy; Mammalian target of rapamycin

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Core tip: Recent studies have demonstrated a complex relationship between ghrelin, ghrelin O-acyltransferase (GOAT) and the mammalian target of rapamycin (mTOR) pathway. In our study, we examined the changes of the ghrelin/GOAT axis and the mTOR pathway in the hypothalamus after sleeve gastrectomy (SG). The mRNA and protein levels of ghrelin and GOAT decreased after SG, while NPY mRNA expression did not change. We also found that SG increased the protein levels of p-Akt, p-mTOR and p-S6. The reduction of ghrelin expression and activation of the mTOR pathway might have opposite effects on food intake. These findings might explain the weight, glucose and food intake regain after SG.

Wang Q, Tang W, Rao WS, Song X, Shan CX, Zhang W. Changes of Ghrelin/GOAT axis and mTOR pathway in the hypothalamus after sleeve gastrectomy in obese type-2 diabetes rats. *World J Gastroenterol* 2017; 23(34): 6231-6241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6231.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6231

INTRODUCTION

Obesity and type-2 diabetes mellitus (T2DM) are closely related epidemic diseases with increasing incidence rates worldwide. Lifestyle factors such as high-fat diet, lack of physical activity, genetic and environmental factors are important causes of diabetes. As one of the most commonly used bariatric surgeries, sleeve gastrectomy (SG) can effectively reduce obesity and improve metabolic symptoms by weight loss and hormonal change. Recent studies have found that weight loss and T2DM remission may be related to ghrelin, bile acids and microbiota changes after SG[1].

Ghrelin, mainly found in the stomach, is an endogenous ligand for the growth hormone secretagogue receptor type 1a (GHS-R1a) and has two major molecular forms, acylated ghrelin (AG) and unacylated ghrelin (UAG)[2-4]. As the activated form of ghrelin, AG is best known for its effect on growth hormone-releasing neurons. For instance, AG was shown to increase neuropeptide Y (NPY) mRNA expression levels by the mammalian target of rapamycin (mTOR) in the hypothalamus[5,6]. The enzyme responsible for transforming UAG to AG was identified in 2008 and named ghrelin O-acyltransferase (GOAT)[7]. A recent study found that AG could regulate GOAT expression in the stomach[8] and that the widespread expression of GOAT corresponded to the widespread distribution of ghrelin expression, *e.g.*, in the pancreas, stomach and hypothalamus[8,9]. Multiple studies have demonstrated that both ghrelin and GOAT play important roles in the regulation of obesity, blood glucose and insulin[5,10,11]. However, it is not clear whether the ghrelin/GOAT axis in the hypothalamus (*via* autocrine/paracrine signaling) is involved in the maintenance of blood glucose concentrations and energy homeostasis by restricting food intake after SG.

Recent studies suggested that the mTOR pathway may play an important role and have complex impact on several cellular functions, such as cell growth, weight loss, food intake and even insulin resistance[12-16]. It was also demonstrated that both energy deprivation and AG can activate the mTOR pathway to affect food intake[6,17]. Meanwhile, insulin inhibits GOAT expression *via* the mTOR pathway in islet cells[3] and SG can suppress the mRNA levels of preproghrelin and GOAT in a remnant stomach[18]. However, changes of the Ghrelin/GOAT axis and the mTOR pathway in the hypothalamus after SG have not yet been investigated. The aim of this study was to evaluate these changes to explain the mechanism of SG from a central point of view.

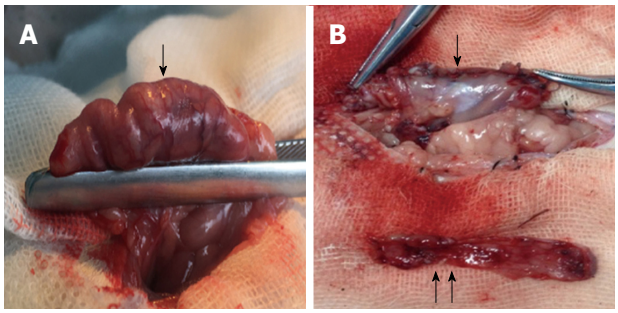


Figure 1 An intact stomach before SG (A) and the remnant stomach (↓) and resected gastric fundus (↑↑) after SG (B). The ↓ appearing in the figure A means that the gastric fundus and a large portion of the gastric body should be resected.

MATERIALS AND METHODS

Animals and experimental protocol

Seventy-seven male Sprague-Dawley rats (6 wk of age, weight: 150-180 g) were obtained from the Laboratory Animal Center of the Second Military Medical University. Equipment and reagents were obtained from the following sources: electronic analytical balance (Libror AEL-200 type, Japan); Roche glucometer (ACCU-CHEK® Performa, Roche Diagnostics); streptozotocin (Sigma-Aldrich, China); rat total ghrelin, AG and Insulin ELISA assay kit (R&D system, United States); ghrelin, GHSR, GOAT, p-AKT(s473), mTOR, p-mTOR(s2448), P70s6k, GAPDH antibody (Abcam, United States); and p-S6 (s235/236) antibody (Sigma-Aldrich, United States). All procedures conformed to the institutional standards of The Second Military Medical University Animal Care and Use Committee (approval no. SCXK(H) 2013-0016).

Establishment of the animal model

Rats had a mean body mass of 173.82 ± 14.32 g and were randomly divided into NF (fed normal fodder, n = 20) or HF groups (fed high-sugar and high-fat fodder, n = 57). During the 2-mo experiment, all rats were housed at 20-25 ℃ and 40%-60% relative humidity. Rats with a 20% higher body mass than the average weight of the NF group[19] were defined as obese rats. Noninsulin-dependent diabetes mellitus was induced in overnight-fasted rats by a single intraperitoneal injection of 45 mg/kg streptozotocin. Blood glucose was measured from the tail vein once daily within 3 d after injecting streptozotocin. Only the rats with an average blood glucose concentration > 16.7 mmol/L were included as successful T2DM models. Of all rats, 32 models were successfully established, with a success rate of 56.14%.

Animal grouping

Of the 32 T2DM model rats, two rats died after SG. The remaining 30 rats were randomly divided into three groups: non-operation (S0 group, n = 10), sham operation (Sh group, n = 10) or sleeve gastrectomy group (SG group, n = 10).

Surgical operation and intervention study

All rats of the operation groups (both Sh and SG) were food- and water-deprived for 12 h before surgery. The rats were anesthetized by an i.p. injection of 0.3% sodium pentobarbital (50 mg/kg) and observed until corneal and pain reflexes were absent. A hypodermic injection of 0.9% sodium chloride solution (20 mL) and an intramuscular injection of penicillin sodium (800000 IU) were given to prevent dehydration and infection before the surgery began.

A 3-cm incision was made 1 cm below the xiphoid process. After opening the abdominal cavity and exposing the stomach, the ligaments surrounding the stomach and gastroepiploic tissues were broken. A 3-5-mm transverse incision was made along the greater curvature to empty the stomach contents. This step could avoid the contents outflowing quickly to prevent abdominal infection. After empting the stomach, the gastric fundus and a large portion of the gastric body (70% of the total stomach) were resected (Figure 1A). The residual stomach was then closed by a single-layer continuous suture using 6-0 absorbable lines (Figure 1B). Before closure of the skin, the abdominal cavity was rinsed with a warm 0.9% sodium chloride solution (20-30 mL).

Sham-operated rats underwent a similar procedure as the SG group, including anesthesia, a 3-5-mm transverse incision was made along the greater curvature near the pylorus to empty stomach contents. After empting the stomach, the incision was extended to 2 cm, with a waiting time that corresponded to the resection time in SG rats, followed by stomach closure. Rats that were operated on were allowed access to water 2 h after surgery, but were fasted for 24 h after operation. After 2-3 d, they were fed a non-residue diet; and after recovery, they consumed their preoperative diet.

Body mass, food intake, oral glucose tolerance test, ghrelin measurements

Body mass was measured once every 5 d preoperatively and once a day postoperatively for 8 wk. Food intake was measured by recording the total amount of fodder consumed per week. To perform the oral glucose tolerance test (OGTT), all rats were fasted overnight and received a 50% glucose solution (1 g/kg) by oral gavage[20]. Blood was collected from the orbital vein to measure the glucose and insulin concentrations at 0, 15, 30, 60, 90 and 120 min after glucose infusion at the first day (rats were 6 wk old), preoperative day 3 and postoperative week 8. The area under the glucose curve (AUC-glucose), AUC-insulin and HOMA-IR (fasting insulin × fasting glucose/22.5) were calculated. The blood collected from the OGTT at 0 min was also used to measure AG and TG.

Hypothalamic tissue was collected for Western

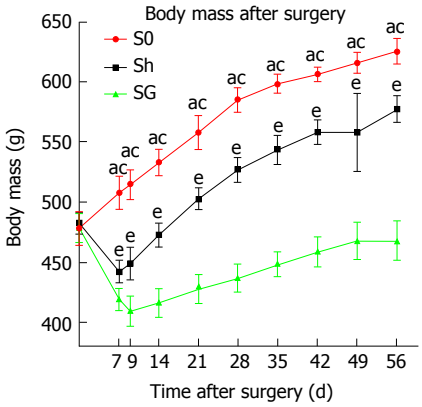


Figure 2 Body mass before and after surgery. Statistical differences were analyzed by one-way ANOVA followed by the LSD-*t* test. $^{a}P < 0.05$, S0 *vs* SG; $^{c}P < 0.05$, S0 *vs* Sh; $^{e}P < 0.05$, Sh *vs* SG. LSD: Least significant difference; Sh: Sham operation group; SG: Sleeve gastrectomy.

blotting and real-time polymerase chain reaction (PCR). All rats were sacrificed by an overdose of anesthesia. Sections containing the hypothalamus were cut according to the rat brain atlas of Paxinos and Watson and immediately frozen in liquid nitrogen[21].

Western blot analysis

The hypothalamus was crushed in PMSF lysate (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Triton X-100, 100 μg/mL PMSF) for 30 min and then centrifuged at 12000 rpm for 5 min. Protein concentration was measured by the BCA protein assay reagent. A total of 40 μg protein was separated by a 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. All membranes were incubated overnight at 4 ℃ with primary antibodies. They were then incubated with secondary antibodies at room temperature for 1 h and visualized using the GChemiDoc MP imaging system and image lab software (Bio-Rad, United States). All results were expressed as target protein/GAPDH ratio.

Real-time PCR analysis

Total RNA was extracted with Trizol Reagent (Invitrogen, United States). Then, 2 μg total RNA was reversely transcribed with Oligo (dT) primer and M-MLV Reverse Transcriptase (Promega, United States), according to the manufacturer's instructions. The complementary DNA was used for real-time PCR, and a final volume of 20 μL was made as follows: 10 μL 2 × SYBR premix exTaq (TaKaRa, China), 0.8 μL forward and reverse primers (2.5 μmol/L, 5 μL cDNA, 4.2 μL ddH_2O). PCR and data collection were performed on a CFX Connect™ Real-Time PCR Detection System (Takara), and data analysis was operated with the $2^{-\Delta\Delta Ct}$ method normalized to the endogenous control β-actin. Primers used in real-time PCR were as follows: Rat preproghrelin: 5′-AAGCCCAGCAGAGAAAGGAATC-3′ (forward); 5′-CAACATCGAAGGGAGCATTGAAC-3′ (reverse). Rat GOAT: 5′-ATTTGTGAAGGGAAGGTGGAG-3′ (forward); 5′-CAGGAGAGCAGGGAAAAAGAG-3′ (reverse). Rat NPY: 5′-GGCCAGATACTACTCCGCTC-3′ (forward); 5′-GTCTTCAAGCCTTGTTCTGGG-3′ (reverse). Rat β-actin: 5′-GAGACCTTCAACACCCCAGCC-3′ (forward); 5′-TCGGGGCATCGGAACCGCTCA-3′ (reverse).

Statistical analysis

Data are presented as the mean ± SD. SPSS 20.0 (IBM, New York, United States) was used for statistical analyses, and GraphPad Prism 6.0c (GraphPad Software, San Diego, CA, United States) was used to edit images. Statistical indicators between the three groups (S0, Sh and SG) were determined by one-way ANOVA, followed by least significance difference (LSD) *post hoc* comparison. The statistical indicator between two time points in one group was determined by a paired *t* test, whereas Student's *t* test was used to compare the means of two independent groups at the same time point.

RESULTS

Body mass and food intake

Baseline body mass before surgery did not differ between groups ($P > 0.05$). Rats in the Sh group and SG group weighed significantly less in the first postoperative week, which gradually normalized and increased in the second week after surgery when rats continued with their preoperative diet. The minimum weight was 442.17 ± 9.57 g in Sh rats at postoperative day 7 and 409.25 ± 12.7 g in SG rats at postoperative day 9. The weight in the sham group was restored to preoperative levels at postoperative day 17. The weight of SG rats remained low and did not reach baseline levels (Figure 2).

Food intake was significantly decreased in the Sh group and the SG group but was consistently higher in the Sh group compared to the SG group (Figure 3).

OGTT

Fasting blood glucose: Fasting blood glucose did not differ between treatment groups at the first day ($P > 0.05$) and significantly increased in obese model rats ($P < 0.05$). Eight weeks after surgery, the fasting blood glucose levels in the SG group were significantly lower than in the S0 and Sh groups ($P < 0.05$; Table 1)

Blood glucose and AUC-glucose: Blood glucose levels of the S0, Sh and SG groups did not differ among all the time points at the first day but significantly increased as rats became obese. Blood glucose levels and AUC-glucose in the SG group significantly decreased after surgery and were significantly lower than in the S0 and Sh groups (Figure 4 and Table 2).

Blood insulin and AUC-insulin: Fasting insulin and

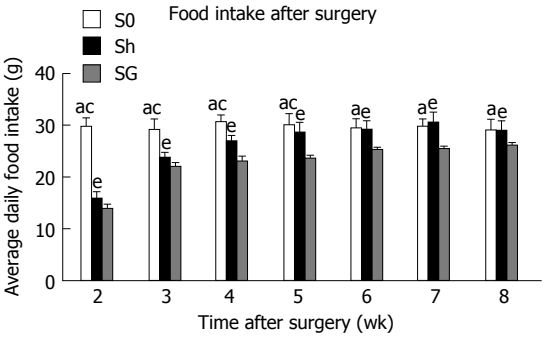


Figure 3 Food intake after surgery. Statistical differences were analyzed by one-way ANOVA followed by the least significant difference-*t* test. $^{a}P < 0.05$, S0 *vs* SG; $^{c}P < 0.05$, S0 *vs* Sh; $^{e}P < 0.05$, Sh *vs* SG. LSD: Least significant difference; Sh: Sham operation group; SG: Sleeve gastrectomy.

Table 1 Changes in fasting blood glucose

Time points	Fasting blood glucose (mmol/L)		
	S0	Sh	SG
First day	6.18 ± 1.19	5.89 ± 1.12	6.19 ± 0.94
Preoperative	15.03 ± 2.4[a]	15.9 ± 1.2[a]	15.88 ± 2.32[a]
Postoperative wk 8	14.55 ± 1.29	14.29 ± 1.69	9.00 ± 1.17[c,e]

Statistical differences among the three groups were analyzed by one-way ANOVA followed by LSD-*t* and between two time points in one group using a paired *t* test. $^{a}P < 0.05$, preoperative *vs* first day levels in the same group; $^{c}P < 0.05$, postoperative *vs* preoperative levels in the SG group; $^{e}P < 0.05$, SG *vs* S0 and Sh postoperatively. LSD: Least significant difference; Sh: Sham operation group; SG: Sleeve gastrectomy.

maximal insulin were significantly increased, and the maximal insulin excretion time was at the 90th min in all groups after glucose loading at preoperative day 3, while the maximal insulin excretion time was at the 60th min after glucose loading on the first day. Compared with the S0 and Sh groups, the SG group had lower fasting insulin and maximal insulin concentrations. The maximal insulin excretion time in SG rats was at the 60th min again. AUC-glucose and AUC-insulin were increased in all obese model groups and were significantly reduced in the SG group at 8 weeks after surgery. HOMA-IR, which reflects insulin resistance, was also significantly reduced in the SG group (Table 3, Figures 5 and 6).

TG and AG

The obese rats showed a lower serum TG level and no change in AG, but the ratio of AG/TG was increased. When compared with the S0 and Sh groups, the SG group showed decreased TG (1482.03 ± 26.55, 1481.49 ± 23.30 and 1206.63 ± 52.02 ng/L, respectively, $P < 0.05$) but unchanged AG (153.06 ± 13.74, 155.37 ± 19.30 and 144.44 ± 16.689 ng/L, respectively, $P > 0.05$). As a result, the ratio of AG/TG was further increased in the SG group (0.103 ± 0.009, 0.105 ± 0.013 and 0.12 ± 0.016, respectively, $P < 0.05$) (Figure 7).

mRNA expression levels of preproghrelin, GOAT and NPY in the hypothalamus

The mRNA expression levels of preproghrelin and GOAT were lower in the SG group than in the S0 group, while NPY mRNA expression did not differ between groups (Table 4).

Ghrelin, GHSR and GOAT protein in the hypothalamus

Ghrelin and GOAT protein expression was significantly reduced in SG rats, while the level of GHSR protein expression was increased (Figure 8).

Protein expression of the mTOR pathway in the hypothalamus

The levels of p-Akt, p-mTOR and p-S6 protein expression were higher in the SG group, while the protein expression levels of mTOR and P70S6K did not differ between the Sh and SG groups (Figure 9).

DISCUSSION

As a bariatric surgery, SG can not only significantly reduce body weight but also effectively improve T2DM and obesity-induced metabolic complications[1]. More and more surgeons worldwide choose SG, especially in Asia, because of the less difficulty and complications of SG when compared with other bariatric surgeries. As the number of SGs is increasing, the surgeons' viewpoint of SG changes from bariatric surgery to metabolic surgery. However, when compared with Roux-en-Y gastric bypass (RYGB), which is regarded as the "gold standard" in metabolic surgery, the effect of SG on improving metabolic symptoms and weight loss is seemingly imperfect[22-24]. Weight and blood glucose regain is still the most disputed problem after SG. Our data showed that body mass, food intake, fasting blood glucose, postprandial blood glucose, AUC-glucose, AUC-insulin and HOMA-IR were significantly reduced after SG. However, the body mass levels at postoperative week 8 were comparable to the preoperative level. Remarkably, hyperglycemia and insulin resistance were significantly improved. The mechanism of SG is still unclear; the most important mechanisms are believed to be the restriction of calories and the complex hormonal changes. One of these hormonal changes include the markedly reduced ghrelin levels after removal of the gastric fundus.

Previous studies demonstrated that low plasma ghrelin levels are beneficial in elevating fasting insulin levels and improving hyperglycemia, insulin resistance and obesity, while higher levels of ghrelin inhibit insulin release in the pancreas and enhance the food intake signals in the hypothalamus[25,26]. Furthermore, ghrelin gene knock-out mice had lower blood glucose and higher blood insulin levels[5]. As one of the two existing forms of ghrelin in the body, AG is commonly considered the active form due to its "negative" effect

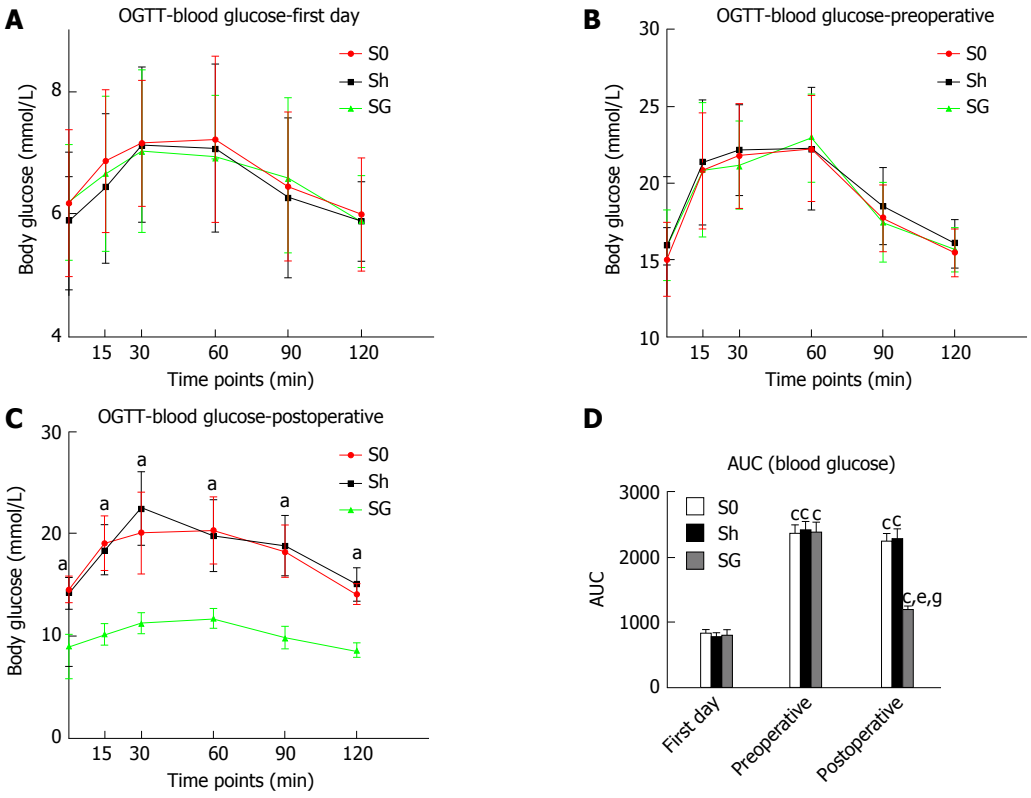


Figure 4 Oral glucose tolerance test curve of blood glucose at the first day (A), preoperatively (B) and postoperatively (C) and AUC-blood glucose (D). Statistical differences among the three groups at the same time point were analyzed by one-way ANOVA followed by LSD-*t* test and between two time points in one group using paired *t* test. C: $^{a}P < 0.05$, SG *vs* S0 and Sh at the same time point; $^{c}P < 0.05$, preoperative or postoperative *vs* first day levels in the same group; $^{e}P < 0.05$, postoperative *vs* preoperative levels in the SG group; $^{g}P < 0.05$, SG *vs* S0 and Sh postoperatively. OGTT: Oral glucose tolerance test; Sh: Sham operation group; SG: Sleeve gastrectomy; LSD: Least significant difference.

Table 2 Oral glucose tolerance test of blood glucose

Time points (min)	Blood glucose (mmol/L)								
	First day			Preoperative			Postoperative		
	S0	Sh	SG	S0	Sh	SG	S0	Sh	SG
0	6.18 ± 1.19	5.89 ± 1.12	6.19 ± 0.94	15.03 ± 2.40[a]	15.90 ± 1.20[a]	15.88 ± 2.32[a]	14.55 ± 1.29	14.29 ± 1.69	9.00 ± 1.17[c,e]
15	6.18 ± 1.17	6.42 ± 1.22	6.66 ± 1.27	20.82 ± 3.80[a]	21.35 ± 4.13[a]	20.84 ± 4.41[a]	19.06 ± 2.68	18.39 ± 2.42	10.12 ± 1.07[c,e]
30	7.15 ± 1.03	7.13 ± 1.27	7.03 ± 1.32	21.76 ± 3.45[a]	22.13 ± 2.96[a]	21.15 ± 2.86[a]	20.09 ± 4.02	22.47 ± 3.63	11.21 ± 1.02[c,e]
60	7.22 ± 1.36	7.07 ± 1.38	6.93 ± 1.02	22.23 ± 3.47[a]	22.23 ± 4.03[a]	22.96 ± 2.78[a]	20.32 ± 3.26	19.80 ± 3.49	11.75 ± 0.95[c,e]
90	6.45 ± 1.21	6.27 ± 1.31	6.58 ± 1.31	17.72 ± 2.19[a]	18.48 ± 2.54[a]	17.43 ± 2.61[a]	18.30 ± 2.50	18.81 ± 2.90	8.76 ± 1.01[c,e]
120	6.00 ± 0.92	5.89 ± 0.65	5.58 ± 0.75	15.47 ± 1.55[a]	15.04 ± 1.58[a]	15.67 ± 1.44[a]	14.08 ± 1.00	15.07 ± 1.63	6.98 ± 1.13[c,e]

Statistical differences among the three groups were analyzed by one-way ANOVA followed by LSD-*t* test and between two time points in one group using a paired *t* test. $^{a}P < 0.05$, preoperative *vs* first day levels in the same group at the same time point; $^{c}P < 0.05$, postoperative *vs* preoperative levels in the SG group; $^{e}P < 0.05$, SG *vs* S0 and Sh postoperatively. LSD: Least significant difference; Sh: Sham operation group; SG: Sleeve gastrectomy.

Table 3 Oral glucose tolerance test of blood insulin

Time points (min)	Blood insulin (mU/L)								
	First day			Preoperative			Postoperative		
	S0	Sh	SG	S0	Sh	SG	S0	Sh	SG
0	13.06 ± 2.21	13.03 ± 2.01	12.97 ± 2.63	24.79 ± 3.06[a]	26.48 ± 2.19[a]	24.37 ± 2.51[a]	23.33 ± 1.81	27.58 ± 2.24	15.21 ± 2.87[c,e]
15	19.16 ± 1.57	19.04 ± 1.83	20.63 ± 1.17	28.66 ± 3.20[a]	29.38 ± 3.33[a]	28.72 ± 2.39[a]	29.78 ± 2.86	32.95 ± 2.65	25.71 ± 7.73[c,e]
30	40.90 ± 3.15	42.65 ± 9.89	41.64 ± 12.51	35.47 ± 3.36[a]	36.27 ± 3.21[a]	35.33 ± 4.68[a]	36.49 ± 3.25	36.78 ± 2.05	47.93 ± 3.31[c,e]
60	63.46 ± 5.30	62.43 ± 6.01	65.38 ± 3.45	54.76 ± 2.85[a]	59.37 ± 2.12	59.81 ± 4.63[a]	57.36 ± 3.51	58.08 ± 2.77	69.05 ± 1.74[c,e]
90	22.55 ± 1.80	21.81 ± 2.65	21.68 ± 3.23	80.35 ± 2.42[a]	82.68 ± 2.02[a]	80.68 ± 4.13[a]	79.79 ± 5.66	82.14 ± 3.46	30.55 ± 7.66[c,e]
120	14.30 ± 1.82	13.25 ± 1.67	13.14 ± 2.02	66.15 ± 4.38[a]	70.11 ± 8.05[a]	71.52 ± 4.20[a]	71.26 ± 7.88	71.76 ± 6.63	18.23 ± 4.95[c,e]

Statistical differences among the three groups were analyzed by one-way ANOVA followed by LSD-*t* and between two time points in one group using a paired *t* test. $^{a}P < 0.05$, preoperative *vs* first day levels in the same group at the same time point; $^{c}P < 0.05$, postoperative *vs* preoperative levels in the SG group; $^{e}P < 0.05$, SG *vs* S0 and Sh postoperatively. LSD: Least significant difference; Sh: Sham operation group; SG: Sleeve gastrectomy.

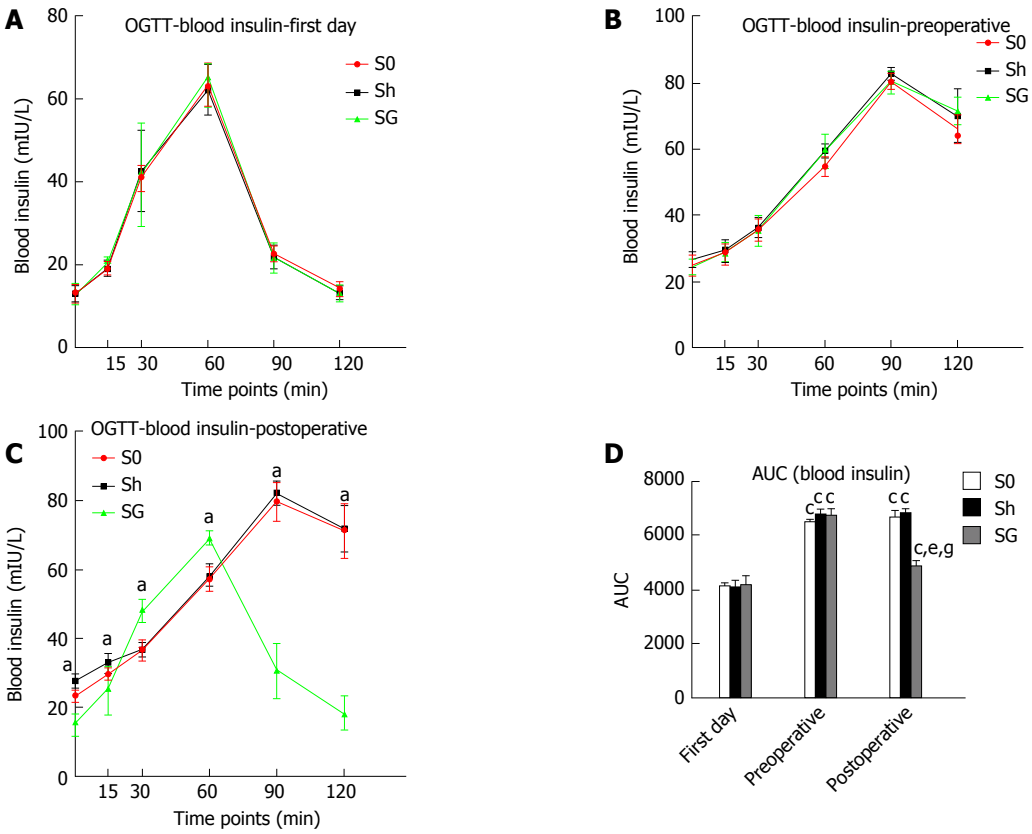


Figure 5 **Oral glucose tolerance test curve of blood insulin at the first day (A), preoperatively (B) and postoperatively (C) and AUC-blood insulin (D).** Statistical differences among the three groups at the same time point were analyzed by one-way ANOVA followed by LSD-*t* test and between two time points in one group using paired t test. [a]*P* < 0.05, SG *vs* S0 and Sh at the same time point; [c]*P* < 0.05, preoperative or postoperative *vs* first day levels in the same group; [e]*P* < 0.05, postoperative *vs* preoperative levels in the SG group; [g]*P* < 0.05, SG *vs* S0 and Sh postoperatively. OGTT: Oral glucose tolerance test; Sh: Sham operation group; SG: Sleeve gastrectomy; LSD: Least significant difference.

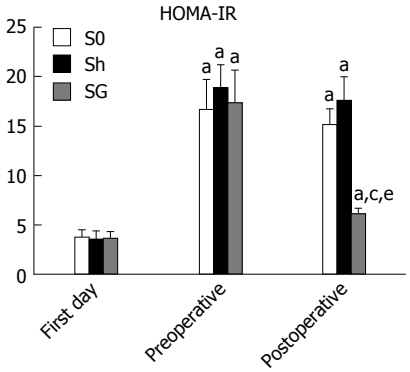


Figure 6 HOMA-IR in the S0, sham operation group and sleeve gastrectomy at the first day, preoperatively and postoperatively. Statistical differences among the three groups at the same time point were analyzed by one-way ANOVA followed by the LSD-*t* test and between two time points in the same group using paired *t* test. [a]*P* < 0.05, preoperative or postoperative *vs* first day levels in the same group; [c]*P* < 0.05, postoperative *vs* preoperative levels in the SG group; [e]*P* < 0.05, SG *vs* S0 and Sh postoperatively. Sh: Sham operation group; SG: Sleeve gastrectomy.

on blood glucose metabolism and positive effect on food intake signals in the hypothalamus[27,28], whereas UAG can antagonize AG's effects[29-31]. Recent studies found that obese individuals have lower blood ghrelin levels but no change in serum AG levels or the ratio of AG/UAG. SG can further reduce serum UAG but not AG levels and increase the ratio of AG/UAG[32]. Our data demonstrated that the level of TG was significantly decreased in obese rats; however, AG levels were unchanged and the ratio of AG/TG was increased. As shown in Figure 7, SG can further reduce the serum TG levels after SG-induced resection of the gastric fundus. However, AG was still not changed, but the ratio of AG/TG was further increased. Since SG can only reduce the levels of serum TG or UAG, it is unclear how SG can improve obesity and T2DM with increasing ratios of AG/TG or AG/UAG. Previous studies mentioned that ghrelin secretion depends on a specific secretion cue related to fasting and feeding[33], which should be abolished by SG. However, ours and other studies did not confirm whether SG really abolished ghrelin's special cyclic secretion. We propose that altered ghrelin secretion cues might be more important than plasma TG, AG or UAG in SG-induced improvement of obesity and metabolic symptoms.

AS is required for active ghrelin to bind with and activate its receptor; GOAT is essential for octanoylation of ghrelin[34]. A number of studies demonstrated that GOAT mainly exists in tissues that play an important role in the regulation of food intake, blood glucose metabolism and energy homeostasis, such

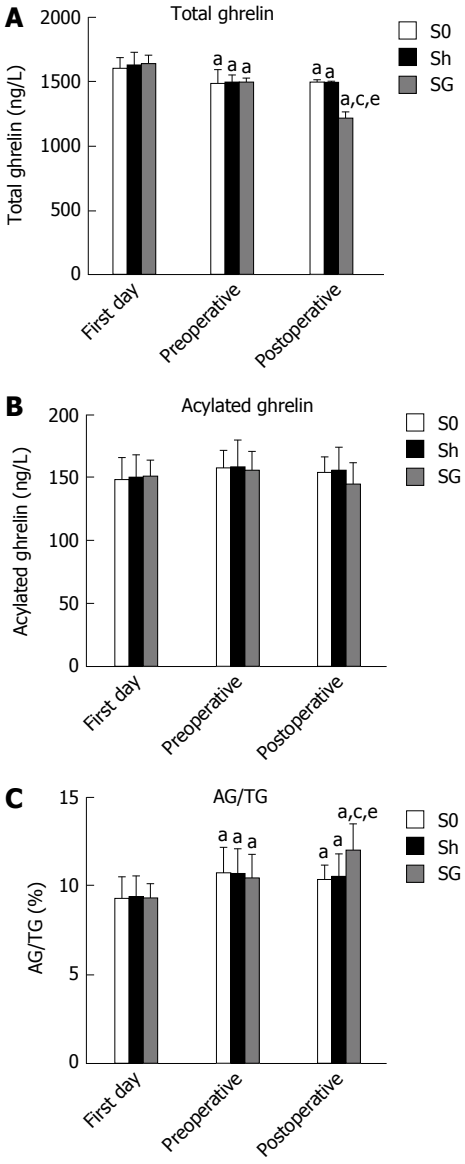


Figure 7 Changes of total ghrelin (A), acylated ghrelin (B) and acylated ghrelin/total ghrelin (C) at the first day, preoperatively and postoperatively. Statistical differences among the three groups at the same time point were analyzed by one-way ANOVA followed by the LSD-*t* test and between two time points in the same group using paired *t* test. $^{a}P < 0.05$, preoperative or postoperative *vs* first day levels in the same group; $^{c}P < 0.05$, postoperative *vs* preoperative levels in the SG group; $^{e}P < 0.05$, SG *vs* S0 and Sh postoperatively. AG: Acylated ghrelin; TG: Total ghrelin; Sh: Sham operation group; SG: Sleeve gastrectomy; LSD: Least significant difference.

as the stomach, hypothalamus and pancreas[8]. However, the relationship between GOAT expression and energy homeostasis is inconsistent. Gonzalez[35] found that a 30% food reduction for 21 d increased GOAT expression in the gastric mucosa, while a 48-h fasting period had no effect on GOAT expression in rats. On the contrary, other studies reported that GOAT expression was decreased after food restriction[36,37]. Body energy level is not the only factor determining GOAT expression. Gahete *et al*[8] found that AG could increase GOAT expression in the stomach, whereas insulin inhibited the expression of GOAT mRNA *via*

Table 4 Hypothalamic mRNA expression of preproghrelin, ghrelin O-acyltransferase and neuropeptide Y

X-mRNA/β-acting-mRNA	S0	SG
Preproghrelin	0.63 ± 0.12	0.50 ± 0.11[a]
GOAT	0.96 ± 0.09	0.87 ± 0.08[a]
NPY	0.61 ± 0.04	0.65 ± 0.07

Hypothalamic mRNA expression of preproghrelin, GOAT and NPY was quantified using PCR and expressed as the ratio to the β-actin mRNA. Statistical differences between the two groups were analyzed using Student's *t* test. $^{a}P < 0.05$, S0 *vs* SG. GOAT: Ghrelin O-acyltransferase; SG: Sleeve gastrectomy; NPY: Neuropeptide Y.

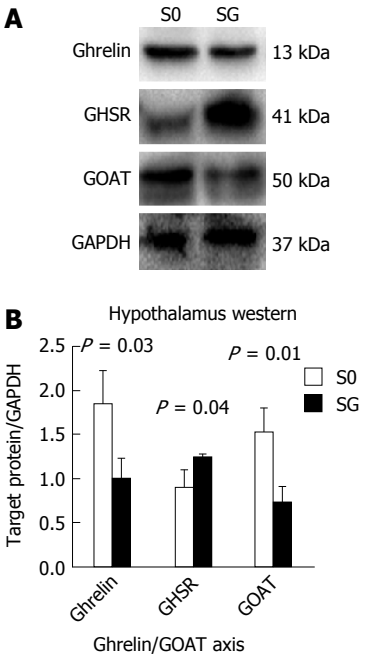


Figure 8 Ghrelin/ghrelin O-acyltransferase axis in hypothalamus. A: Expression of ghrelin, GOAT and GHSR protein in the hypothalamus. GAPDH was used as an internal control; B: Ratio of target protein gray value to GAPDH gray value. Statistical differences between the two groups were analyzed by Student's *t* test. GOAT: Ghrelin O-acyltransferase; SG: Sleeve gastrectomy.

the mTOR pathway[3]. In our study, both mRNA and protein expression levels of ghrelin and GOAT significantly decreased after SG, while GHSR protein expression significantly increased. As mentioned above, this phenomenon may be related to the abolishment of ghrelin's special cyclic secretion. The "override inhibition" theory[38] describes a phenomenon in which hormones, normally secreted in response to an episodic stimulus, are paradoxically inhibited when that stimulus occurs continuously. Therefore, SG may eliminate the periodic effect of cyclic ghrelin on the hypothalamus, resulting in a reduced expression level of preproghrelin mRNA and ghrelin protein, as well as a GHSR compensatory increase. The reduction of GOAT mRNA and protein may be due to a local ghrelin decrease and energy deprivation after SG. Although ghrelin was significantly decreased, the level of NPY mRNA expression in SG rats did not change, which

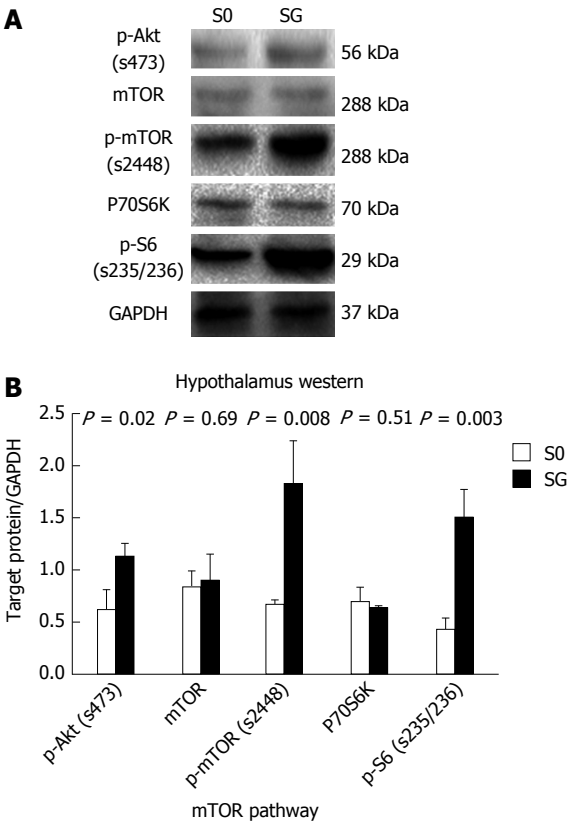


Figure 9 The mammalian target of rapamycin pathway in hypothalamus. A: Protein expression of the mTOR pathway in the hypothalamus. GAPDH was used as an internal control; B: Ratio of target protein gray value to GAPDH gray value. Statistical differences between the two groups were analyzed by Student's *t* test. SG: Sleeve gastrectomy; mTOR: Mammalian target of rapamycin.

may be related to the activation of the mTOR pathway in the negative energy balance of the hypothalamus after SG.

Several studies have shown that the hypothalamic mTOR pathway plays a major role in the regulation of energy homeostasis and food intake[6]. A recent study demonstrated that energy deprivation and administration of ghrelin into the 4th ventricle led to increased levels of p-mTOR and increased NPY mRNA expression and promoted feeding through the activation of the hypothalamic mTOR pathway. Moreover, rapamycin could significantly inhibit mTOR activity and reduce food intake[6,17]. Our data suggest that SG can significantly increase the protein levels of p-Akt, p-mTOR and p-S6 in the hypothalamus. As a classic restrictive metabolic surgery, it is possible that SG first reduces the calorie intake, which then activates the mTOR pathway. Therefore, the activation of the mTOR pathway may be the reason for the increased NPY level after SG.

One of the limitations of our study is that we did not compare SG with other restrictive metabolic surgeries. Kawasaki *et al*[21] found no difference in hypothalamic mRNA expression of NPY between normally fed rats and SG rats. However, when compared with gastric banding rats, the NPY mRNA expression in SG rats was significantly lower. The reduction of ghrelin expression and activation of the mTOR pathway might have an opposite effect on food intake after SG.

Overall, altered glucose metabolism and food intake changes following SG should be divided into peripheral and central changes. In the periphery, SG can significantly reduce food intake by decreasing gastric volume and changing blood ghrelin levels. Further studies should focus on how SG changes ghrelin's special cyclic secretion, especially AG's. In the center, as mentioned above, we found two opposite factors for the regulation of food intake. A recent study has shown that a residual stomach expansion occurred in more than 50% of the SG patients over a short-term period[39,40]. Increased food intake may be the most important factor for the residual stomach expansion. The results of our study provide a clue for further studies on SG and its regulation of food intake from a central point of view.

COMMENTS

Background

Sleeve gastrectomy (SG), which is a classic restrictive metabolic surgery, becomes more and more popular worldwide because of its less difficulty and complications. However, weight and blood glucose regain is still the most disputed problems after SG. Increasing food intake might be the most important reason for these problems.

Research frontiers

This study aimed to examine the changes of the ghrelin/ghrelin O-acyltransferase (GOAT) axis and the mammalian target of rapamycin (mTOR) pathway in the hypothalamus after SG. These changes provide a clue for further studies on SG and its regulation of food intake from a central point of view.

Innovations and breakthroughs

The authors compared between SG rats and non-operation rats in ghrelin/GOAT axis and the mTOR pathway in the hypothalamus, and found that SG could suppress mRNA and protein levels of preproghrelin and GOAT but did not change neuropeptide Y mRNA expression. The protein levels of p-Akt, p-mTOR and p-S6 were higher in the SG group, which indicates that the hypothalamic mTOR pathway was activated after SG on the postoperative week 8.

Applications

The findings in this study suggest that patients who underwent SG still need to control food intake for better improvement of obesity and glucose metabolism.

Terminology

SG is a novel procedure of metabolic surgery to remove at least 70% of stomach from the greater curvature. Ghrelin, mainly found in the stomach, could increase food intake and should be inhibited after SG. GOAT is essential for transforming unacylated ghrelin to acylated ghrelin. The mTOR pathway has an important and complex impact on several cellular functions, such as cell growth, weight loss, food intake and even insulin resistance.

Peer-review

The authors examined changes of the ghrelin/GOAT axis and the mTor pathway in the hypothalamus after SG. This is a carefully done study and the findings are of considerable interest.

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ORIGINAL ARTICLE

Basic Study

Dihydromyricetin-mediated inhibition of the Notch1 pathway induces apoptosis in QGY7701 and HepG2 hepatoma cells

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Abstract

AIM

To investigate whether Dihydromyricetin (DHM) inhibits cell proliferation and promotes apoptosis by downregulating Notch1 expression.

METHODS

The correlation between Notch1 and Hes1 (a Notch1

target molecule) expression in hepatoma samples was confirmed by qRT-PCR. In addition, MTT assays, flow cytometry and TUNEL analysis showed that DHM possessed strong anti-tumor properties, evidenced not only by reduced cell proliferation but also by enhanced apoptosis in QGY7701 and HepG2 hepatocellular carcinoma (HCC) cells. The expressions of Notch1, Hes1, Bcl-2 and Bax were determined by Western blot.

RESULTS

Among the tested samples (n = 64), the expression levels of Notch1 (75% of patients) and Hes1 (79.7% of patients) mRNA in tumor tissues were higher than in the normal liver tissues. There was a negative correlation between the expression of Notch1 and the degree of differentiation and positively correlated with the Alpha Fetal Protein concentration. The viability of HCC cells treated with DHM was significantly inhibited in a dose and time-dependent manner. Apoptosis was induced in HepG2 and QGY7701 cell lines following 24 h of DHM treatment. After treatment with DHM, the protein expression of Notch1 was downregulated, the apoptosis-related protein Bax was upregulated and Bcl2 was downregulated. Notch1 siRNA further enhanced the anti-tumor properties of DHM.

CONCLUSION

Notch1 is involved in the development of HCC and DHM inhibits cell proliferation and promotes apoptosis by down-regulating the expression of Notch1.

Key words: Dihydromyricetin; Apoptosis; Hepatocellular carcinoma; Notch1

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Core tip: The novel findings of this study are that Notch1 is highly expressed in tumor cells with higher degrees of malignancy and proliferative activity, Notch1 is involved in the development of hepatocellular carcinoma (HCC) and may serve as a potential diagnostic marker for HCC. Dihydromyricetin can strongly induce apoptosis of the hepatic cancer cell lines QGY7701 and HepG2 by downregulating the expression of Notch1.

Lu CJ, He YF, Yuan WZ, Xiang LJ, Zhang J, Liang YR, Duan J, He YH, Li MY. Dihydromyricetin-mediated inhibition of the Notch1 pathway induces apoptosis in QGY7701 and HepG2 hepatoma cells. *World J Gastroenterol* 2017; 23(34): 6242-6251 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6242.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6242

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks sixth and third worldwide in tumor incidence and mortality, respectively[1]. Despite dramatic improvements in surgical treatment and chemotherapy, the one-year survival rate of patients diagnosed with HCC is not high[2,3]. In addition, traditional chemotherapy drugs have obvious side effects. Therefore, seeking an effective drug treatment that reduces the mortality of patients with HCC is of great significance.

Dihydromyricetin (DHM, $C_{15}H_{12}O_8$, PubChem CID: 161557) is a component of wild woody vines of the genus *Vitis*, which is a flavonoid compound[4]. DHM was reported to have antioxidant[5,6], anti-inflammatory[7], anti-hypertensive[8], hypoglycemic[9], hepatoprotective[10,11], and anti-carcinogenic effects[12]. Previous studies have reported that DHM can inhibit the invasion and metastasis of HCC cells[13]. Combined application of DHM and nedaplatin induced apoptosis of HCC cells through the p53/Bcl-2 pathway, and enhanced sensitivity to nedaplatin chemotherapy[14]. DHM has great potential as a treatment option for liver cancer.

The Notch signaling pathway plays an important role in the fate of many cells and is crucial for the biological development of embryos. The Notch pathway is composed of a transmembrane family of receptors (Notch 1-4), their ligands (Jagged 1-2, Delta-like 1-3), and downstream target genes in the HES and HEY protein families[15]. Notch receptors bind to ligands in the membranes of adjacent cells, causing the intracellular segment of the Notch receptor to release, and the receptor then travels to the nucleus, resulting in activation of Notch target genes, thus successfully mediating cell growth and development[16].

Studies of Notch and liver diseases have been conducted in recent years. Notch3 is highly expressed in HCC, can promote the self-renewal of HCC cells and plays a significant role in maintaining stem cells by downregulating β-catenin and upregulating the expression of Nanog[17]. High expression of Notch1 and Notch4 is closely associated with poor prognosis of patients with HCC[18]. Suppression of the Notch signaling pathway averts cholestatic liver fibrosis by lessening the differentiation of hepatic progenitor cells into cholangiocytes[19]. Inhibition of Notch1 expression promoted pancreatic cancer and prostate cancer cell apoptosis[20,21], but whether downregulation of Notch1 induces apoptosis in hepatoma cells has rarely been reported. Moreover, the anti-tumor activity of DHM and its regulation of the Notch pathway are still unclear.

In the present study, we demonstrated that aberrant Notch1 expression was involved in tumor progression. DHM could downregulate the expression of Notch1 to induce apoptosis in HepG2 and QGY7701 HCC cells and restrain their proliferation. Inhibition of Notch1 signaling may serve as a novel target for HCC treatment.

MATERIALS AND METHODS

Reagents and chemicals

DHM (Sigma, United States) was solubilized in 100% dimethyl sulfoxide to prepare a primary solution of 50 mmol/L and stored at -20 ℃. Rabbit anti-human

primary antibodies against Notch1, Hes1, Bcl-2 and Bax were obtained from Cell Signaling Technology (Beverly, MA, the United States of America). Notch1 siRNA was obtained from Santa Cruz Biotechnology (the United States of America). FITC Annexin V staining Kit was brought from BD Biosciences (the United States of America). MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was obtained from Sigma-Aldrich Biotechnology (United States).

Clinical specimens

All patients were diagnosed with pathological primary HCC. Tissue samples were obtained from these patients during hepatectomy surgery. HCC was confirmed by pathology. All tissue samples were frozen in liquid nitrogen. This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

Cell lines and cell culture

The cells (SK-Hep-1, QGY7703, MHCC97L, HepG2, QGY7701 and HL7702) were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) in a humidified 5% CO_2 incubator. Upon reaching 80% confluence, the cells were treated with various concentrations of DHM (0, 5, 25, 50, 100 or 150 μmol/L) for 12 h, 24 h and 36 h and DMSO treatment served as a control.

Cell inhibition and cytotoxicity assay

The cells were placed into 96-well plates and treated with different concentrations of DHM. DMSO treatment served as a control. Twenty μL of MTT (5 mg/mL) solution was transferred to each well, and then the plate was incubated at 37 ℃ for 4 h. Next, the cell supernatants were removed, and 150 μL of DMSO was added to each well to solubilize the formed crystals. The optical density values were measured at 570 nm with a spectrophotometer (PerkinElmer, the United States of America).

Apoptosis assay

The HepG2 and QGY7701 cells were seeded in the 6-well plates. Following overnight growth, the cells were pretreated with 50, 100, or 150 μmol/L DHM and incubated for 24 h. Following careful collection and suspension in binding buffer, the HepG2 and QGY7701 cells were incubated with 5 mL of annexin V-FITC and 5 mL of PI for 15 min in darkness prior to flow cytometric analysis.

Western blot

The tissues protein samples were prepared with Fastprep-24 Sample Preparation equipment (MP, United States). The cells treated with DHM were harvested and lysed in lysis buffer to assess the expression of Notch1, Hes1, Bcl-2 and Bax. Equal amounts of protein samples were loaded per well. Membranes containing protein blots were incubated in blocking buffer (5% non-fat milk) for 1 h at room temperature, and then the membranes were incubated with the primary antibodies (1:1000 dilution) at 4 ℃ overnight. Following a wash with TBST, membranes were incubated with goat anti-rabbit secondary antibodies for 1 h. Detection was performed using an Odyssey Infrared Imaging System (LI-COR Biosciences Inc., Lincoln, NE, the United States of America).

qRT-PCR analysis

Frozen tissues were used to isolate messenger RNA (mRNA) using TRIzol reagent (Invitrogen, Guangzhou, China), according to the manufacturer's protocol. Reverse transcription was performed with 1 μg of RNA using the PrimeScript RT Reagent kit with a gDNA Eraser kit (Takara Bio, Inc, Otsu, Japan). The primer sequences used were as follows: Notch1 forward, 5'-CACCCATGACCACTACCCAGTT-3' and reverse, 5'-CCTCGGACCAATCAGAGATGTT-3'; Hes1 forward, 5'-AGATCTATGCCAGCTGATAAT-3'and reverse, 5'-AAGCTTCACTTAATACAGCT-3', and 18s forward, 5'-CGGCGACGACCCATTCGAAC-3' and reverse, 5'-GAATCGAACCCTGATTCCCCGTC-3'. Data were evaluated using the comparative count method and normalized to the corresponding 18S value.

TUNEL assay

In situ cell death detection kit-POD (Roche, Switzerland) was used to detect the late-stage apoptosis. Cells were seeded in 96-well plates and treated with various concentrations of DHM. Cells were fixed with 4% paraformaldehyde, and then counterstained with 4',6-diamidino-2-phenylindole for 5 min at room temperature in the dark and observed under a fluorescence microscope (Olympus IX70, Japan) to detect the apoptotic cells.

siRNA transfection

QGY7701 and HepG2 cells were plated into 6-well plates and transfection procedures were performed according to the manufacturer's protocol. Three different Notch1 siRNA sequences were tested in both QGY7701 and HepG2 cell lines for screening the efficacy of Notch1 knockdown and selecting the best performing siRNA transcript. Notch1 siRNA-2 showed the best degree of knockdown and was used in subsequent experiments. Before each experiment, we transiently transfected cells using an Notch1 siRNA gene knockdown kit (JIMA Biotechnology Company, Shanghai, China) and the negative control siRNA.

Colony formation assay

Cells in each logarithmic growth phase were detached from culture flasks using 0.25% trypsin. Then, the cells were percussed into a single cell suspension, and 1000 cells were cultured in each well of a 6-well plate, and humidity was saturated for 1 wk. The cells were stained with crystal violet. Clones were directly counted by the naked eye or counted using a microscope, and

data were analyzed using ImageJ software.

Statistical analysis

The data were obtained from at least three independent experiments, and all results are presented as the mean ± SD. The results were evaluated using Student's *t* test, Pearson correlation analyses were used to examine the correlation of two parameters. The significance levels were defined as $P < 0.05$.

RESULTS

Notch1 expression is activated in HCC tissues and reflects differentiation properties

The expression of Notch1 protein by Western blots and found that Notch1 accumulated predominantly in HCC tissues (Figure 1A). In addition, the expression of Notch1 and Hes1 genes in HCC and normal liver tissue samples was analyzed by qRT-PCR (Figure 1B and C). Among the tested samples ($n = 64$), the expression levels of Notch1 (75% of patients) and Hes1 (79.7% of patients) mRNA in tumor tissues were higher than in the normal liver tissues (Figure 1D). A positive correlation between Notch1 and Hes1 expression suggests that high expression of Notch1 in tumors may result in an upregulation of the target gene Hes1 (Figure 1E). To clarify the role of Notch1 in tumorigenesis and tumor differentiation, we analyzed the association among Notch1 expression, AFP concentration, and the degree of tumor differentiation. There was a negative correlation between the expression of Notch1 and the degree of differentiation and a positive correlation with the AFP concentration (Figure 1F and G). The results showed that Notch1 was highly expressed in tumor cells with higher degrees of malignancy and proliferative activity. Based on the results above, Notch1 was involved in the development and progression of HCC.

DHM inhibits the proliferation of HCC cells

We also screened the Notch1 protein expression profiles in several hepatoma cell lines. Notch1 exhibited higher expression levels in hepatoma cells compared with the immortalized normal liver cell line HL7702 (Figure 2B). Among the hepatoma cell lines, we found that QGY7701 and HepG2 cells highly express Notch1. Therefore, we chose these two lines for the next experiment. MTT assays showed that the proliferation of DHM-treated QGY7701 and HepG2 cells was inhibited. After 12 h, 24 h and 36 h, the viability of HCC cells treated with DHM was significantly inhibited in a dose- and time-dependent manner (Figure 2C and D). As shown in Figure 2, the proliferation of cells was significantly inhibited after treatment with 150 μmol/L and 50 μmol/L DHM for 12 h ($P < 0.01$), and the cell viability of both QGY7701 and HepG2 cells treated with 50 μmol/L DHM for 48 h and 25 μmol/L DHM for 24 h was significantly inhibited ($P < 0.001$). The microscopy suggested that the proliferation of the two cell lines was inhibited and partial apoptosis occurred compared with the control group (Figure 2E). A colony formation assay was utilized to further determine the proliferative ability of cells treated with various concentrations of DHM, and similarly, DHM suppressed colony-forming capability in a dose-dependent manner (Figure 2F-H).

DHM induces apoptosis in HCC cells

Flow cytometry revealed that apoptosis was induced in HepG2 and QGY7701 cell lines following 24 h of DHM treatment (Figure 3A-C). A TUNEL assay was utilized to detect late-stage apoptosis of the cell lines treated with various concentrations of DHM for 24 h. In the fluorescence images, there were cells in late-stage apoptosis in both the HepG2 and QGY7701 cell lines (Figure 3D-F).

Effects of DHM treatment on proteins in the Notch1 pathway and on apoptosis-related proteins in HCC cells

In this study, Notch1, Hes1 and apoptosis-related proteins were detected by Western blotting. The results demonstrated that DHM downregulated the expression of Notch1 and Hes1 in QGY7701 and HepG2 cells. Meanwhile, Bax was upregulated, and Bcl-2 was downregulated. The results suggested that down-regulation of Notch1 activated the mitochondrial apoptotic pathway (Figure 4A and C). It is known that a lower ratio of Bcl-2/Bax leads to apoptosis, and our data showed that the ratio of Bcl-2/Bax was reduced (Figure 4B and D).

DHM induces apoptosis in HCC cells by downregulating the Notch1 pathway

To further elucidate whether the inhibition of proliferation and the promotion of apoptosis of QGY7701 and HepG2 cells were caused by the down-regulation of Notch1 following DHM treatment, siRNA was used to silence Notch1 expression, and Notch1-silenced cells were treated with DHM. The knockout efficiency of the three different Notch1 siRNA sequences is shown in Figure 5A. The expression levels of Notch1, Hes1 and the apoptotic proteins Bcl-2 and Bax were analyzed by Western blot. Compared with the control group, Bax was upregulated, and Hes1 and Bcl-2 were downregulated after the depletion of Notch1 *via* siRNA. The results were consistent with the DHM 100 μmol/L treatment group. However, the effect of the Notch1-siRNA and DHM 100 μmol/L combination on protein expression was more obvious (Figure 5B and D). As expected, the ratio of Bcl2/Bax decreased the most in the Notch1-siRNA + DHM 100 μmol/L group (Figure 5C and E). In summary, DHM exerted anti-tumor activity by downregulating the expression of Notch1.

DISCUSSION

As a multifunctional protein, Notch1 plays a role in regulating cell differentiation, development, proliferation and survival in various contexts[15,16]. Abnormal expression of Notch1 often leads to tumors. For example,

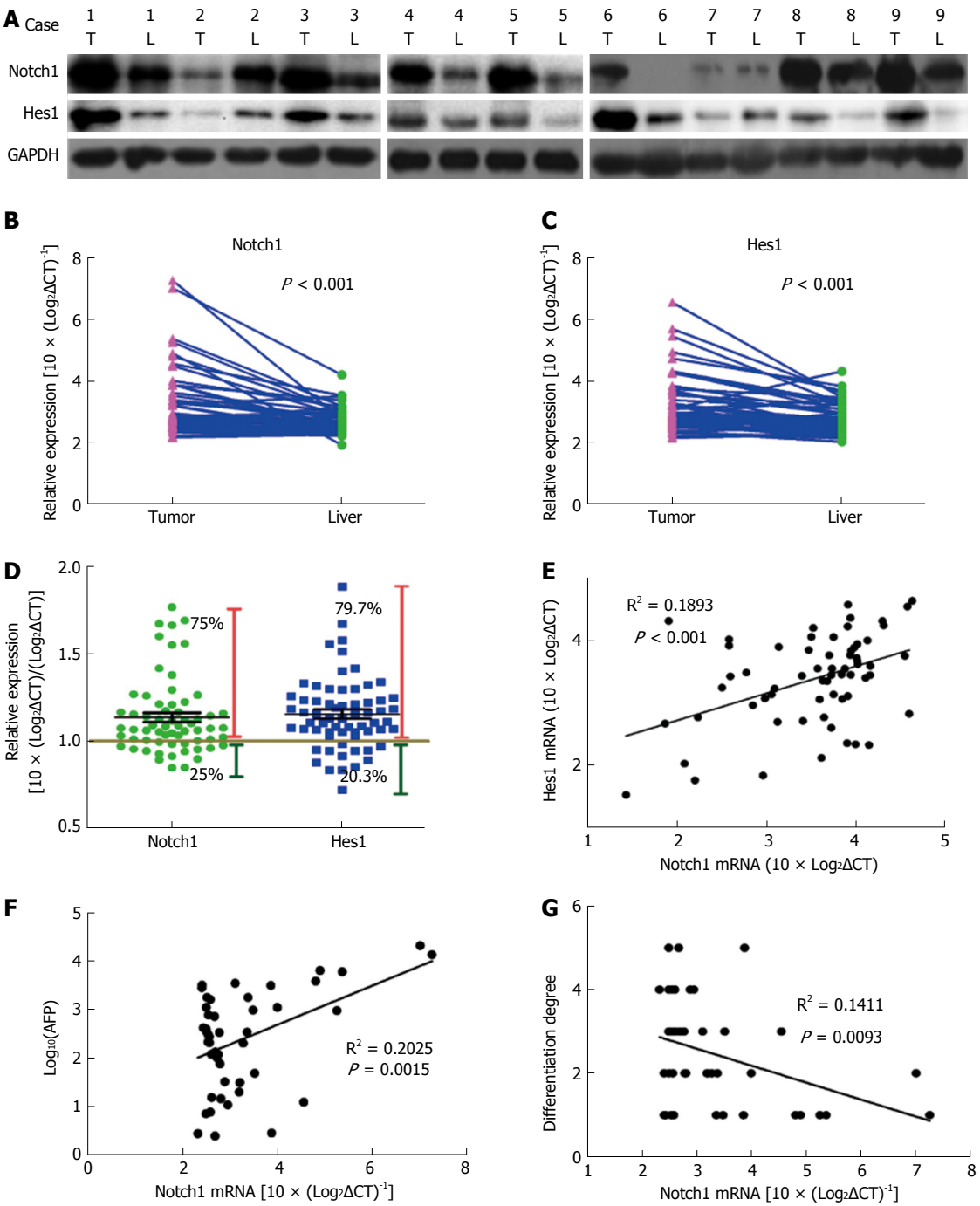


Figure 1 Notch1 expression is activated in hepatocellular carcinoma tissues and reflects differentiation properties. Protein levels were detected by Western blot analysis. A: Notch1 and Hes1 proteins were obviously abundant in most of the tumor tissues; B and C: Gene expression levels were measured with qRT-PCR. *Notch1* and *Hes1* genes were expressed at higher levels in tumor tissues compared with normal liver tissues; D: *Notch1* and *Hes1* gene expression levels were higher in 75% and 79.7% of the hepatocellular carcinoma patients, respectively; E: A Pearson correlation analysis revealed a correlation between Notch1 and Hes1 expression (r^2 = 0.1893, P < 0.001); E and G: Notch1 is positively correlated with the AFP level (r^2 = 0.2025, P = 0.0015) and negatively correlated with the degree of differentiation (r^2 = 0.1411, P = 0.0093).

in pancreatic cancer, colon cancer, non-small cell lung cancer, cervical cancer and other tumors, Notch1 expression is increased, and Notch1 participates in the promotion of cell proliferation, inhibition of apoptosis and cellular differentiation[22-24]. Overexpression of Hes1 has also been reported in the development of HCC[17]. Western blotting and qRT-PCR analysis of clinical HCC samples indicated that Notch1 and Hes1 were highly expressed in tumor samples, and the expression rates of Notch1 and Hes1 accounted for 75% and 79.7% of the total data, respectively. The above results show that Notch1-mediated activation of Notch signaling is a hallmark of HCC. This further explains the correlation among Notch1 expression, AFP (a clinical diagnostic marker of HCC) expression, and the degree of differentiation. The expression of Notch1

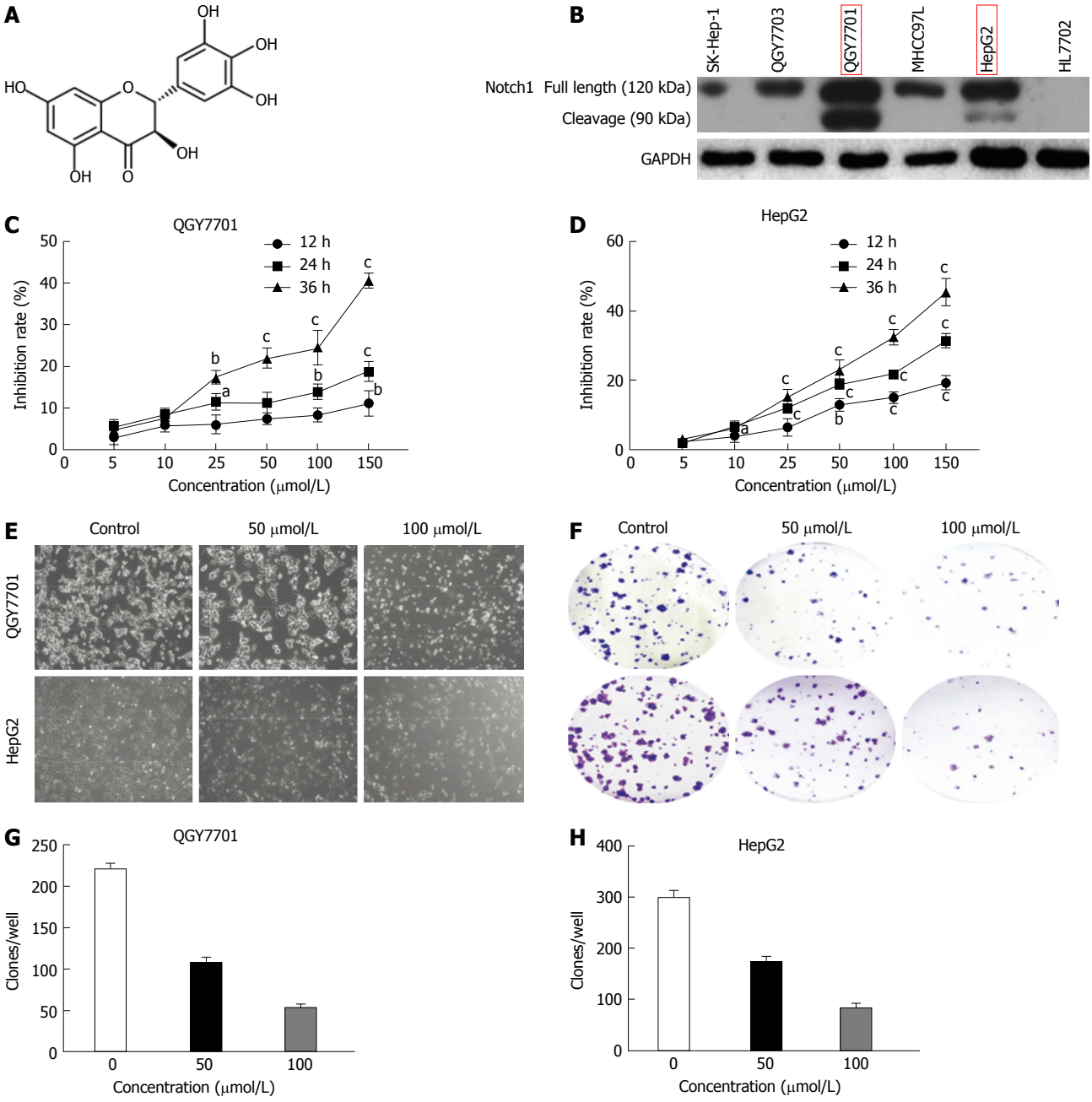


Figure 2 Dihydromyricetin inhibits the proliferation of QGY7701 and HepG2 hepatocellular carcinoma cells. A: Chemical structure of DHM; B: The Notch1 expression profiles of several hepatocellular carcinoma (HCC) cell lines were screened by Western blotting; C and D: MTT assays were used to analyze the rate of cell inhibition (means ± SD) of QGY7701 and HepG2 cells treated with dihydromyricetin (DHM) at different concentrations (5, 10, 25, 50, 100 and 150 μmol/L) at 12 h, 24 h and 36 h; E: The proliferation of QGY7701 and HepG2 cells treated with different concentrations of DHM (0, 50, or 100 μmol/L) for 24 h was observed under microscope (× 200 magnification); F: Colony formation capability assay with different concentrations of DHM in HCC QGY7701 and HepG2 cells; G and H: The clones were quantified, and the data are presented as a statistical figure; Each experiment was repeated at least three times. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$, *vs* 0 μmol/L (control) group.

was positively correlated with AFP expression and negatively correlated with the degree of differentiation. This indicated that Notch1 is highly expressed in tumor cells with higher degrees of malignancy and proliferative activity. Experienced clinicians frequently find that tumors with higher growth activity seem to be more susceptible to chemotherapeutic treatment. These facts suggest that Notch1 is involved in the development of HCC and Notch1 may be a potential diagnostic indicator of HCC and target for chemotherapeutic treatment.

The inhibition of cell proliferation and promotion of apoptosis are common targets by many chemotherapeutic agents[25]. DHM inhibits the proliferation of diverse types of cells, including osteosarcoma, melanoma, leukemia and gastric cancer cells[26-29]. Many molecules and signaling pathways are involved in the anti-tumor effect of DHM, including the Chk1/Chk2/Cdc25C pathway[29], the NF-κB and MAPK signaling pathways[7], and the transforming growth factor-β pathway[30]. Nevertheless, the effect of DHM

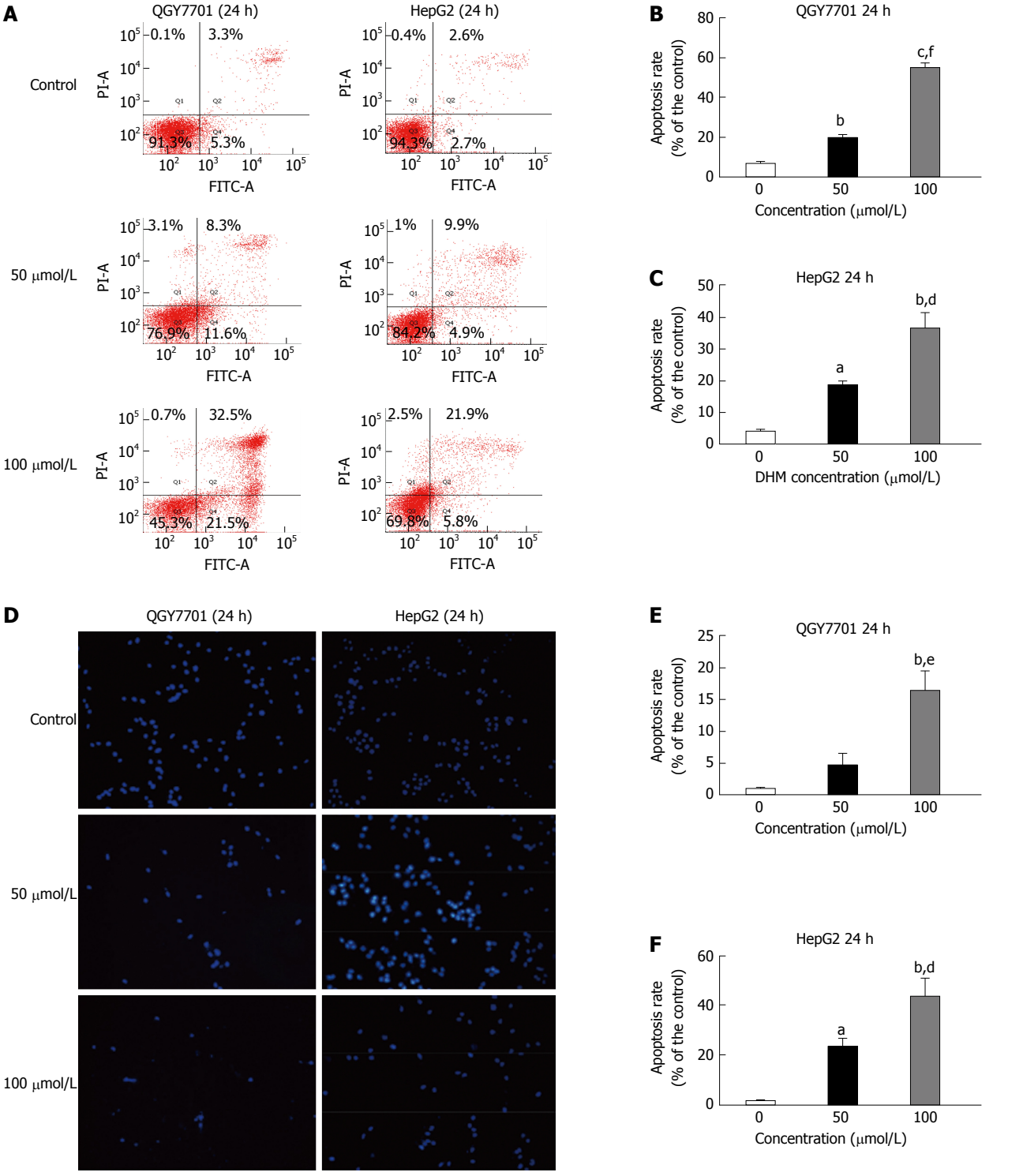


Figure 3 Dihydromyricetin induces apoptosis in QGY7701 and HepG2 hepatocellular carcinoma cells. A: Apoptosis of QGY7701 and HepG2 cells treated with DHM at different concentrations (0, 50, or 100 μmol/L) for 24 h was analyzed by flow cytometry; B and C: Apoptosis was quantified and presented as a statistical figure; D: A TUNEL assay was utilized to examine the apoptosis of QGY7701 and HepG2 cells treated with different concentrations (0, 50, or 100 μmol/L) of DHM for 24 h; E and F: Apoptosis was quantified and presented as a statistical figure; Each experiment was repeated more than three times; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* 0 μmol/L (control) group; $^{e}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* 50 μmol/L group.

on liver cancer and the specific underlying mechanism remain unclear. We found that DHM treatment of the QGY7701 and HepG2 hepatoma cell lines led to cell proliferation inhibition in a time- and dose-dependent manner. The colony-forming capability of QGY7701 and HepG2 cells treated with different concentrations of DHM was inhibited. Cell images demonstrated the inhibition of cell proliferation and induction of apoptosis in cells treated with high concentrations of DHM. Flow cytometry and TUNEL assays further verified the occurrence of significant apoptosis in QGY7701 and HepG2 cells treated with DHM. In view of the high

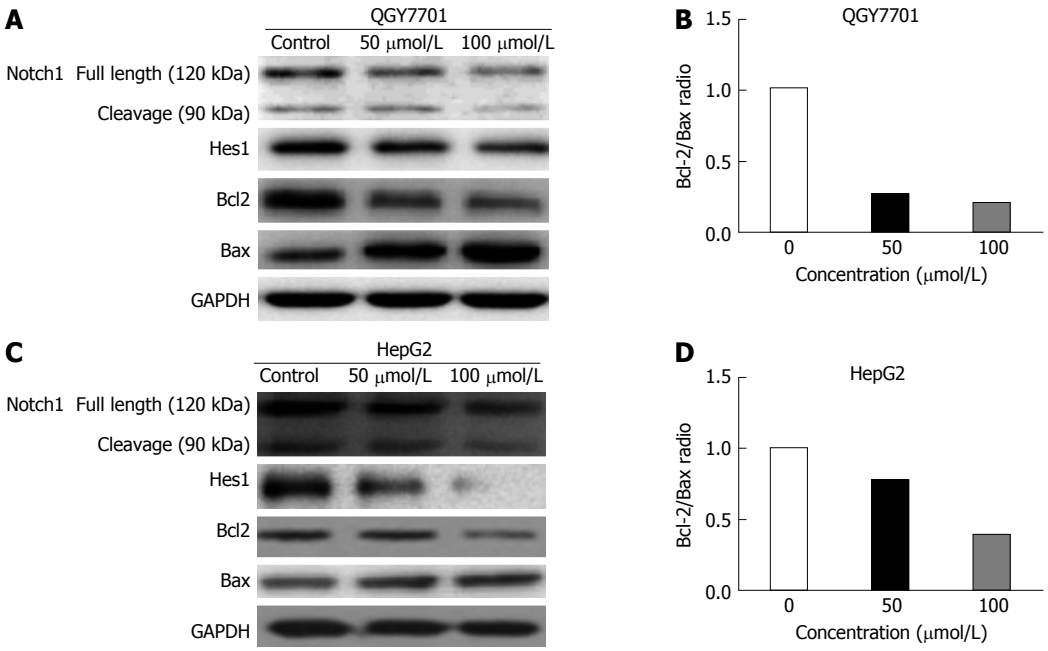


Figure 4 Effects of dihydromyricetin treatment on proteins in the Notch1 pathway and on apoptosis-related proteins in hepatocellular carcinoma cells. A and C: The levels of the Notch1, Hes1, Bcl-2, and Bax proteins in QGY7701 and HepG2 cells treated with DHM at different concentrations (0, 50, or 100 μmol/L) were analyzed by western blot; B and D: The Bcl-2/Bax protein ratios were measured by optical density analysis with ImageJ software.

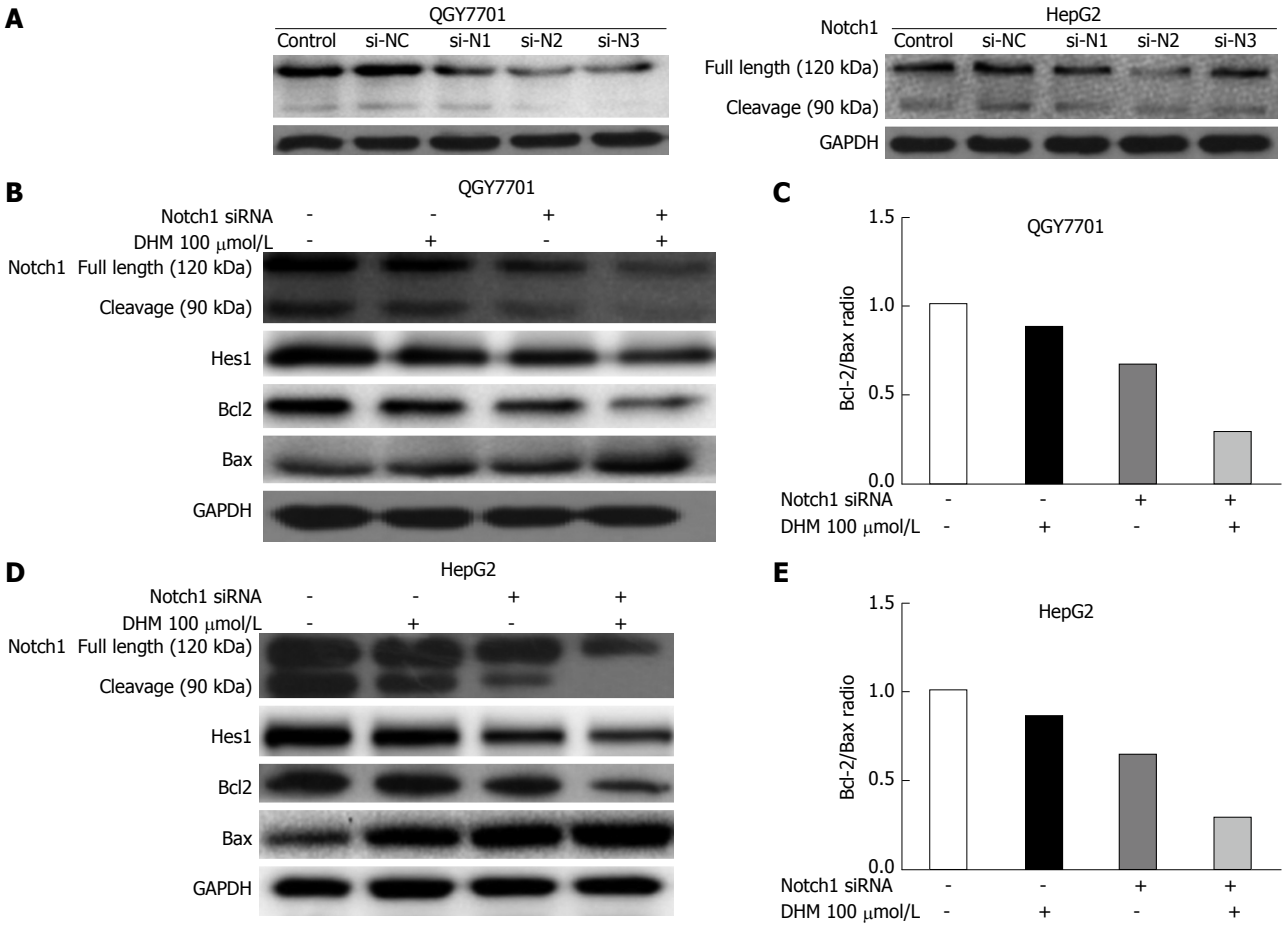


Figure 5 Dihydromyricetin induced apoptosis in hepatocarcinoma cells by downregulating the Notch1 pathway. A: Knockdown efficiency of 3 different Notch1 siRNA sequences was tested in both QGY7701 and HepG2 cell lines by Western blot; B and D: The effects of different treatment methods (Notch1-siRNA, dihydromyricetin 100 μmol/L, and combined treatment) on Notch1, Hes1, Bcl-2, and Bax protein levels as determined by Western blot; C and E: The Bcl-2/Bax protein ratios were measured by optical density analysis with ImageJ software. si-NC: si-negative control; si-N1: si-Notch1-1; si-N2: si-Notch1-2; si-N3: si-Notch1-3.

expression of Notch1 in HCC samples, we examined the effect of various concentrations of DHM on the expression of Notch1, and found that Notch1 and Hes1 were downregulated. Bcl-2 and Bax proteins play major roles in the regulation of mitochondria-mediated apoptosis, which is a common apoptosis pathway. The Bcl-2 (pro-apoptotic protein) and Bax (anti-apoptotic protein) ratio can further reflect the anti-apoptotic tendencies. The ratio of anti-apoptotic to pro-apoptotic molecules, such as the Bcl-2/Bax ratio, indicates the threshold sensitivity of cells to the induction of apoptosis *via* the intrinsic (mitochondria-mediated) pathway[31,32]. The data showed that Bcl-2 and Bax were downregulated in DHM-treated QGY7701 and HepG2 cells, and the Bcl-2/Bax ratio was also decreased. Additionally, we knocked out the expression of Notch1 and treated these Notch1-deficient cells with DHM. The results showed that the effects of Notch1 silencing and DHM treatment were not different. However, when used in combination, Bcl-2 was downregulated, Bax was upregulated, and the reduction in the ratio of Bcl-2/Bax was more significant. These results show that the anti-tumor capacity of DHM in the QGY7701 and HepG2 hepatocarcinoma cell lines is partially exerted through inhibition of the Notch1 pathway.

In summary, our experiments confirmed that Notch1 is involved in the development of HCC and may serve as a potential diagnostic marker for HCC and an indicator of malignancy. DHM inhibits cell proliferation and promotes apoptosis by down-regulating the expression of Notch1, indicating that it can be used as a candidate drug for the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a highly malignant type of solid tumor which is a serious threat to human health. It is urgent to develop novel chemotherapeutic drugs and therapeutic targets for the treatment of HCC.

Research frontiers

Notch-1 was detected to be overexpressed in HCC; Notch1 is involved in the development and progression of HCC. Dihydromyricetin (DHM) has numerous pharmacological activities, such as antiinflammatory, antioxidation and anticarcinogenic effects. However, whether DHM can induce hepatoma cell apoptosis by Notch1 is not yet known.

Innovations and breakthroughs

This is the first study to demonstrate that DHM inhibits cell proliferation and promotes apoptosis by down-regulating the expression of Notch1.

Applications

DHM may be used a potential effective candidate agent for the treatment of HCC.

Peer-review

This article is well written. The authors investigate whether DHM inhibits cell proliferation and promotes apoptosis by downregulating Notch1 expression. These results show that the anti-tumor activity of DHM in the QGY7701 and HepG2 HCC cell lines is partially exerted through inhibition of the Notch1 pathway.

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ORIGINAL ARTICLE

Basic Study

Curcumin inhibits hepatitis B virus infection by down-regulating cccDNA-bound histone acetylation

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Author contributions: Wei ZQ, Zhang YH and Ke CZ performed the experiments; Meng ZJ and Luo J designed and coordinated the research; Cheng HX and Ma DQ analyzed the data; Wei ZQ and Meng ZJ wrote the paper; Ren P and Hu P reviewed and edited the manuscript; all authors read and approved the manuscript.

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Abstract

AIM

To investigate the potential effect of curcumin on

hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and the underlying mechanism.

METHODS

A HepG2.2.15 cell line stably transfected with HBV was treated with curcumin, and HBV surface antigen (HBsAg) and e antigen (HBeAg) expression levels were assessed by ELISA. Intracellular HBV DNA replication intermediates and cccDNA were detected by Southern blot and real-time PCR, respectively. The acetylation levels of histones H3 and H4 were measured by Western blot. H3/H4-bound cccDNA was detected by chromatin immunoprecipitation (ChIP) assays. The deacetylase inhibitors trichostatin A and sodium butyrate were used to study the mechanism of action for curcumin. Additionally, short interfering RNAs (siRNAs) targeting HBV were tested along with curcumin.

RESULTS

Curcumin treatment led to time- and dose-dependent reductions in HBsAg and HBeAg expression and significant reductions in intracellular HBV DNA replication intermediates and HBV cccDNA. After treatment with 20 μmol/L curcumin for 2 d, HBsAg and cccDNA levels in HepG2.2.15 cells were reduced by up to 57.7% ($P < 0.01$) and 75.5% ($P < 0.01$), respectively, compared with levels in non-treated cells. Meanwhile, time- and dose-dependent reductions in the histone H3 acetylation levels were also detected upon treatment with curcumin, accompanied by reductions in H3- and H4-bound cccDNA. Furthermore, the deacetylase inhibitors trichostatin A and sodium butyrate could block the effects of curcumin. Additionally, transfection of siRNAs targeting HBV enhanced the inhibitory effects of curcumin.

CONCLUSION

Curcumin inhibits HBV gene replication *via* down-regulation of cccDNA-bound histone acetylation and has the potential to be developed as a cccDNA-targeting antiviral agent for hepatitis B.

Key words: Curcumin; Hepatitis B virus; Covalently closed circular DNA; Histone deacetylation

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Core tip: We showed that curcumin inhibited hepatitis B virus (HBV) replication and expression *via* reductions in covalently closed circular DNA-bound histone acetylation. Furthermore, siRNAs targeting HBV acted synergistically with curcumin, resulting in enhanced inhibition of HBV.

Wei ZQ, Zhang YH, Ke CZ, Chen HX, Ren P, He YL, Hu P, Ma DQ, Luo J, Meng ZJ. Curcumin inhibits hepatitis B virus infection by down-regulating cccDNA-bound histone acetylation. *World J Gastroenterol* 2017; 23(34): 6252-6260 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6252.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6252

INTRODUCTION

Hepatitis B virus (HBV) is a species of the genus *Orthohepadnavirus*. HBV infection leads to severe diseases, including hepatitis, liver cirrhosis and hepatocellular carcinoma[1]. HBV has also been suggested to be involved in the development of pancreatic cancer[2]. Approximately 350 million individuals are infected with HBV, and more than 0.6 million people with HBV infection die every year as a result of end-stage liver disease and hepatocellular carcinoma worldwide[1,3]. The HBV genome is made of partially double-stranded relaxed circular DNA (rcDNA), 3020-3320 bp in size[4]. After the virus infects hepatocytes, rcDNA is released into the nucleus and converted to covalently closed circular DNA (cccDNA), which, along with histone binding, constitutes a minichromosome. The mean copies of cccDNA per HBV-infected hepatocyte range from 5 to 50[5,6]. Moreover, cccDNA serves as the critical template for viral replication and mRNA synthesis, which is the source of persistent and recurrent HBV infection. Nucleos(t)ide analogues (NAs) can efficiently inhibit HBV replication; however, they do not promote clearance of residual cccDNA[7].

Several treatment strategies are available to target cccDNA by inhibiting cccDNA minichromosome formation or inducing cccDNA degradation. Disubstituted sulfonamide (DSS) compounds have been reported to be specific inhibitors that prevent rcDNA conversion into cccDNA; however, DSS cannot promote cccDNA decay[8]. Lucifora *et al*[9] showed that inducing apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3A (APOBEC3A) and 3B (APOBEC3B) cytidine deaminase by interferon-α and lymphotoxin-β receptor activation induces cccDNA degradation *via* cytidine deamination and apurinic/apyrimidinic site formation. However, the absence of specificity of these cytidine deaminases results in genomic damage and cell-cycle arrest[10]. Recently, with the aid of DNA-cleaving enzymes, including zinc-finger nucleases (ZFN), TAL effector nucleases (TALENs), and CRISPR-associated system 9 (Cas9) proteins, specific targeting of HBV cccDNA was shown to cleave cccDNA[11-15]. Nevertheless, chronic expression of enzymes leads to off-target cleavage at homologous sequences in the human genome and represents a major limitation.

Furthermore, cccDNA-bound acetylated histones can modulate HBV replication and expression[16,17]. Hepatitis B virus X (HBx) protein can be recruited onto a cccDNA minichromosome to accelerate acetylation. Using a cccDNA chromatin immunoprecipitation (ChIP)-Seq assay, Tropberger *et al*[18] reported that low levels of histone posttranslational modifications (PTMs)

were associated with transcriptional repression and promoter silencing.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] was isolated from the rhizome of *Curcuma longa* L. (Zingiberaceae family), which exhibits antimicrobial activities against various bacteria, viruses, fungi, and parasites[19-23]. Curcumin can inhibit HBV *via* down-regulation of the gluconeogenesis gene coactivator PGC-1α[24] or transactivation of transcription and increased stability of p53[25].

Based on findings that curcumin can inhibit p300 histone acetyltransferase activity[26,27], we hypothesized that deacetylation of cccDNA-bound histones may contribute to the inhibitory activities of curcumin on HBV. Therefore, the effects of curcumin on cccDNA-bound histones and on steady-state levels of HBV cccDNA were investigated in detail in the present study.

MATERIALS AND METHODS

Cell culture and transfection

HepG2.2.15 cells (an HBV stably transfected human hepatocarcinoma cell line) were maintained in DMEM medium (Gibco, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Gibco), 1% GlutaMAX-I (Gibco) and 1% MEM Non-Essential Amino Acids Solution (Gibco). Transfection of siRNAs into HepG2.2.15 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States). The sequences were 5′-GAAUCCUCACAAUACCGCAtt and 5′-UGAGAGUCCAAGAGUCCUCtt for HBx-siRNA and 5′-GAAUCCUCACAAUACCGCAtt and 5′-UGCGGUAUU GUGAGGAUUCtt for hepatitis B virus S antigen (HBs)-siRNA[28].

Curcumin and deacetylase inhibitor treatments

Cells were seeded at approximately 60%-80% confluence 24 h prior to treatment with different concentrations of curcumin (Sigma, St. Louis, MO, United States) dissolved in dimethyl sulphoxide (DMSO). For histone acetylation blocking assays, cells were co-treated with 20 μmol/L curcumin and either 5 mmol/L sodium butyrate (Sigma) or 1 μmol/L trichostatin A (TSA, Sigma).

Nucleoprotein extraction and Western blot analysis

Nucleoproteins were extracted using a Nucleoprotein Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. For Western blot analysis, proteins were subjected to SDS-polyacrylamide gel electrophoresis on 12.5% gels and were then electrophoretically transferred to nitrocellulose membranes (Millipore, Billerica, MA, United States). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS; pH 7.5) with 0.05% Tween 20 for 2 h at RT and were then probed with rabbit polyclonal anti-acetyl-histone H3 (Abnova, diluted 1:1000) overnight at 4 ℃. Mouse monoclonal anti-histone H3 (Beyotime Biotechnology, diluted 1:1000) served as an internal control protein. Horseradish peroxidase-conjugated goat anti-mouse antibody (Biosharp, 1:5000) was used as a secondary antibody. Protein brands were visualized by enhanced chemiluminescence (ECL) using an ECL kit (Millipore).

Extraction and quantification of HBV cccDNA

HepG2.2.15 cells were lysed in 800 μL of lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 150 mmol/L NaCl and 1% SDS] and incubated for 30 min at 4 ℃. Cell lysates were adjusted to 0.5 mol/L KCl and centrifuged for 1 min at 10000 *g* to precipitate protein-bound DNA. Supernatants were digested with 0.5 mg/mL proteinase K for 2 h at 55 ℃. The cccDNA was purified by phenol/chloroform (1:1) extraction and isopropanol precipitation in the presence of 15 μg of tRNA and 200 mM NaAc (pH 5.2).

Purified DNA was digested with Plasmid-Safe ATP-Dependent DNase (Epicenter, Madison, WI, United States) to degrade contaminating HBV inserted in cellular genomic DNA and OC (open circular) species and was then subjected to PCR amplification to select HBV cccDNA forms, as previously described[15]. The cccDNA was later subjected to real-time-PCR using SYBR Green Real-time PCR Master Mix (Roche, Mannheim, Germany) and cccDNA-specific primers: 5′-TGCACTTCGCTTCACCT (forward) and 5′-AGGGGC ATTTGGTGGTC (reverse). PCR was performed using an Applied Biosystems StepOne Real-Time PCR System. cccDNA copy numbers were quantified according to a standard curve generated from an HBV plasmid in a concentration range of 10^2-10^8 copies.

Extraction and quantification of HBV mRNA

Total RNA was extracted from HepG2.2.15 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions and was then subjected to real-time-PCR using primers for cccDNA quantification; β-actin mRNA transcript levels were used to normalize the expression of each RNA sample.

cccDNA acetyl-histone H3 and acetyl-histone H4 ChIP assays

cccDNA ChIP assays were performed using EpiQuik Acetyl-Histone H3 ChIP and EpiQuik Acetyl-Histone H4 ChIP kits (EpiGentek, Farmingdale, NY, United States). Briefly, cells were collected and *in vivo* cross-linked in fresh culture medium containing 1% formaldehyde for 10 min at RT and were then lysed in 200 μL CP3A for 10 min at RT to isolate nuclear pellets. Chromatin solutions were sonicated for 4 pulses of 12 s each at level 5 using a Branson Microtip probe, followed by a 40-s rest on ice between each pulse to generate 200- to 1000-bp DNA fragments. Supernatants were diluted with CP4 at a 1:1 ratio, and 5 μL was removed as "input DNA". Chromatin was then subjected to

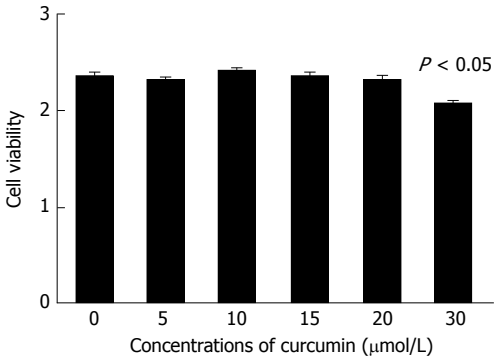


Figure 1 Cell cytotoxicity of curcumin. HepG2.2.15 cells were treated with 0, 5, 10, 15, 20 or 30 μmol/L curcumin for 2 d and then subjected to CCK-8 assay to detect toxic effect. The experiment was performed in duplicate and repeated at least three times.

immunoprecipitation for 1 h at RT using strip wells pre-coated with antibodies specific to acetyl-histone H3, acetyl-histone H4 or normal mouse IgG. After six washes with CP1, immunoprecipitated chromatins and input DNA coated on the strip wells were digested with proteinase K and then purified using collection tubes. Purified DNA was subjected to Plasmid-Safe ATP-Dependent DNase digestion and real-time PCR amplification, as described above.

Quantification of HBV antigens

Culture supernatants of HepG2.2.15 cells were harvested and analysed for HBV surface antigen (HBsAg) and e antigen (HBeAg) levels using a Thermo Scientific Multiskan FC Microplate Photometer and ELISA kits (InTec, Xiamen, China).

Purification of HBV DNA from intracellular core particles and Southern blot analysis

HepG2.2.15 cells were washed twice with ice-cold PBS and lysed in 800 μL of lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA and 1% NP-40]. Cell lysates were centrifuged for 1 min at 10000 *g* to precipitate cell nuclei. Cellular genomic DNA and cccDNA were removed by the addition of 10 mM $MgCl_2$ and 100 μg/mL DNase I, and the mixture was incubated for 30 min at 37 ℃. Digestion was stopped by the addition of 25 mmol/L EDTA (pH 8.0). Proteins were digested with 0.5 mg/mL proteinase K and 1% SDS for 2 h at 55 ℃. HBV DNA from intracellular core particles was purified by phenol/chloroform (1:1) extraction and isopropanol precipitation in the presence of 15 μg of tRNA and 200 mmol/L NaAc (pH 5.2)[28,29].

For Southern blot analysis, HBV DNA was subjected to agarose gel electrophoresis, followed by denaturation and transfer to nylon membranes using a Model 785 Vacuum Blotter (Bio-Rad, Hercules, CA, United States). DNA was fixed to membranes using an Ultraviolet Crosslinker (UVP, Upland, CA, United States). DNA hybridization and detection were performed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche).

Statistical analysis

Statistical analyses were performed using unpaired *t*-tests. The results were evaluated with GraphPad Prism 5 and are expressed as the mean ± SD. *P* values < 0.05 (a) or < 0.01 (b) were set as the level of significance.

RESULTS

Curcumin inhibits HBV replication and expression

To confirm whether curcumin directly inhibits HBV expression, HepG2.2.15 cells were treated with various concentrations of curcumin for three consecutive days. Cell cytotoxicity was assessed using the CCK-8 assay, which revealed that there was no detectable toxic effect when cells were treated with less than 20 μmol/L curcumin (Figure 1). Cell culture supernatants from each day were collected and analysed for levels of HBsAg and HBeAg. Curcumin decreased HBsAg levels both dose- and time-dependently; HBsAg levels were reduced by up to 57.7%, 2 d after treatment with 20 μmol/L curcumin (Figure 2A). HBeAg was not reduced by 20 μmol/L of curcumin (Figure 2B). HBV cccDNA and HBV DNA from intracellular core particles were assayed 2 d after treatment with 20 μmol/L curcumin. RT-PCR and Southern blot experiments revealed strong reductions in cccDNA (75.5%; Figure 2C), mRNA (24.4%; Figure 2D) and HBV replication intermediates (Figure 2E).

Curcumin mediates the deacetylation of chromosomal and cccDNA-bound histones

To investigate the effects of curcumin on chromosomal histone acetylation, equivalent numbers of HepG2.2.15 cells were treated with various concentrations of curcumin for 2 d or with 20 μmol/L curcumin for different durations. As shown in Figure 3A and B, curcumin decreased histone acetylation levels both dose- and time-dependently. We found that 5 μmol/L curcumin was sufficient to decrease histone acetylation. When cells were treated with 20 μmol/L curcumin, acetylated histone H3 was reduced 30 min after treatment. Using a cccDNA ChIP assay, we found that the acetylation levels of cccDNA-bound histone H3 and histone H4 were significantly reduced when cells were treated with 20 μmol/L curcumin (Figure 3C). This finding is similar to our observed reduction of chromosomal histone acetylation.

Histone deacetylase inhibitors block the inhibitory effect of curcumin

The histone deacetylase inhibitors sodium butyrate and TSA were used to test whether curcumin inhibits HBV by decreasing histone acetylation. HepG2.2.15 cells were treated with 20 μmol/L curcumin for 2 d, which resulted in significant reductions in the

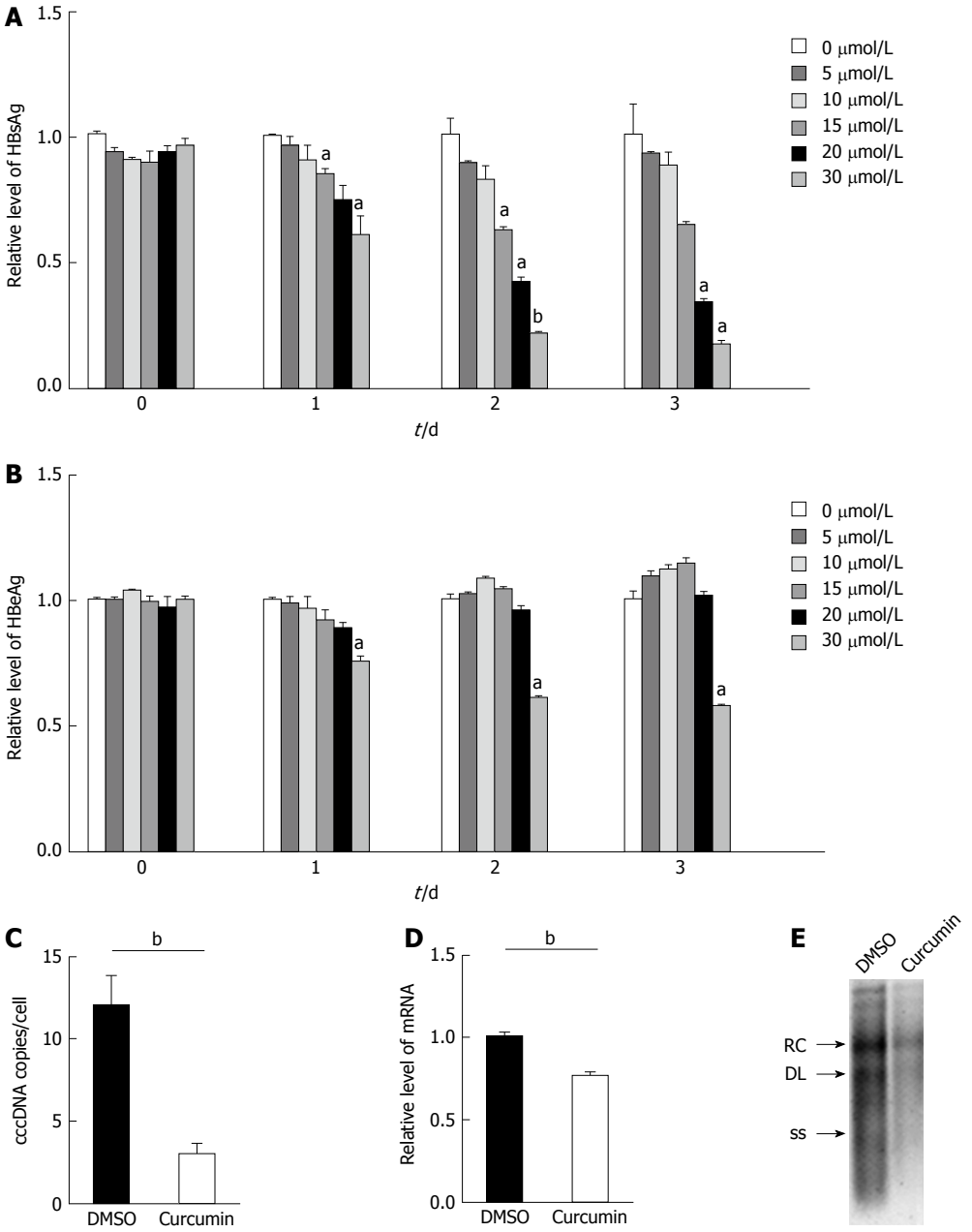


Figure 2 Curcumin inhibits hepatitis B virus replication and expression. A: HepG2.215 cells were treated with 0, 5, 10, 15, 20 or 30 μmol/L curcumin for three consecutive days. Culture medium from each day was collected and analysed for levels of HBsAg; B: Culture medium from each day was collected and analysed for levels of HBeAg; C: Hepatitis B virus (HBV) cccDNA accumulation in HepG2.215 cells treated with 20 μmol/L curcumin or DMSO for 2 d. HBV cccDNA was digested with Plasmid-Safe ATP-Dependent DNase to degrade contaminating HBV that had inserted in cellular genomic DNA and OC species and was then subjected to PCR amplification to amplify HBV cccDNA forms. Results are expressed as numbers of cccDNA copies per cell; D: Total RNA was extracted from HepG2.215 cells treated with 20 μmol/L curcumin or DMSO for 2 d and was subjected to real-time PCR to quantify HBV mRNA transcript levels; E: HBV DNA was extracted from intracellular core particles in HepG2.215 cells treated with 20 μmol/L curcumin or DMSO for 2 d. Southern blot analysis of HBV DNA replicative intermediates. RC, DL and SS represent relaxed circular, double linear and single-stranded forms of HBV DNA, respectively. All experiments were repeated at least three times; ELISA and RT-PCR were performed in duplicate. $^{a}P < 0.05$; $^{b}P < 0.01$. cccDNA: Covalently closed circular DNA; DMSO: Dimethyl sulphoxide.

levels of HBsAg (Figure 4A) and cccDNA (Figure 4B). HBsAg and cccDNA levels increased by 31.3% and 2.4-fold, respectively, when cells were co-treated with curcumin and sodium butyrate compared with cells treated with curcumin alone. The histone deacetylase inhibitor TSA also partially blocked the inhibitory effect of curcumin on HBV, although the effect was much weaker than that of sodium butyrate. These findings suggest that curcumin inhibits HBV by inducing histone deacetylation.

siRNAs against HBV enhance the inhibitory effects of curcumin

Because HBx also regulates epigenetic modifications of cccDNA-bound histones[16], HBx-siRNA and HBs-siRNA were used to enhance the inhibitory effects of curcumin. HepG2.2.15 cells were transfected with 20 nmol/L siRNAs and treated with 20 μmol/L curcumin

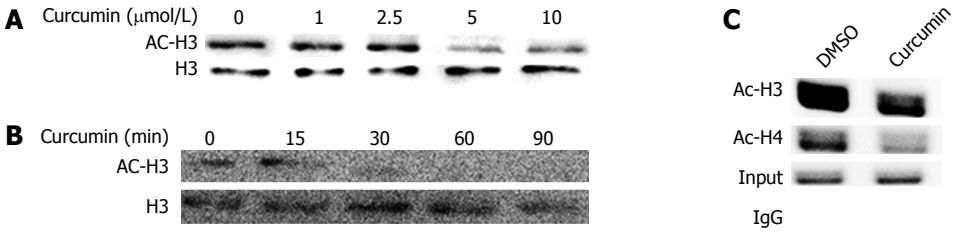


Figure 3 Curcumin mediates chromosomal and covalently closed circular DNA-bound histone deacetylation. A: HepG2.215 cells were treated with 0, 1, 2.5, 5 or 10 μmol/L curcumin and incubated at 37 ℃ for 2 d. The acetylation status of cellular H3 histones was analysed by Western blot; B: HepG2.215 cells were treated with 20 μmol/L curcumin for the indicated periods of time; C: ChIP on HepG2.215 cells treated with 20 μmol/L curcumin or DMSO for 2 d was performed using specific antibodies to acetyl-histone H3 (AcH3), acetyl-histone H4 (AcH4) or control IgG. Immunoprecipitated chromatins were digested with Plasmid-Safe ATP-Dependent DNase to degrade contaminating HBV that had inserted in cellular genomic DNA and OC species and then were subjected to PCR amplification to select HBV cccDNA forms. All experiments were repeated at least three times. cccDNA: Covalently closed circular DNA; HBV: Hepatitis B virus; DMSO: Dimethyl sulphoxide.

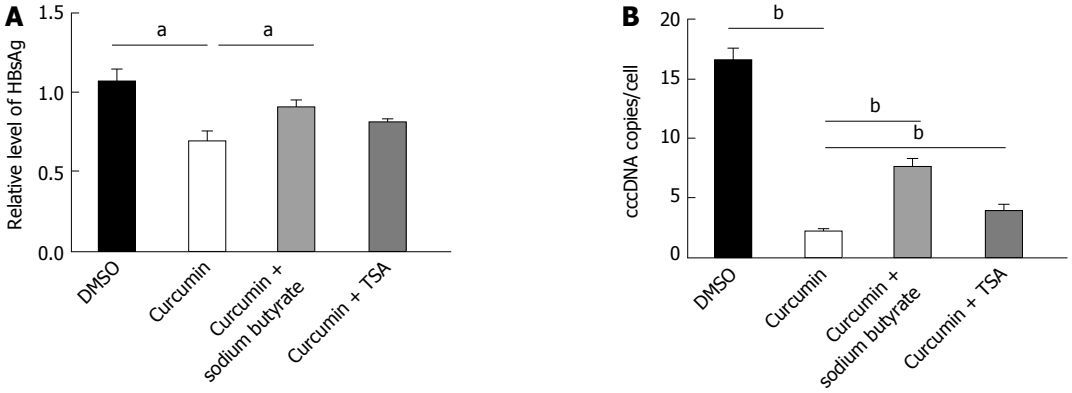


Figure 4 Histone deacetylase inhibitors block the inhibitory effect of curcumin. A: HepG2.215 cells were treated with 20 μmol/L curcumin alone or with either 5 mmol/L sodium butyrate or 1 μmol/L TSA for 2 d. Medium was collected and analysed for levels of HBsAg; B: HBV cccDNA was extracted and subjected to real-time qPCR. All experiments were performed in duplicate and repeated at least three times. $^{a}P < 0.05$; $^{b}P < 0.01$. cccDNA: Covalently closed circular DNA; DMSO: Dimethyl sulphoxide; TSA: Trichostatin A; HBV: Hepatitis B virus; HBsAg: HBV surface antigen.

for 2 subsequent days. Transfection with siRNAs alone significantly reduced HBsAg expression to a level below the limit of detection (Figure 5A). Moreover, a further reduction was observed in HBV DNA levels from intracellular core particles extracted from cells that received combined treatment with siRNAs and curcumin compared with cells treated with curcumin alone (Figure 5B).

DISCUSSION

The present study showed that curcumin not only inhibited intracellular HBV replication and HBsAg and HBeAg expression but also exhibited potent inhibitory effects on HBV cccDNA. Moreover, curcumin also reduced levels of both chromosomal and cccDNA-bound histones H3/H4, and addition of the histone deacetylase inhibitors sodium butyrate and TSA blocked the inhibitory effects of curcumin on HBV. These findings suggest that curcumin may induce the deacetylation of cccDNA-bound histones H3/H4, disrupt the steady state of HBV cccDNA and lead to the potent inhibition of HBV mRNA transcription and protein expression, along with a reduction in DNA replication. Furthermore, enhanced inhibition of intracellular HBV replication was revealed when curcumin was combined with siRNAs against HBV. Since HBx is pivotal for the steady state of cccDNA by regulating epigenetic modifiers of cccDNA-bound histones[16,30], the combination of curcumin with siRNAs, especially those targeting HBx, may lead to a synergistic effect in modulating the cccDNA steady state.

Several host-related factors, including histone proteins, regulate transcription and translation processes of cccDNA minichromosomes. Acetylation status changes of cccDNA-bound histones can regulate cccDNA transcription[16]. Previous studies have indicated that acetyltransferase inhibitors and deacetylase activators inhibit cccDNA transcription[16,31]. Curcumin has been reported to be an inhibitor of histone acetyltransferase (HAT), which specifically represses the activity of the p300/CREB-binding protein (CBP) HAT[26,27]. In the present study, curcumin mediated reductions in the levels of chromosomal and cccDNA-bound histone acetylation, which might result from the induction of histone deacetylation, because the deacetylase inhibitors TSA and sodium butyrate could block the inhibitory effects of curcumin on HBV.

HBV cccDNA is the primary template that allows for HBV gene expression and viral replication, and the steady state of cccDNA minichromosomes in the nuclei of hepatocytes contributes to persistent infection by

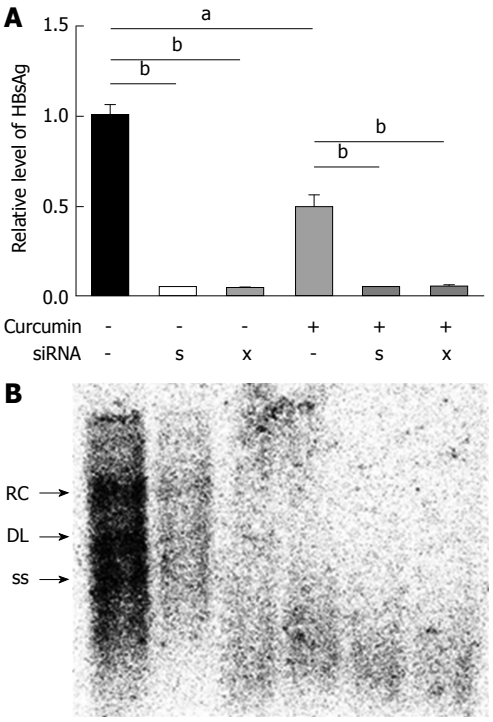


Figure 5 siRNAs against hepatitis B virus enhance the inhibitory effects of curcumin. HepG2.215 cells were transfected with 20 nmol/L siRNAs or negative control (HC) siRNA and were treated with 20 μmol/L curcumin or dimethyl sulphoxide for the next 2 d. HBsAg in cell culture supernatants and intracellular HBV replicative intermediates were detected by ELISA (A) and Southern blot analysis (B), respectively. All experiments were repeated at least three times; ELISA was performed in duplicate. $^{a}P < 0.05$; $^{b}P < 0.01$. HBV: Hepatitis B virus; HBsAg: HBV surface antigen.

HBV[32]. While there are no therapeutics currently available that target cccDNA, targeted therapeutics are attractive because the elimination of cccDNA results in the genomic cure of HBV infection[5]. Although genomic editing techniques, such as the CRISPR/CAS9 system, have shown promise in their capacity to edit HBV cccDNA, translating this finding into the clinic will be a challenge, mostly because of the need for the development of safe vectors for gene therapies.

Curcumin has been widely used as a dietary supplement for food colouring and flavouring, suggesting that it is safe for humans. However, poor bioavailability represents the biggest challenge for the clinical application of curcumin[33]. Poor absorption and rapid metabolism of curcumin in the body lead to low levels in the plasma and tissue. Fortunately, various efforts have been undertaken to promote its bioavailability. Removing the β-diketone moiety prevents curcumin from retro-aldol decomposition at physiological pH values[34]. Curcumin conjugated to two folic acid molecules (curcumin-2FA) increases water solubility and exhibits more efficient targeting of cancer cells that overexpress folic acid receptors[35]. Additionally, many other curcumin analogues can improve its bioavailability[36-40]. Moreover, curcumin-modified silver nanoparticles exhibited more efficient inhibition of respiratory syncytial virus[21]. Improving the bioavailability of curcumin will extend its clinical applications, especially in the treatment of HBV infection. Nevertheless, the long-term effects of curcumin on HBV cccDNA and its role in the elimination of cccDNA, along with combined effects of curcumin with other therapeutics, should be investigated in detail.

Taken together, the present study demonstrates that curcumin inhibits HBV by reducing cccDNA-bound histone acetylation and could potentially be developed as a cccDNA-targeting therapeutic for anti-HBV therapy.

COMMENTS

Background

Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) is the critical template for viral replication and mRNA synthesis. The elimination of cccDNA is indicative of complete cure of hepatitis B. Nucleos(t)ide analogues (NAs) can efficiently inhibit HBV replication but do not result in clearance of residual cccDNA. Curcumin can inhibit HBV; however, its mechanism is unclear. The potential role of curcumin in the regulation of the steady state of cccDNA is thus of interest.

Research frontiers

The steady state of cccDNA minichromosomes can be regulated through deacetylation of cccDNA-bound histones. Hepatitis B virus X (HBx) protein is recruited onto cccDNA minichromosomes and plays a pivotal role in the steady state of cccDNA by regulating epigenetic modifiers of cccDNA-bound histones.

Innovations and breakthroughs

This is the first study indicating that curcumin inhibits HBV gene replication by reducing cccDNA-bound histone acetylation.

Applications

Curcumin has the potential to be developed as a cccDNA-targeting antiviral agent for hepatitis B.

Terminology

HBV covalently closed circular DNA (cccDNA): After the HBV infects hepatocytes, virus genome is released into the nucleus and converted to cccDNA, which, along with histone binding, constitutes a minichromosome and serves as the critical template for viral replication and mRNA synthesis.

Peer-review

The authors have evaluated the effects of curcumin on HBV DNA replication and gene expression in the HepG2.2.15 cell line. Interestingly, curcumin showed robust inhibitory effects on HBV cccDNA. In addition, the authors have described the mechanism underlying the effect of curcumin on HBV cccDNA, which might be through histone deacetylation or reducing histone acetylation.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Systemic immune-inflammation index for predicting prognosis of colorectal cancer

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Abstract

AIM

To investigate the clinical significance of preoperative systemic immune-inflammation index (SII) in patients with colorectal cancer (CRC).

METHODS

A retrospective analysis of 1383 cases with CRC was performed following radical surgery. SII was calculated with the formula SII = (P × N)/L, where P, N, and L refer to peripheral platelet, neutrophil, and lymphocyte counts, respectively. The clinicopathological features

and follow-up data were evaluated to compare SII with other systemic inflammation-based prognostic indices such as the neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) in patients with CRC.

RESULTS

The optimal cut-off point for SII was defined as 340. The overall survival (OS) and disease-free survival (DFS) were better in patients with low NLR, PLR, and SII ($P < 0.05$). The SII was an independent predictor of OS and DFS in multivariate analysis. The area under the receiver-operating characteristics (ROC) curve for SII (0.707) was larger than those for NLR (0.602) and PLR (0.566). In contrast to NLR and PLR, SII could effectively discriminate between the TNM subgroups.

CONCLUSION

SII is a more powerful tool for predicting survival outcome in patients with CRC. It might assist the identification of high-risk patients among patients with the same TNM stage.

Key words: Colorectal cancer; Systemic immune-inflammation index; Neutrophil-lymphocyte ratio; Platelet-lymphocyte ratio; Prognosis

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Core tip: A preoperative systemic immune-inflammation index based on peripheral lymphocyte, neutrophil, and platelet counts was established, and better prognostic predictive abilities for overall survival and recurrence were found when compared with neutrophil-lymphocyte ratio and platelet-lymphocyte ratio in patients with colorectal cancer. This index might assist the identification of high-risk patients among patients with the same TNM stage in clinical practice.

Chen JH, Zhai ET, Yuan YJ, Wu KM, Xu JB, Peng JJ, Chen CQ, He YL, Cai SR. Systemic immune-inflammation index for predicting prognosis of colorectal cancer. *World J Gastroenterol* 2017; 23(34): 6261-6272 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6261.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6261

INTRODUCTION

Colorectal cancer (CRC), the third most frequently diagnosed cancer in men, and the second in women, is the third most common cause of cancer-related mortality worldwide[1]. The incidence of CRC in the United States has decreased owing to the improvements in cancer screening and the removal of precancerous adenomas[2]. However, an increase in the incidence of CRC was observed in many developing countries[3]. Owing to the absence of early symptoms and a hesitation in performing colonoscopy, a considerable number of CRC patients are diagnosed at an advanced stage, with an unfavorable overall survival (OS)[4]. Currently, the TNM staging system for CRC is the most commonly used predictor of OS and recurrence. However, prognostic heterogeneity was observed among patients with the same TNM stage[5], which causes confusion among clinicians when making therapeutic choices. Hence, more potential biomarkers should be included in clinical practice to improve prognostic prediction.

The interplay between systemic inflammation and the local immune response was recognized as the seventh hallmark of cancer, and it has been demonstrated to be involved in the initiation, development, and progression of several types of malignancies[6,7]. Cancer-related inflammation encompasses tumor-derived and host-derived cytokines, immune cells, and small inflammatory protein mediators[8,9], and is determined by the levels of serum leukocytes, neutrophils, lymphocytes, platelets, and acute-phase proteins such as C-reactive protein. Recently, the combinations of these systemic inflammation parameters, including neutrophil-lymphocyte ratio (NLR)[10] and platelet-lymphocyte ratio (PLR)[11], were reported as prognostic factors in some malignant solid tumors, including CRC. However, Hu *et al*[12] reported that systemic immune-inflammation index (SII), an integrated indicator based on peripheral lymphocyte, neutrophil, and platelet counts, was a powerful prognostic marker for patients with hepatocellular carcinoma. However, the SII for CRC has not been reported to date, and little is known about its prognostic value for CRC.

The aim of the present study was to investigate and compare the clinical significance and prognostic value of NLR, PLR, and SII in patients with CRC who underwent radical surgery.

MATERIALS AND METHODS

Ethics statement

The present study was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University. The requirement for informed consent was waived owing to the retrospective nature of this study.

Patients

We retrospectively analyzed the patients with primary CRC who underwent radical surgery at the Department of Gastrointestinal Surgery, the First Affiliated Hospital of Sun Yat-sen University between January 1994 and December 2010. 5-fluorouracil (5-FU)-based adjuvant chemotherapy was administered to stage III/IV patients and high-risk stage II patients. The inclusion criteria for patient enrollment were as follows: (1) primary colorectal adenocarcinoma confirmed by histopathology; (2) patients who underwent radical surgery; and (3) the availability of complete peripheral blood counts and follow-up data. The exclusion criteria

were as follows: (1) clinical evidence of infection; (2) the presence of hematological system diseases; (3) previous treatment with neoadjuvant chemotherapy or radiochemotherapy; (4) bowel obstruction or enterobrosis resulting in emergency surgery; (5) concurrent cancers or CRC recurrence; and (6) the use of anti-inflammatory or immunosuppressive medicines. Finally, 1383 cases were enrolled in the present study.

Data collection

The following variables were analyzed: demographics (age and sex), clinicopathological features (tumor location, tumor size, histological type, and tumor stage), and treatment with chemotherapy. We defined cecum carcinoma, ascending colon cancer, and right-half transverse colon as right-sided CRC, whereas the rest were classified as left-sided CRCs. The well and moderately differentiated adenocarcinomas were histologically categorized as the well-differentiated type, and the poorly differentiated adenocarcinomas included poorly differentiated adenocarcinoma, mucinous adenocarcinoma, signet ring cell cancer, and undifferentiated cancer. Tumor staging was performed according to the 7th edition of the Union for International Cancer Control-American Joint Committee on cancer classification for CRC.

Preoperative blood sampling was performed to measure the neutrophil, lymphocyte, and platelet levels for the calculation of the NLR, PLR, and SII indices. NLR and PLR were defined as the total number of neutrophils or platelets divided by the total number of lymphocytes. SII was calculated with the formula SII = (P × N)/L, where P, N, and L refer to peripheral platelet, neutrophil, and lymphocyte counts, respectively.

Follow-up

Patients were followed every 3 mo in the first 2 years following surgery, every 6 mo in years 3-5, and annually thereafter. As previously described[13], clinical history was taken, physical examination was performed, peripheral tumor biomarker levels were measured, and chest radiography, abdominal and pelvic computed tomography or ultrasonography, and colonoscopy were performed in the follow-up period according to the NCCN Clinical Practice Guidelines in Oncology. The OS and disease-free survival (DFS) were defined as the interval between surgery and time of death or the time from the last follow-up to the time of first confirmed recurrence, respectively.

Statistical analysis

Data was analyzed using the SPSS statistical software for Windows, version 17 (SPSS Inc., Chicago, United States). Receiver operating characteristic (ROC) curve analysis was performed to analyze the area under the ROC curve (AUC), and the Youden Index was used to identify the optimal cut-off values for NLR, PLR, and SII. Pearson χ^2 test or Fisher exact test were performed to compare the different categorical variable groups. Survival analysis was performed using the Kaplan-Meier method and the Log-Rank test was used to compare the survival differences. Univariate and multivariate analyses were performed with the Cox proportional hazards regression model. A P value < 0.05 was considered statistically significant.

RESULTS

ROC analysis

Using cancer-specific death as the end point, ROC analysis was performed to identify the optimal cut-off point with the highest sensitivity and specificity, which was 2.7 for NLR, 210 for PLR, and 340 for SII (sensitivity and specificity: 0.414 and 0.750 for NLR, 0.425 and 0.708 for PLR, and 0.857 and 0.524 for SII, respectively). For each immune-inflammation index, patients were divided into two groups for further analysis [NLR $\leqslant$ 2.7 (low) and NLR > 2.7 (high); PLR $\leqslant$ 210 (low) and PLR > 210 (high); SⅡ $\leqslant$ 340 (low) and SⅡ > 340 (high)].

Baseline characteristics of patients

In total, 1383 cases were enrolled in the present study. Patients in the high NLR group were more elderly compared to the low NLR group (> 60 years old: 54.7% *vs* 46.8%, respectively); however, associations between age and the levels of PLR and SII were not identified. Moreover, there were significant sex distribution differences in the three groups. In addition, cases in the high NLR and PLR groups were more likely to have left-sided CRC; however, the tumor location did not differ significantly between the high and low SII groups. High levels of NLR, PLR, and SII correlated with poor histological differentiation, larger tumor size, advanced T stage, N stage, M stage, TNM stage, and chemotherapy. The associations of NLR, PLR, and SII with clinicopathological parameters are demonstrated in Table 1.

Prognostic value of NLR, PLR, and SII

In the present study, the 5-, 10-, 15-, and 20-year OS rates were 61.2%, 45.1%, 35.6%, and 28.1%, respectively. The patients in the high NLR, PLR, and SII groups showed poorer OS compared to patients in the low NLR, PLR, and SII groups, respectively (Figure 1A-C). In order to identify the prognostic parameters for OS, 13 variables were included in the univariate Cox regression analysis, which showed that the NLR, PLR, SII, age, histological type, tumor invasion, lymph node involvement, distant metastasis, TNM stage, and chemotherapy were the variables that had significant impact on OS. After the exclusion of variables that showed no impact on the OS in univariate analysis, Cox multivariate regression analysis was performed, which identified SII (95%CI: 2.616-3.824), PLR (95%CI: 1.123-1.492), age (95%CI: 1.355-1.798), distant metastasis (95%CI: 1.512-2.517), and TNM stage (95%CI: 1.191-1.518) as the independent

Table 1 Baseline patient characteristics based on neutrophil-lymphocyte ratio, platelet-lymphocyte ratio and systemic immune-inflammation index *n* (%)

	Cases	NLR		*P* value	PLR		*P* value	SII		*P* value
		≤ 2.7	> 2.7		≤ 210	> 210		≤ 340	> 340	
Age (yr)				0.005			0.904			0.063
≤ 60	698 (50.5)	480 (53.2)	218 (45.3)		437 (50.3)	261 (50.7)		233 (54.2)	465 (48.8)	
> 60	685 (49.5)	422 (46.8)	263 (54.7)		431 (49.7)	254 (49.7)		197 (45.8)	488 (51.2)	
Gender				0.067			0.422			0.927
Male	808 (58.4)	511 (56.7)	297 (61.7)		500 (57.6)	308 (59.8)		252 (58.6)	536 (58.3)	
Female	575 (41.6)	391 (43.3)	184 (38.3)		368 (42.4)	207 (40.2)		178 (41.4)	397 (41.7)	
Tumor location				< 0.001			0.008			0.060
Right-sided	324 (23.4)	185 (20.5)	139 (28.9)		183 (21.1)	141 (27.4)		87 (20.2)	237 (24.9)	
Left-sided	1059 (76.6)	717 (79.5)	342 (71.1)		685 (78.9)	374 (72.3)		343 (79.8)	716 (75.1)	
Histological type				< 0.001			0.020			< 0.001
Well-differentiated	1126 (81.4)	761 (84.4)	365 (75.9)		723 (83.3)	403 (78.3)		376 (87.4)	750 (78.7)	
Poorly differentiated	257 (18.6)	141 (15.6)	116 (24.1)		145 (16.7)	112 (21.7)		54 (12.6)	203 (21.3)	
Tumor size				< 0.001			< 0.001			0.003
≤ 5 cm	936 (67.7)	660 (73.2)	276 (57.4)		617 (71.1)	319 (61.9)		315 (73.3)	621 (65.2)	
> 5 cm	447 (32.3)	242 (26.8)	205 (42.6)		251 (28.9)	196 (38.1)		115 (26.7)	332 (34.8)	
T stage				< 0.001			< 0.001			< 0.001
T1	67 (4.8)	45 (5.0)	22 (4.6)		54 (4.9)	13 (4.8)		38 (8.8)	29 (3.0)	
T2	256 (18.5)	197 (21.8)	59 (12.3)		170 (19.9)	86 (16.7)		104 (24.2)	152 (15.9)	
T3	748 (54.1)	507 (56.2)	241 (50.1)		475 (54.7)	273 (53.0)		232 (54.0)	516 (54.1)	
T4	312 (22.6)	153 (17.0)	159 (33.1)		169 (19.5)	143 (27.8)		56 (13.0)	256 (26.9)	
N stage				< 0.001			0.021			< 0.001
N0	487 (35.2)	353 (39.1)	134 (27.9)		325 (37.4)	162 (31.5)		196 (45.6)	291 (30.5)	
N1	416 (30.1)	277 (30.7)	139 (28.9)		261 (30.1)	155 (30.1)		121 (28.1)	295 (31.0)	
N2	308 (22.3)	188 (20.8)	120 (24.9)		190 (21.9)	118 (22.9)		77 (17.9)	231 (24.2)	
N3	172 (12.4)	84 (9.3)	88 (18.3)		92 (10.6)	80 (15.5)		36 (8.4)	136 (14.3)	
M stage				< 0.001			< 0.001			< 0.001
M0	1115 (80.6)	774 (85.8)	341 (70.9)		726 (83.6)	389 (75.5)		386 (89.8)	729 (76.5)	
M1	268 (19.4)	128 (14.2)	140 (29.1)		142 (16.4)	126 (24.5)		44 (10.2)	224 (23.5)	
TNM stage				< 0.001			0.001			< 0.001
I	187 (13.5)	141 (15.6)	46 (9.6)		133 (15.3)	54 (10.5)		92 (21.4)	95 (10.0)	
II	515 (37.2)	368 (40.8)	147 (30.6)		335 (38.6)	180 (35.0)		171 (39.8)	344 (36.1)	
III	413 (29.9)	265 (29.4)	148 (30.8)		258 (29.7)	155 (30.1)		123 (28.6)	290 (30.4)	
IV	268 (19.4)	128 (14.2)	140 (29.1)		142 (16.4)	126 (24.5)		44 (10.2)	224 (23.5)	
Chemotherapy				< 0.001			< 0.001			< 0.001
No	684 (49.5)	488 (54.1)	196 (40.7)		472 (54.4)	212 (41.2)		243 (56.5)	441 (46.3)	
Yes	699 (50.5)	414 (45.9)	285 (59.3)		396 (45.6)	303 (58.8)		187 (43.5)	512 (53.7)	

NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio; SII: Systemic immune-inflammation index.

prognostic factors of OS (Table 2).

Similarly, the 5-, 10-, 15-, and 20-year DFS rates were 56.5%, 39.1%, 31.3%, and 26.5%, respectively. The DFS rates were lower in patients with high NLR, PLR, and SII compared to those of the patients with low NLR, PLR, and SII, respectively (Figure 1D-F). The DFS data for the three patient groups are demonstrated in Table 3. Univariate Cox proportional hazards regression analysis revealed that age, histological type, tumor invasion, lymph node involvement, distant metastasis, TNM stage, chemotherapy, NLR, PLR, and SII had statistically significant associations with DFS. In addition, multivariate analysis indicated that SII was a significant independent prognostic parameter for DFS, whereas PLR and NLR were not (Table 3).

The AUCs of the NLR, PLR, and SII for OS were 0.602, 0.566, and 0.707, respectively (Figure 2A) and the AUCs for DFS were 0.597, 0.558, and 0.701, respectively (Figure 2B). Hence, among the immune-inflammation indices analyzed in the present study, SII was the best predictor of long-term survival and recurrence in cases with CRC.

Prognostic value of NLR, PLR, and SII stratified according to TNM stage

Further subgroup analyses were performed to investigate the prognostic value of SⅡ, NLR, and PLR in patients with CRC who were stratified according to the TNM stage. The results of the analyses showed that only SⅡ was able to distinguish the OS and DFS for each TNM stage (Figure 3A-F). On the other hand, NLR could identify the survival differences between TNM stages Ⅱ-Ⅳ, while PLR could only detect the prognostic differences of stage Ⅱ-Ⅲ cancers (Figure 4A-F for NLR and Figure 5A-F for PLR). Hence, the results indicated that only SII had prognostic significance for the CRC cases stratified according to TNM stage.

DISCUSSION

In the present study, we established an immune-inflammation-based prognostic index (SII) based on

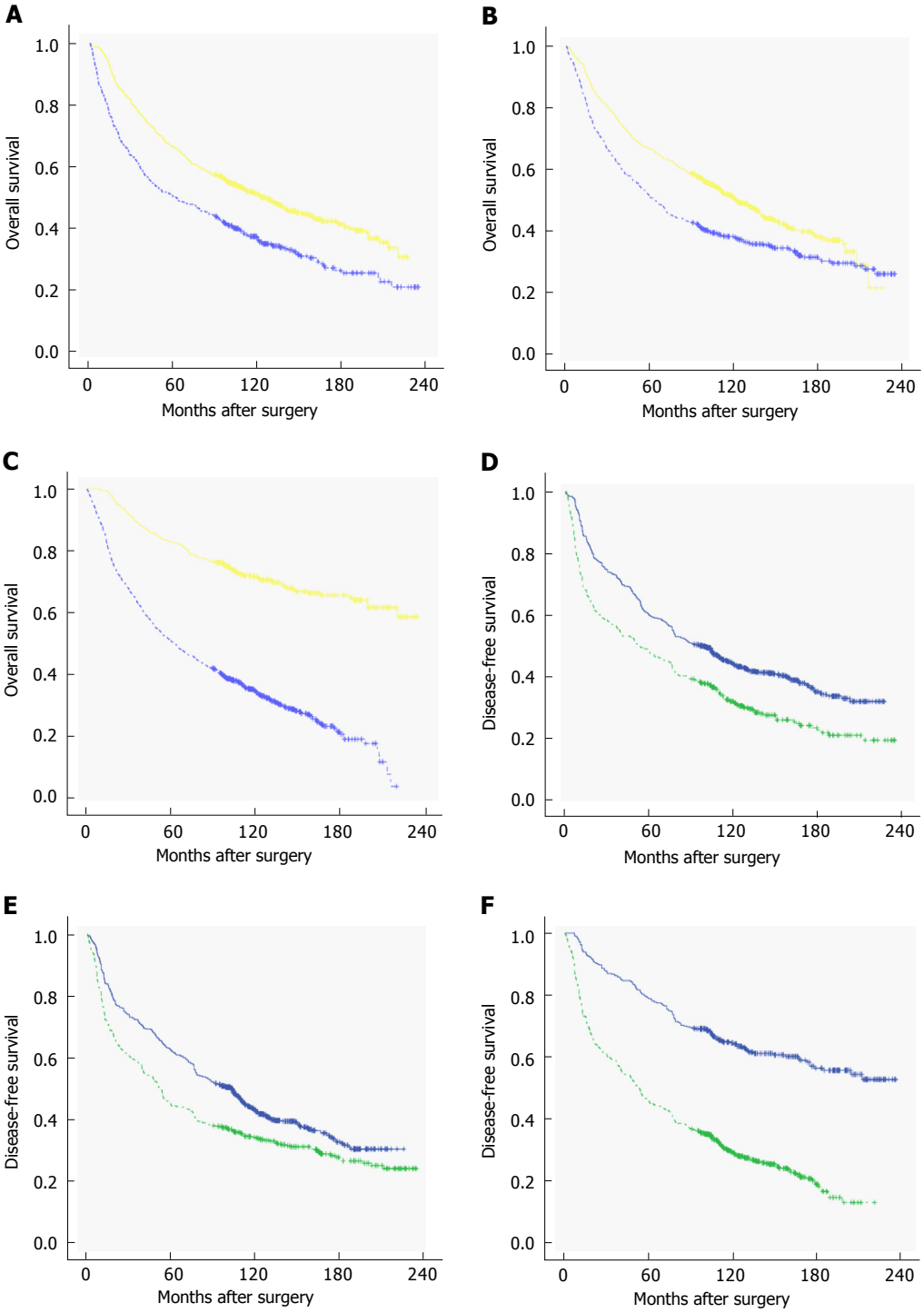


Figure 1 Kaplan-Meier curves of overall survival and disease-free survival of colorectal cancer patients based on neutrophil-lymphocyte ratio (A and D), platelet-lymphocyte ratio (B and E), and systemic immune-inflammation index (C and F).

peripheral neutrophil, platelet, and lymphocyte counts and demonstrated that elevated SII was correlated with poor OS and recurrence in patients with CRC. In addition, SII was a superior prognostic factor for survival outcome compared to NLR and PLR.

It was recognized that inflammatory-based indices were associated with poor tumor behavior and survival outcome in various malignant solid tumors, including CRC. Several combinations such as NLR[14], PLR[15], prognostic nutritional index[16], Glasgow Prognostic score[17], and lymphocyte monocyte ratio (LMR)[18] showed positive correlations between elevated inflammation-based factors and poor survival outcome in patients with CRC. To our knowledge, this was the first report investigating the prognostic value of SII in patients with CRC after radical surgery. Using an integrated index based on peripheral neutrophil, platelet, and lymphocyte counts, Hu *et al*[12] found

Table 2 Univariate and multivariate Cox regression analysis of the associations between clinical parameters and overall survival in patients with colorectal cancer

	Univariate analysis				Multivariate analysis			
	χ^2 value	HR	95%CI	*P* value	χ^2 value	HR	95%CI	*P* value
Age	22.965	1.405	1.223-1.614	< 0.001	38.131	1.561	1.355-1.798	< 0.001
Gender	0.017	-	-	0.896				
Tumor location	0.269	-	-	0.604				
Histological type	22.178	1.493	1.264-1.764	< 0.001				
Tumor size	1.166	-	-	0.280				
T stage	71.702	1.498	1.364-1.645	< 0.001				
N stage	113.905	1.432	1.340-1.529	< 0.001				
M stage	239.937	3.415	2.923-3.989	< 0.001	26.390	1.951	1.512-2.517	< 0.001
TNM stage	207.252	1.785	1.650-1.932	< 0.001	22.971	1.345	1.191-1.518	< 0.001
NLR status	40.824	1.582	1.374-1.821	< 0.001				
PLR status	23.604	1.416	1.231-1.649	< 0.001	12.618	1.294	1.123-1.492	< 0.001
SII status	173.330	3.529	2.925-4.258	< 0.001	141.427	3.163	2.616-3.824	< 0.001
Chemotherapy	76.931	1.894	1.642-2.184	< 0.001				

NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio.

Table 3 Univariate and multivariate Cox regression analysis of the associations between clinical parameters and PFS in patients with colorectal cancer

	Univariate analysis				Multivariate analysis			
	χ^2 value	HR	95%CI	*P* value	χ^2 value	HR	95%CI	*P* value
Age	21.774	1.373	1.202-1.569	< 0.001	34.479	1.499	1.310-1.716	< 0.001
Gender	0.144	-	-	0.705				
Tumor location	0.509	-	-	0.476				
Histological type	20.091	1.447	1.231-1.701	< 0.001				
Tumor size	1.095	-	-	0.295				
T stage	59.847	1.419	1.299-1.550	< 0.001				
N stage	86.718	1.354	1.270-1.443	< 0.001				
M stage	221.926	3.172	2.725-3.692	< 0.001	31.580	2.024	1.583-2.589	< 0.001
TNM stage	179.589	1.673	1.552-1.804	< 0.001	15.414	1.255	1.121-1.406	< 0.001
NLR status	36.441	1.518	1.325-1.738	< 0.001				
PLR status	20.826	1.369	1.196-1.567	< 0.001				
SII status	159.123	2.988	2.521-3.542	< 0.001	129.495	2.717	2.287-3.228	< 0.001
Chemotherapy	61.159	1.716	1.499-1.965	< 0.001				

NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio.

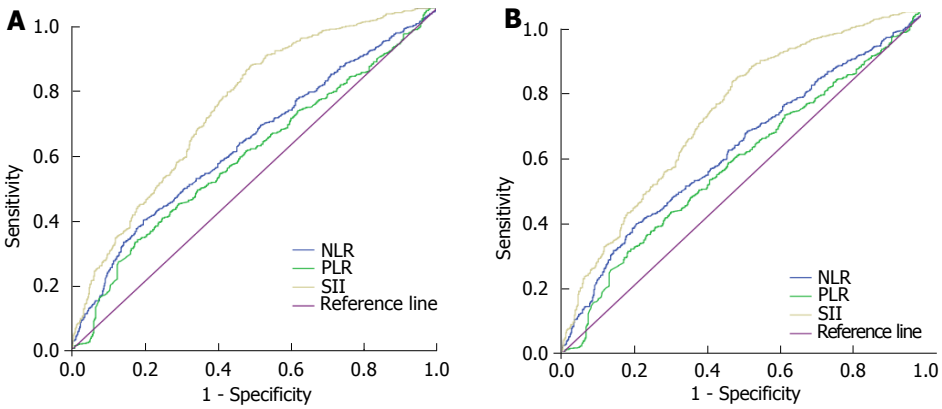


Figure 2 Receiver operating curve analysis of overall survival (A) and disease-free survival (B). NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio; SⅡ: Systemic immune-inflammation index.

that patients having elevated preoperative SⅡ were usually diagnosed with thrombocytosis, neutropenia, or lymphopenia. They believed that a better understanding of the roles of neutrophils, platelets, and lymphocytes in cancer development and progression would help clarify the association between SⅡ and its clinical impact. Neutrophils do not only alter the tumor microenvironment *via* the extrinsic pathway, but they

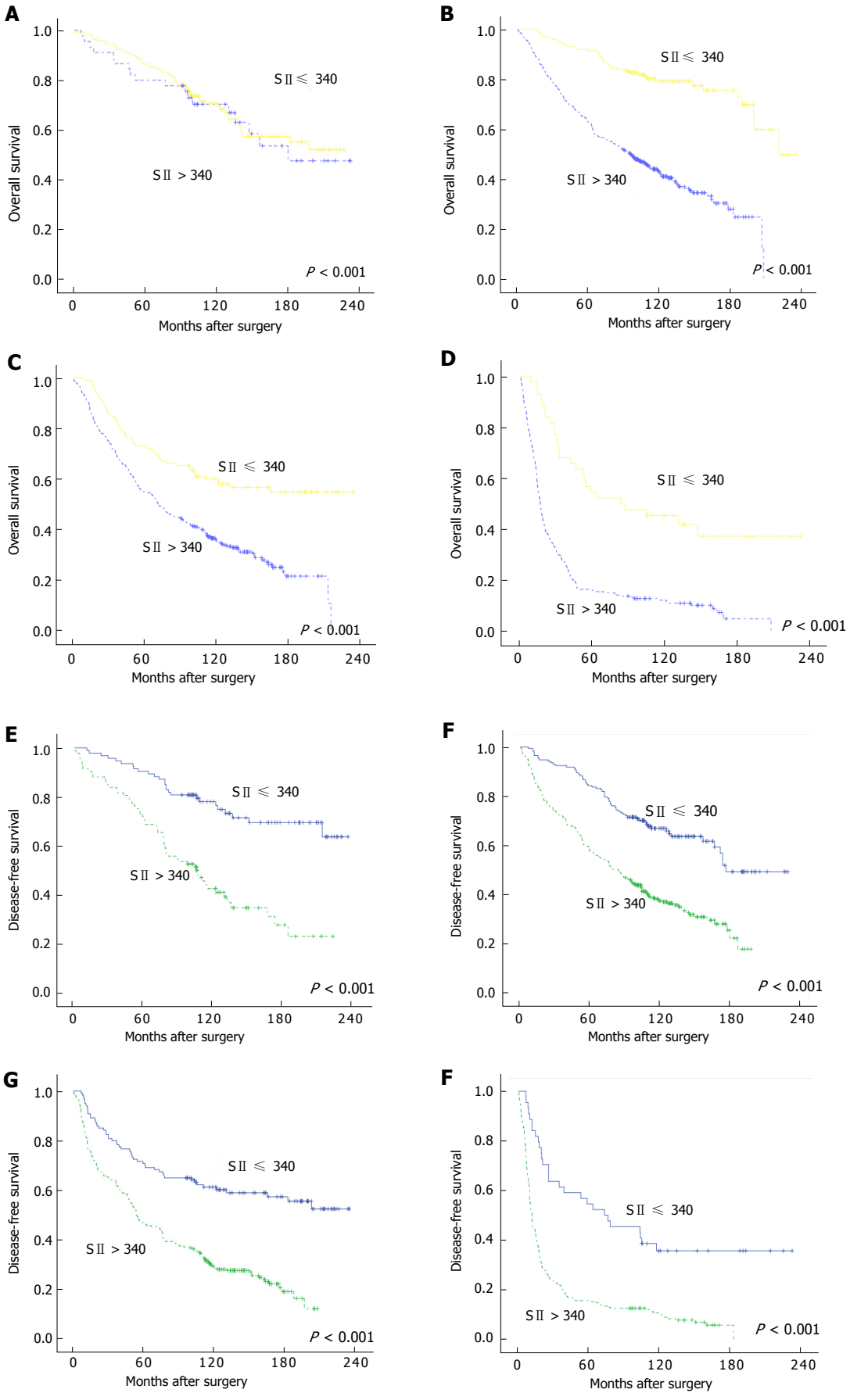


Figure 3 Kaplan-Meier curves of overall survival and disease-free survival of colorectal cancer patients based on different levels of systemic immune-inflammation index. A-D: OS for cases with stages I, II, III, and IV CRC, respectively; E and F: DFS for cases with stages I, II, III, and IV CRC, respectively. OS: Overall survival; DFS: Disease-free survival; CRC: Colorectal cancer.

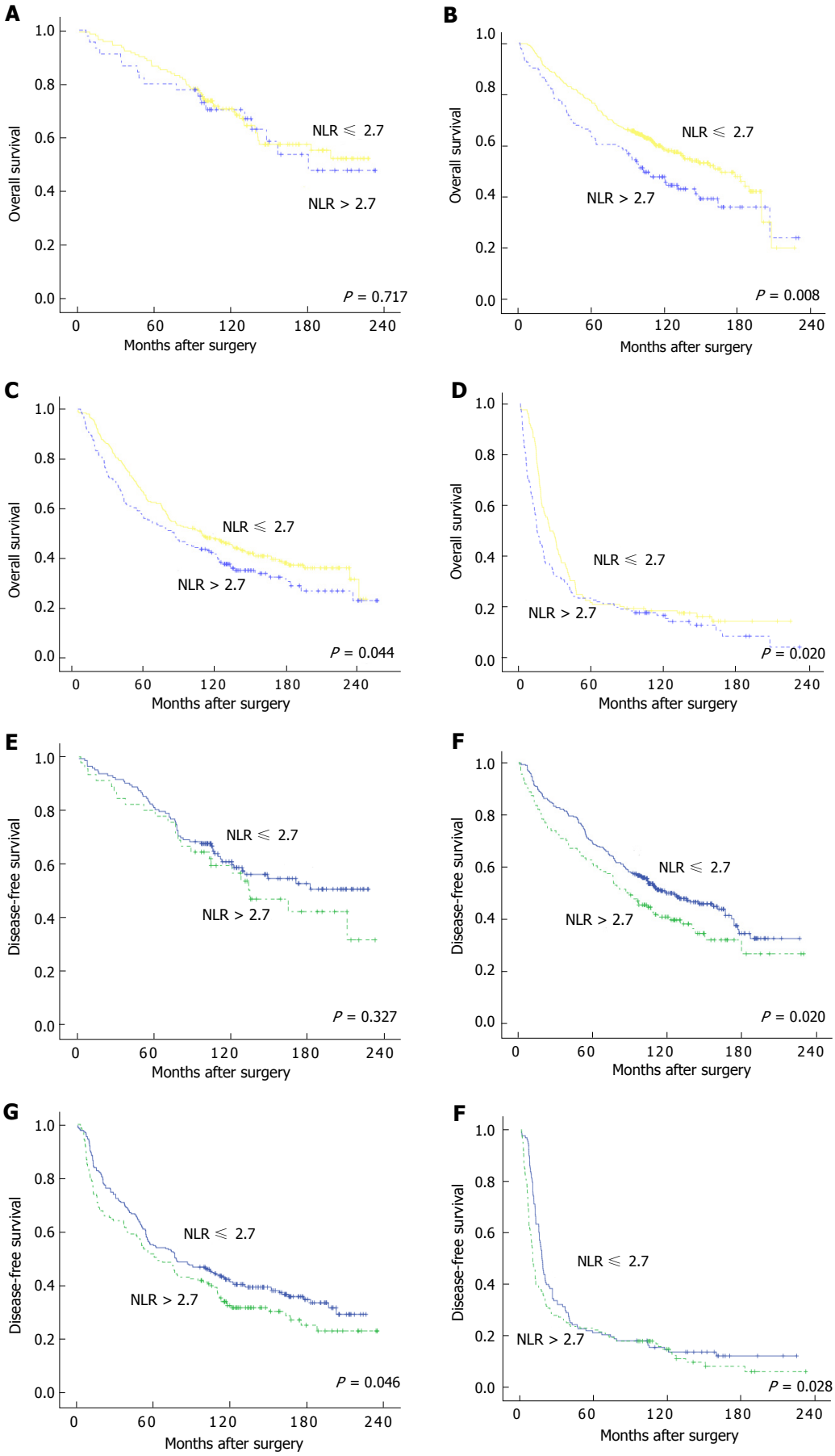


Figure 4 Kaplan-Meier curves of overall survival and disease-free survival of colorectal cancer patients based on different levels of neutrophil-lymphocyte ratio. A-D: OS for cases with stages I, II, III, and IV CRC, respectively; E and F: DFS for cases with stages I, II, III, and IV CRC, respectively. OS: Overall survival; DFS: Disease-free survival; CRC: Colorectal cancer; NLR: Neutrophil-lymphocyte ratio.

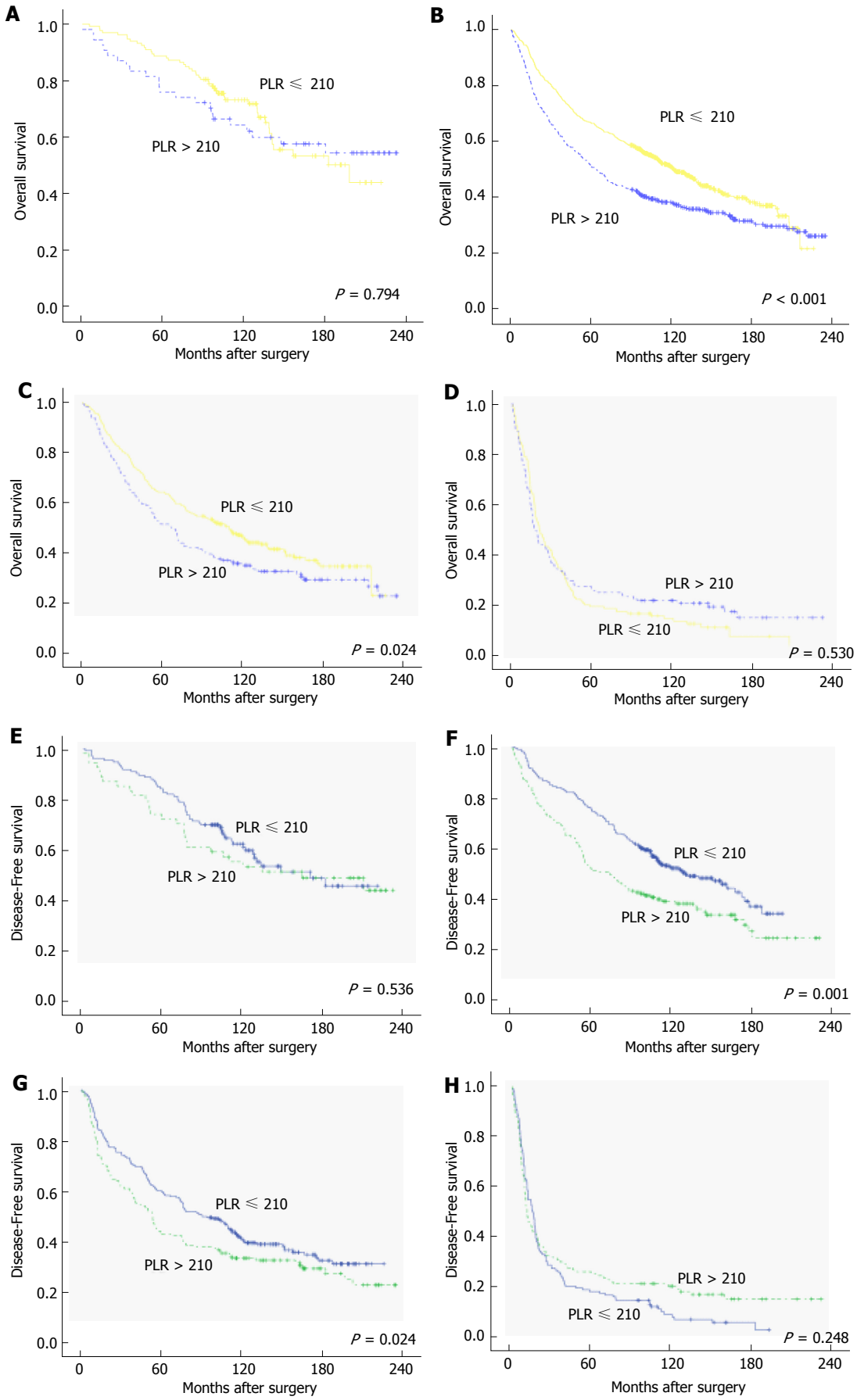


Figure 5 Kaplan-Meier curves of overall survival and disease-free survival of colorectal cancer patients based on different levels of platelet-lymphocyte ratio. A-D: OS for cases with stages I, II, III, and IV CRC, respectively; E-F: DFS for cases with stages I, II, III, and IV CRC, respectively. OS: Overall survival; DFS: Disease-free survival; CRC: Colorectal cancer; PLR: Platelet-lymphocyte ratio.

also secrete some inflammatory mediators to promote tumor cell proliferation, invasion, metastasis to lymph nodes or distant organs, and cellular senescence *via* the intrinsic pathway[19,20]. Accumulating experimental and clinical evidence showed that platelet activation could act as chemoattractants for cancer cells, induce the formation of optimized conditions for metastatic foci, promote the epithelial to mesenchymal transition in tumor cells, and increase the level of circulating tumor cells[21,22]. Lymphopenia was commonly accompanied by leukocytosis and thrombocytosis, which might help tumor cells to escape immune surveillance and prevent damage from the autoimmune response by cytotoxic T cells[23]. There was a good and a bad inflammatory reaction. In other words if the inflammation was based on the production of simply growth factors, the inflammatory reaction has a negative effect. But if the inflammatory reaction consists on neutralizing antibodies produced by activated lymph nodes, this reaction can have a positive effect. Thus, a high SII level reflected alterations in the cancer microenvironment that favor cancer initiation, progression, and metastasis.

The present study revealed interesting associations between inflammation-based indices and clinicopathological features. Consistent with the clinicopathological features associated with NLR and PLR, which are the most common indices, SII was also associated with poor histological differentiation, larger tumor size, more advanced T stage, N stage, M stage, and TNM stage, validating the above hypothesis that the elevated inflammatory response might promote tumor proliferation, progression, and metastasis.

As a simple, convenient, easily obtained, cheap, and non-invasive marker, SII was first described by Hu *et al*[12] in hepatocellular carcinoma. They concluded that preoperative SII might be related to circulating tumor cells and act as a powerful prognostic predictor in patients with hepatocellular carcinoma. Consistent with the results of previous studies, Yang *et al*[24] also reported that elevated SII with a cut-off value of 300 was negatively associated with OS in HBV-related hepatocellular carcinoma[25]. Moreover, SII was reported as a predictor of metastatic CRC in patients who received first-line chemotherapy with bevacizumab[26]. To our knowledge, the present study was the first to investigate the prognostic value of SII in CRC. Confirmed by the Kaplan-Meier analysis using the log-rank method, all the inflammation-based indices were significantly associated with OS and recurrence. However, SII was identified in Cox multivariate analysis to be a superior predictor of OS and DFS compared to other inflammation-based prognostic indices. The discriminative abilities of these three indices were further evaluated and compared; based on the AUC values obtained from ROC curves, SII was the most effective predictor of long-term survival outcome compared to NLR and PLR. The potential explanation of a better prognostic value might be that SII was more comprehensive in reflecting the status of inflammatory and immune response than the other factors.

Pathological TNM staging is presently the gold standard for predicting survival outcome and the treatment choice. However, because TNM staging was performed postoperatively, survival prediction before surgery and decision of further treatment strategies became difficult. Moreover, TNM stage can only reflect the biological behavior of the tumor. To our knowledge, prognosis was not only associated with the clinicopathological characteristics of the tumor, but also with the host inflammatory response[27]. SII is based on peripheral neutrophil, platelet, and lymphocyte counts, and it reflects the status of the tumor microenvironment and the preoperative host inflammatory response, serving as a complementary to the TNM stage for predicting OS. Our findings demonstrated that preoperative SII had powerful prognostic discriminative abilities in terms of each TNM subgroup compared to NLR and PLR. Therefore, using a combination of parameters that reflect both the tumor characteristics and the host systemic inflammatory status might be important for accurately predicting survival outcome in patients with CRC.

The present study had a few limitations. First, it was a retrospective, single-center study. Therefore, a large-scale prospective validation study is required to validate the results of the present study. Second, only the patients who received radical surgery were enrolled and thus, the results of the present study are not applicable in incurable patients or in those for whom the treatment was terminated because of various reasons.

In conclusion, this was the first study to demonstrate that preoperative SII is a simple and powerful prognostic indicator of OS and DFS in patients with CRC. SII might be used along with the TNM staging for individualized treatment in future clinical practice. A larger prospective study is warranted for the validation of the preliminary results obtained in the present study.

COMMENTS

Background

Recently, there were many papers describing mathematical formulas, such as neutrophil-lymphocyte ratio and platelet-lymphocyte ratio, which were considered important prognostic factors for colorectal cancer (CRC). In this article, the authors want to investigate the clinical value of systemic immune-inflammation index (SII), an integrated indicator based on peripheral lymphocyte, neutrophil, and platelet counts.

Research frontiers

The interplay between systemic inflammation and the local immune response was recognized as the seventh hallmark of cancer, and it has been demonstrated to be involved in the initiation, development, and progression of several types of malignancies. Inflammation can produce several cytokines and growth factors which can facilitate the occurrence and development of cancer. We established a systemic inflammation parameter, SII and found that SII had better prognostic predictive abilities for overall survival and recurrence of CRC in this study.

Innovations and breakthroughs

Preoperative SII based on peripheral lymphocyte, neutrophil, and platelet counts was established, and no study investigated the clinical value of SII in CRC before. We found that SII had better prognostic predicting abilities for overall survival and recurrence when compared with neutrophil-lymphocyte ratio and platelet-lymphocyte ratio in patients with CRC. It might assist the identification of high-risk patients among patients with the same TNM stage in clinical practice.

Applications

Patients with high SII showed aggressive tumor biological behavior, poor overall survival and early tumor recurrence. Hence, SII may give help to identify the high-risk patients among patients with the same TNM stage in clinical practice.

Terminology

SII is based on the peripheral lymphocyte, neutrophil, and platelet counts, and is calculated using the formula SII = (P × N)/L, where P, N, and L refer to the preoperative peripheral platelet, neutrophil, and lymphocyte counts, respectively.

Peer-review

This is an interesting study. Obvious conclusions were drawn from a well-known phenomenon: Correlation between inflammation and cancer development and progression. I think the paper should be published to address an important point: To cure or prevent inflammation can prevent cancer formation and progression. The result may give help to clinical doctors.

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ORIGINAL ARTICLE

Retrospective Study

Predictive factors for the failure of endoscopic stent-in-stent self-expandable metallic stent placement to treat malignant hilar biliary obstruction

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Author contributions: Sugimoto M designed research; Sugimoto M and Takagi T performed research; Sugimoto M, Takagi T and Ohira H analysed data; Sugimoto M, Takagi T and Ohira H wrote the paper; Suzuki R, Konno N, Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Sato Y and Hikichi T provided clinical advice; Hikichi T and Ohira H supervised the report.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Fukushima Medical University Hospital.

Informed consent statement: Patients were not required to give informed consent for this study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Fukushima Medical University.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM

To investigate the factors predictive of failure when

placing a second biliary self-expandable metallic stents (SEMSs).

METHODS

This study evaluated 65 patients with an unresectable malignant hilar biliary obstruction who were examined in our hospital. Sixty-two of these patients were recruited to the study and divided into two groups: the success group, which consisted of patients in whom a stent-in-stent SEMS had been placed successfully, and the failure group, which consisted of patients in whom the stent-in-stent SEMS had not been placed successfully. We compared the characteristics of the patients, the stricture state of their biliary ducts, and the implemented endoscopic retrograde cholangiopancreatography (ERCP) procedures between the two groups.

RESULTS

The angle between the target biliary duct stricture and the first implanted SEMS was significantly larger in the failure group than in the success group. There were significantly fewer wire or dilation devices (ERCP catheter, dilator, or balloon catheter) passing the first SEMS cell in the failure group than in the success group. The cut-off value of the angle predicting stent-in-stent SEMS placement failure was 49.7 degrees according to the ROC curve (sensitivity 91.7%, specificity 61.2%). Furthermore, the angle was significantly smaller in patients with wire or dilation devices passing the first SEMS cell than in patients without wire or dilation devices passing the first SEMS cell.

CONCLUSION

A large angle was identified as a predictive factor for failure of stent-in-stent SEMS placement.

Key words: Endoscopic stent-in-stent self-expandable metallic stent placement; Predictive factor; Endoscopic retrograde cholangiopancreatography; Malignant hilar biliary obstruction; Self-expandable metallic stent

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Core tip: We investigated the factors predictive of failure when placing multiple endoscopic self-expandable metallic stents (SEMSs) to relieve a malignant biliary obstruction. The angle between the target biliary duct stricture and the first implanted SEMS was significantly higher in the failure than in the success group. There were significantly fewer wire or dilation devices passing the first SEMS cell in the failure group than in the success group. The angle was significantly smaller in patients with wire or dilation devices passing the first SEMS cell. In conclusion, a large angle was identified as a predictive factor for failure of stent-in-stent SEMS placement.

Sugimoto M, Takagi T, Suzuki R, Konno N, Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Sato Y, Hikichi T, Ohira H. Predictive factors for the failure of endoscopic stent-in-stent self-expandable metallic stent placement to treat malignant hilar biliary obstruction. *World J Gastroenterol* 2017; 23(34): 6273-6280 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6273.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6273

INTRODUCTION

A malignant hilar biliary stricture can be caused by several different types of cancer, such as cholangiocarcinoma, pancreatic cancer, liver cancer, and lymph node metastases. A malignant hilar biliary stricture causes obstructive jaundice, and biliary drainage must be accomplished prior to surgical or chemotherapeutic treatment. Two procedures can be used to achieve biliary drainage to treat malignant obstructive jaundice: endoscopic therapy and percutaneous transhepatic bile drainage. Due to its safety and efficacy, endoscopic therapy is the first-choice treatment[1].

Generally, self-expandable metallic stents (SEMSs) are superior to plastic stents in terms of stent patency, cost, and length of hospital stay[2-7]. SEMSs are likewise superior for the treatment of a malignant hilar biliary stricture[8]. Whether it is necessary to multistent a malignant hilar biliary stricture remains a topic of discussion[8-15]. In fact, some patients require multistenting to treat cholangitis and liver abscesses. However, it is sometimes difficult to place multiple metallic stents to relieve a malignant hilar biliary stricture. Moreover, the predictive factors for the failure of biliary multistenting have not been identified.

Therefore, the aim of the present study was to determine the predictive factors for the failure of biliary multistenting.

MATERIALS AND METHODS

Study design

This study was retrospective in design. To evaluate the predictive factors for the failure to place an endoscopic stent-in-stent SEMS, we compared the patient characteristics and the therapeutic endoscopy factors of the groups with successful and unsuccessful stent-in-stent SEMS placement. This study was approved by the ethics committee of Fukushima Medical University.

Patients

We examined 65 patients with an unresectable malignant hilar biliary stricture in whom an endoscopic stent-in-stent biliary SEMS placement was attempted between April 2003 and August 2016 in our hospital. The patients were not required to give informed consent to participate in this study because the analysis used anonymised data obtained after each patient provided

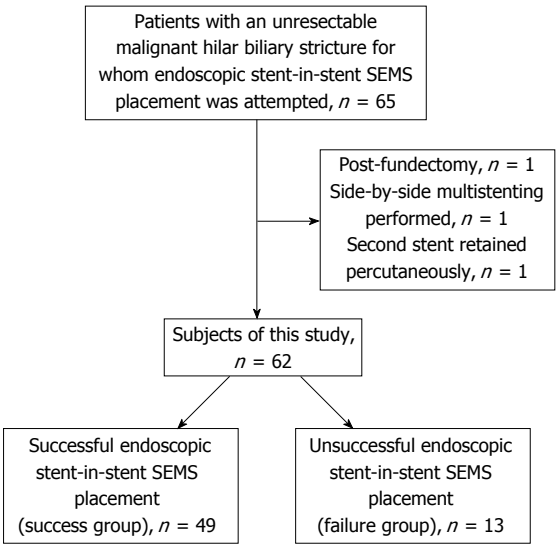


Figure 1 Targets of this study. Among 65 patients with an unresectable malignant hilar biliary obstruction, 62 patients were included. These 62 patients were divided into two groups, the success group, in whom an endoscopic stent-in-stent self-expandable metallic stent (SEMS) placement was successful, and the failure group, in whom an endoscopic stent-in-stent SEMS placement was unsuccessful.

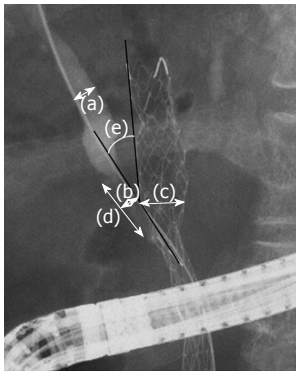


Figure 2 Endoscopic retrograde cholangiography image showing the details of the obstructive state of the biliary duct. We evaluated the obstructive state of the biliary duct by measuring the following four items: (a) The diameter of the target biliary duct, (b) the diameter of the target biliary stricture, (c) the diameter of the first retained self-expandable metallic stent (SEMS), (d) the length of the target biliary duct stricture and (e) the angle between the target biliary duct stricture and the first implanted SEMS.

written consent to undergo the medical examination. We included 62 of the 65 patients in our study, all of whom had Bismuth type II-IV strictures, had not received upper gastrointestinal tract surgery, and had underwent multiple stent-in-stent placements (Figure 1). Moreover, all patients retained the first metallic stent that was placed. We divided the patients into two groups. The 49 patients in whom a stent-in-stent SEMS was successfully placed at the hilar biliary stricture were included in the success group. The 13 patients in whom only one metallic stent was placed at the hilar biliary stricture were included in the failure group.

Endoscopic retrograde cholangiopancreatography procedure

Endoscopic retrograde cholangiopancreatography (ERCP) was performed by specialists of pancreaticobiliary endoscopy who had experience performing at least 2000 ERCP procedures or by trainees under the guidance of specialists. Before the ERCP procedure was initiated, all patients were sufficiently sedated with midazolam. After the ERCP endoscope was placed in the descending portion of the duodenum, biliary duct cannulation was performed, and the strictured region was observed using cholangiography. Endoscopic sphincterotomy (EST) was performed as required. The first metallic stent was placed at the hilar biliary stricture, and a wire was passed to the second targeted biliary duct. If a wire was not passed to the second targeted biliary duct, the procedure was finished. After the mesh was dilated using an ERCP catheter, a dilator catheter, or a balloon catheter, a second metallic stent was placed. Dilation devices were selected randomly by each endoscopist. JF240, TJF240, or JF260V ERCP endoscopes (Olympus, Tokyo, Japan) were used. The guidewires used in this study were Visiglide 1, Visiglide 2 (Olympus, Tokyo, Japan), Jagwire, Hydra Jagwire (Boston Scientific Japan, Tokyo, Japan), RevoWave (Piolax, Kanagawa, Japan), or Tracer Metro (Cook Japan, Tokyo, Japan). A CleverCut 3V or a Needle Knife device (Boston Scientific Japan, Tokyo, Japan) was used for EST. A SMART (Johnson & Johnson, Tokyo, Japan), JOSTENT (Zeon Medical, Tokyo, Japan), Zilver, Zilver 635 (Cook Japan, Tokyo, Japan), Niti-S D-type, Niti-S large-cell D-type (Taewoong-Medical, Gyeoenggi-do, Korea), Wall, or Wall Flex (Boston Scientific Japan, Tokyo, Japan) device was used as a biliary uncovered metal stent deployment system. A Tandem XL or CONTOUR taper tip, CONTOUR ultra-taper tip, CONTOUR 5-4-3 tip (Boston Scientific Japan), PR-233Q (Olympus), or MTW ERCP catheter taper (MTW Endoskopie, Wesel, Germany) was used as the ERCP catheter. A Soehendra biliary 6-Fr or 7-Fr dilation catheter (Cook Japan) was used as the biliary dilation catheter. An RX Hurricane balloon dilatation catheter (Boston Scientific Japan), Zara EPBD catheter (Kaneka Corporation, Tokyo, Japan), or REN biliary dilation catheter (Kaneka Corporation) was used to dilate the first SEMS lumen or mesh.

Variables considered

The following variables of the success and failure groups were considered: age, gender, diagnoses causing the biliary stricture (primary lesions or metastases), bismuth classification, diameter of the targeted biliary duct (Figure 2-a), diameter of the targeted biliary stricture (Figure 2-b), diameter of the first implanted SEMS (Figure 2-c), length of the targeted biliary duct stricture (Figure 2-d), angle between the target biliary duct stricture and the first

Table 1 Comparison of the characteristics and the target biliary duct status of patients in the success and failure groups

	Success (n = 49)	Failure (n = 13)	P value
Age, median (range), yr	74 (42-88)	71 (16-76)	0.45
Sex (male), n (%)	33 (67.3)	8 (61.5)	0.75
Diagnoses			
Primary lesion (Biliary tract or pancreatic cancer)	40	10	0.70
Metastases	9	3	
Pancreatic cancer	1		
Lung cancer	1		
Tracheal cancer	1		
Colon cancer	3	2	
Uterine cancer	1		
Gastric cancer	1	1	
Prostatic cancer	1		
Bismuth classification			0.98
Ⅱ	12	3	
Ⅲ	18	5	
Ⅳ	19	5	
Target biliary duct status,			
Diameter of the target biliary duct, median (range), mm	6.4 (2.0-15.9)	6.6 (4.4-17.3)[1]	0.45
Diameter of the target biliary stricture, median (range), mm	0 (0-1.6)	0 (0-0.9)	0.94
Diameter of the first implanted SEMS, median (range), mm	5.8 (3.1-11.7)	6.7 (3.4-12.6)	0.25
Length of the target biliary stricture, median (range), mm	11.0 (3.0-69.6)	7.9 (1.7-34.2)[1]	0.44
Angle between the target biliary duct stricture and the first implanted SEMS, median (range), degree	44.4 (7-119)	75.3 (28-109.3)[1]	< 0.01

[1]Data of one of the patient were not available. SEMS: Self-expandable metallic stent.

implanted SEMS (Figure 2-e), length of the procedure, clinically effective rate, adverse effects, wire passage of the first SEMS cell, catheter usage to dilate the first SEMS cell, catheter passage of the first SEMS cell, dilator usage to dilate the first SEMS cell, dilator passage of the first SEMS cell, balloon catheter usage to dilate the first SEMS lumen, balloon usage to dilate the first SEMS cell, balloon passage of the first SEMS cell, the number of used dilation devices (0: no dilation device was used-3: catheter, dilator and balloon catheter were all used), type of first SEMS used (braided or laser-cutting type), stenting order, number of ERCP sessions, and the area of the first SEMS cell. Improvement in liver or biliary function (ALT or ALP or bilirubin) within 2 wk after ERCP was determined as "clinically effective". The area of the first SEMS cell was not available for two patients in the failure group and three patients in the success group.

Statistical analysis

Student's t test or Mann-Whitney U test and Fisher's exact test were used to calculate the significance of the differences between the continuous variables and the nominal scales of the two groups. The Bismuth classifications were analysed using the Mann-Whitney U test. A P value of < 0.05 was considered to indicate a significant difference. All statistical analyses were performed using the EZR platform (Saitama Medical Centre, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR is a modified version of R commander that was designed to perform functions that are frequently used in biostatistics[16].

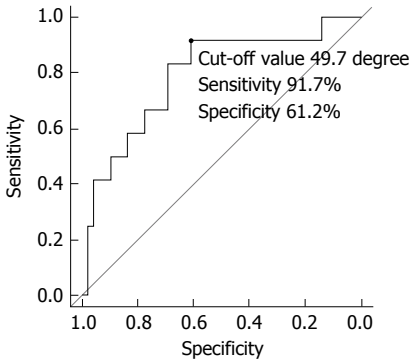


Figure 3 ROC curve of the angle between the target biliary duct stricture and the first implanted self-expandable metallic stent. A cut-off value of 49.7 degrees with a sensitivity of 91.7% and a specificity of 61.2% can be used to predict stent-in-stent self-expandable metallic stent placement failure.

RESULTS

No significant differences in the characteristics of the patients (Table 1) or the details of the target biliary ducts of the success and failure groups (Table 1) were observed with the exception of the following: the angle between the target biliary duct stricture and the first implanted SEMS was significantly different between the success group and the failure group [44.4 (7-119) degree *vs* 75.3 (28-109.3) degree, P < 0.01]. The ROC curve of the angle between the target biliary duct stricture and the first implanted SEMS revealed a cut-off value of 49.7 degrees with a sensitivity of 91.7% and a specificity of 61.2% for predicting stent-in-stent SEMS placement failure (Figure 3).

Regarding the ERCP procedures and outcomes used for the patients in the two groups, clinically effective rate, the rates of wire passage through the first SEMS cell, catheter passage through the first SEMS cell, dilator passage through the first SEMS cell, and balloon catheter passage through the first SEMS cell in the failure group were significantly lower than in the success group (Table 2). No other variables for the ERCP procedures used for the members of the success and failure groups were significantly different.

DISCUSSION

In this study, we investigated the factors predictive of failure in placing a second SEMS. The angle between

Table 2 Comparison of the endoscopic retrograde cholangiopancreatography procedures and outcomes employed in the success and failure groups *n* (%)

	Success (*n* = 49)	Failure (*n* = 13)	*P* value
Procedure time, median (range), min	70 (20-160)	90 (40-150)[1]	0.30
Clinically effective rate	49 (100)	11 (84.6)	0.04
Adverse effects	1 (2)	1 (7.7)	0.38
Post-ERCP pancreatitis	1	0	
Perforation of biliary duct	0	1	
Wire passage of the first SEMS cell	49 (100)	9 (69.2)	0.006
Diameter of wire (0.025/0.035)	32/14[2]	6/6[1]	0.31
Catheter usage to dilate the a first SEMS cell	24	8	0.54
Catheter passage of the first SEMS cell	22 (92)	4 (50)	0.02
Dilator usage to dilate first SEMS cell	18	5	1.00
Dilator passage of the first SEMS cell	17 (94)	2 (40)	0.02
Balloon catheter usage to dilate the first SEMS lumen	5	1	1.00
Balloon catheter usage to dilate the first SEMS cell	18	3	0.51
Balloon catheter passage of the first SEMS cell	18 (100)	0 (0)	< 0.001
The number of used dilation devices, median (range)	1 (0-3)	1 (0-3)	0.79
Type of first SEMS used (braided/laser), *n*	41/8	9/4	0.26
Stenting order			0.22
Left→Left	2	1	
Left→Right	28	6	
Right→Left	12	6	
Right→Right	7	0	
Procedure sessions			0.328
1	42	13	
2	7	0	
Area of first SEMS cell, median (range), mm^2	18.3 (3.5-39.3)	18.3 (3.5-18.3)[3]	0.59

[1]Data for one patient were not available; [2]Data for three patients were not available; [3]Data for two patients were not available. ERCP: Endoscopic retrograde cholangiopancreatography; SEMS: Self-expandable metallic stent.

the target biliary duct stricture and the first implanted SEMS was significantly associated with failure to place a second SEMS. In fact, failure to pass the devices was significantly associated with unsuccessful endoscopic stent-in-stent SEMS placement.

Few reports have examined the relationship between failure to place an endoscopic stent-in-stent SEMS and the characteristics of the patients. In one report, metastatic disease was found to be related to the failure to place an endoscopic stent-in-stent SEMS[17]. Although a similar number of patients who were evaluated in the previous investigation were analysed in this study, different results were obtained. However, the relationship between the failure to place an endoscopic stent-in-stent SEMS and the obstructive state of the biliary duct has not been reported. In this study, the effect of the precise details of the biliary obstructive state on the placement of an endoscopic stent-in-stent SEMS was evaluated. Among several features regarding the biliary obstructive state, only the angle between the target biliary duct stricture and the first implanted SEMS was significantly different between the success and failure groups. An angle less than 49.7 degrees, according to the ROC curve, predicted high probability of successful placement of a second SEMS.

Several reports have considered the devices used to treat patients with a malignant hilar biliary obstruction. The efficacy of using a slimmer stent or a large-cell metal stent for bilateral stent-in-stent placement has been reported[18-21]; the large-cell metal stent that was used was in these studies was a Niti-S large-cell stent (Taewoong-Medical), and the slimmer stent that was used was a Zilver stent (Cook Japan). We generally used these types of stents to initially treat the patients recruited in this study. The areas of the first SEMS cell placed in the patients of the two groups were not significantly different. This result is consistent with those of a previous study showing that the cell size did not affect successful bilateral drainage[22].

In studies reporting a high success rate for bilateral drainage, a 7-Fr Soehendra dilator (Cook Japan), a 6- to 8-mm CRE wire-guided oesophageal/pyloric balloon dilatation catheter, or a 6- or 8-mm Hurricane RX dilatation catheter (Boston Scientific Japan) was used[18,21]. In this study, these devices and ERCP catheters, such as the Tandem XL cannula (Boston Scientific Japan), were used to pass the first SEMS. To identify the cause underlying the failure of these devices to pass, we evaluated the influence of the angle between the target biliary duct stricture and the first implanted SEMS on wire or dilation catheter passage using the Mann-Whitney *U* test (Figure 4). The angle was statistically higher in patients in whom the wire or dilation device was unable to pass the first implanted SEMS than in patients in whom the wire or dilation catheter passed the first implanted SEMS [93.0 degrees (55-109.3) *vs* 44.2 degrees (7-119.0),

Table 3 Comparison of the endoscopic retrograde cholangiopancreatography procedures procedures employed in the success and failure groups (Angle between the target biliary duct stricture and the first implanted self-expandable metallic stent > 49.7 degrees) *n* (%)

	Success (*n* = 19)	Failure (*n* = 12)	*P* value
Diameter of the first implanted SEMS, median (range), mm	6.2 (3.1-15.9)	6.5 (4.4-17.3)	0.16
Wire passage of the first SEMS cell	19 (100)	8 (69.2)	0.02
Diameter of wire (0.025/0.035)	12/6[1]	5/6[1]	0.44
Catheter usage to dilate the a first SEMS cell	8	7	0.47
Catheter passage of the first SEMS cell	87.5 (7/8)	42.9 (3/7)	0.12
Dilator usage to dilate first SEMS cell	6	4	1.00
Dilator passage of the first SEMS cell	6 (100)	1 (25)	0.03
Balloon catheter usage to dilate the first SEMS lumen	1	1	1.00
Balloon catheter usage to dilate the first SEMS cell	7	2	0.42
Balloon catheter passage of the first SEMS cell	7 (100)	0 (0)	0.03
The number of used dilation devices, median (range)	1 (0-2)	1 (0-3)	0.76
Type of first SEMS used (braided/laser)	13/6	8/4	1.00
Stenting order			0.53
Left→Left	1	1	
Left→Right	12	5	
Right→Left	6	6	
Procedure sessions			0.27
1	16	12	
2	3	0	
Area of first SEMS cell, median (range), mm^2	18.3 (3.5-18.3)	18.3 (3.5-18.3)[2]	0.96

[1]Data for one patient were not available; [2]Data for two patients were not available. SEMS: Self-expandable metallic stent.

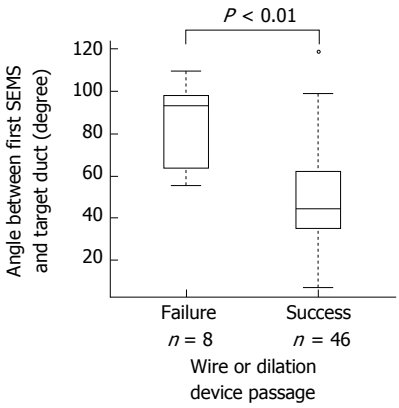


Figure 4 Influence of the angle between the target biliary duct stricture and the first retained self-expandable metallic stent on wire or dilation device passage. The angle between the target biliary stricture and the first implanted self-expandable metallic stent in patients without wire or dilation catheter passage was significantly greater than the corresponding angle in the patients with wire or dilation catheter passage [93.0 degree (55-109.3) *vs* 44.2 degree (7-119.0), $P < 0.01$, median (range)].

$P < 0.01$, median (range)]. Therefore, the findings demonstrate that the angle influences not only the second SEMS placement but also the passage of the wire or dilation device through the first SEMS cell.

Finally, we considered how to increase the success rate of stent-in-stent deployment of SEMSs in patients with larger angles. Upon comparing patients in the success and failure groups with larger angles (Table 3), the diameter of the first implanted SEMS was not significantly different. According to this result, the cause of the failure of the second SEMS insertion was not radial force. The passage of dilation devices was significantly different between the two groups. As described above, we used SEMSs recommended in past reports. Based on this study, we believe that improvements in dilation devices have contributed to overcoming such difficult cases. As the second SEMS insertion was effective in improving liver or biliary function in some patients (clinically effective rate from Table 2), an improvement in dilation devices is desired.

A limitation of this study is that it was a retrospective study that involved a small number of patients at one institution. Most reports regarding stenting to treat malignant hilar biliary obstruction consider the devices used here. It would be difficult to conduct a prospective study of the predictive factors for failure to place an endoscopic stent-in-stent SEMS. However, a larger multicentre study should be performed in the future.

In conclusion, we revealed that the angle between the target biliary duct stricture and the first implanted SEMS has an impact on the passing of both wire and dilation devices through the first SEMS cell and the placement of the second SEMS. We determined that a large angle between the target biliary duct stricture and the first implanted SEMS is a predictive risk factor for endoscopic stent-in-stent SEMS placement failure.

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COMMENTS

Background

Endoscopic biliary drainage is the first choice for biliary drainage in unresectable malignant biliary obstruction patients. Among drainage stents, metallic stents are more effective than plastic stents. The placement of multiple endoscopic self-expandable metallic stents (SEMSs) to relieve a malignant biliary obstruction is often challenging. Therefore, we investigated the factors predictive of failure when placing a second SEMS.

Research frontiers

Regarding endoscopic stent-in-stent SEMS insertion, which stents should be used is major topic of discussion. On the other hand, predictive risk factors of endoscopic stent-in-stent SEMS placement failure have not been sufficiently validated.

Innovations and breakthroughs

This is the first report to investigate the relationship between details of the biliary obstructive state and the failure of endoscopic stent-in-stent SEMS placement. We found that a large angle between the target biliary duct stricture and the first implanted SEMS is a predictive risk factor for endoscopic stent-in-stent SEMS placement failure.

Applications

If the risk factors of endoscopic stent-in-stent SEMS placement failure are known, time spent on procedures will not be wasted. The procedure is completed as a unilateral biliary drainage procedure or another rapidly drainage technique, such as percutaneous transhepatic biliary drainage.

Terminology

Endoscopic retrograde cholangiopancreatography: An endoscope is inserted into the descending portion of the duodenum. After a catheter is inserted into the biliary duct or pancreatic duct, a contrast agent is injected to visualize these ducts under X-ray fluoroscopy. Images of the biliary duct or pancreatic duct are observed. SEMSs: This stent is a metallic stent that can expand after it is placed in the malignant biliary stricture.

Peer-review

This is an interesting retrospective study attempting to identify predictive factors for unsuccessful deployment of a second stent when placement of multiple metal stents (MS) was attempted in the stent-in-stent manner for unresectable malignant perihilar biliary obstruction. The authors evaluated many factors, including etiology, findings of cholangiography, and procedural factors, and concluded that the duller (larger) angle between the first deployed stent and the target duct for next placement was the important factor. This is a very interesting issue and your conclusion seems informative.

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ORIGINAL ARTICLE

Retrospective Study

Assessment of colon polyp morphology: Is education effective?

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Abstract

AIM

To determine the inter-observer variability for colon polyp morphology and to identify whether education can improve agreement among observers.

METHODS

For purposes of the tests, we recorded colonoscopy video clips that included scenes visualizing the polyps. A total of 15 endoscopists and 15 nurses participated in the study. Participants watched 60 video clips of the polyp morphology scenes and then estimated polyp morphology (pre-test). After education for 20 min, participants performed a second test in which the order of 60 video clips was changed (post-test). To determine if the effectiveness of education was sustained, four months later, a third, follow-up test was performed with the same participants.

RESULTS

The overall Fleiss' kappa value of the inter-observer agreement was 0.510 in the pre-test, 0.618 in the post-test, and 0.580 in the follow-up test. The overall diagnostic accuracy of the estimation for polyp morphology in the pre-, post-, and follow-up tests was 0.662, 0.797, and 0.761, respectively. After education, the inter-observer agreement and diagnostic accuracy of all participants improved. However, after four months, the inter-observer agreement and diagnostic accuracy of expert groups were markedly decreased, and those of beginner and nurse groups remained similar to pre-test levels.

CONCLUSION

The education program used in this study can improve inter-observer agreement and diagnostic accuracy in assessing the morphology of colon polyps; it is especially effective when first learning endoscopy.

Key words: Colon polyp; Morphology; Education

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Core tip: Paris classification is used worldwide in clinical practice to categorize the morphology of gastrointestinal polyps. However, few studies regarding the inter-observer variability associated with this classification have been reported. In this study, we identified that an education program could be helpful in improving inter-observer agreement and diagnostic accuracy when determining the morphology of colon polyps, and that the educational program was especially effective when first learning endoscopy. We suggest that daily practice of morphology assessment of colon polyps and the proper education of the Paris classification are essential in maintaining the quality of colonoscopic examination.

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INTRODUCTION

Adenomatous polyps are the most common neoplasm detected during colonoscopic examination, and patients with this type of polyp have a greater risk of developing advanced neoplasia[1-5]. According to the National Polyp Study[6,7], colonoscopy is a useful tool in detecting colorectal polyps, and colonoscopic removal of adenomatous polyps prevents death from colorectal cancer (CRC). The size, location, and grade of polyp dysplasia are correlated with potential risks of malignant evolution[8-10], and the endoscopic appearance of polyps can predict invasion into the submucosa[11-13]. The invasive growth rates of depressed lesions, sessile lesions, and pedunculated lesions are 61%, 34% and 5%, respectively[14,15]. Therefore, polyp morphology is an important factor in determining the therapeutic method of endoscopic treatment or surgery.

When a colon polyp is confined to the mucosa or upper part of the submucosa, it can be safely removed by endoscopic procedure. However, insufficient recognition of lesions, particularly flat lesions including sessile serrated adenoma/polyps (SSA/P), can result in incomplete endoscopic resection, which is strongly associated with interval cancer[16,17]. In 2002, Western and Japanese endoscopists, surgeons, and pathologists gathered and presented the Paris classification of superficial neoplastic lesions in the digestive tract[14]. The Paris classification is now utilized worldwide to classify the morphology of polyps in clinical practice. However, few studies regarding inter-observer variability associated with this classification have been reported. Recently, Van Doorn *et al*[18] assessed the inter-observer agreement for the Paris classification among seven Western expert endoscopists and found that agreement was moderate and did not change after training. They suggested that the usefulness and the educational impact of this classification in clinical practice are questionable, and that the introduction of a simplified classification system is needed. However, additional studies that include Eastern expert endoscopists are necessary in order to generalize these results.

In the current study, we aimed to evaluate inter-observer variability for colon polyp morphology and to determine the effectiveness and persistence of an education program for both Korean experts and beginners.

MATERIALS AND METHODS

Creation of the video clips

For purposes of this study, colon polyps detected by colonoscopy were recorded as photographic images. Two experienced gastroenterologists (Kwon HJ and Choi YJ) performed colonoscopic examinations and recorded several pictures of polyps at various alignments. From these, a study investigator (Nam KS) selected five to seven for each polyp and created a video clip by connecting the pictures using Windows Movie Maker (Microsoft, Redmond, Washington, United States). A total of 80 video clips were created and edited to 10-15 s in length. Sixty video clips were used for the exam and 20 video clips for the learning sets. To minimize any bias in assessing polyp morphology, all video clips were reviewed by two experienced gastroenterologists (Jung K and Kim SE).

Examination and educational program for participants

This study was approved by the Institutional Review

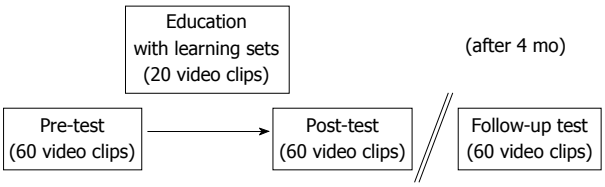


Figure 1 Flowchart of the study.

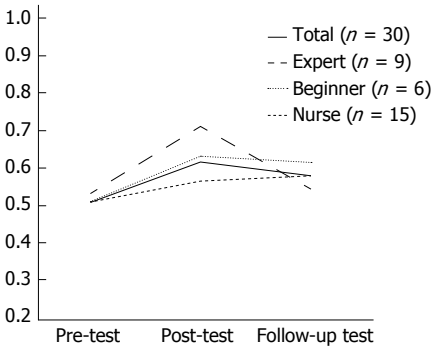


Figure 2 Inter-observer agreement of the estimated polyp morphology.

Board of Kosin University Gospel Hospital. All study participants were provided informed written consent prior to study enrollment. Fifteen endoscopists and 15 nurses participated in the study. Nine of the endoscopists were experienced (expert group) and had performed more than 1000 colonoscopies, six were endoscopic training fellows (beginner group) who had performed fewer than 300 colonoscopies, and 15 were nurses working in endoscopic practice (nurse group). The study included a pre-test, an educational program, a post-test, and a follow-up test, as shown in Figure 1.

On the day of the exam, participants received an explanation about the aim of the study and were asked to assess 60 polyps according to the Paris classification. They watched 60 video clips and recorded (in writing) the polyp morphology (Ip, Isp, Is, Ⅱa, Ⅱb, Ⅱc, or Ⅲ) ("pre-test"). After the first exam, a study investigator presented a 20-min educational program that included a detailed explanation of the Paris classification and 20 learning video clips that consisted of standard pictures illustrating the Paris classification ("education"). Following this, participants completed the second exam, in which the order of 60 video clips ("post-test") was changed. Four months later, we randomly administered the third exam ("follow-up" test) to the same participants to determine if the effectiveness of education was sustained. When analyzing exam results, we assessed inter-observer agreement and the diagnostic accuracy of each exam and compared the results.

Statistical analysis

Inter-observer agreement was evaluated by calculating the Fleiss' kappa value between participants. We assessed inter-observer agreement for all participants and compared expert, beginner, and nurse groups. Kappa values below 0.40 and of 0.41-0.60, 0.61-0.80, and greater than 0.80 were defined as fair agreement, moderate agreement, substantial agreement, and almost perfect agreement, respectively. We also evaluated the assessed category of polyp morphology for each polyp. For example, if the participants all chose Ip for a polyp, it was assessed as one category per one question. If the participants chose Is or IIa for a polyp, it was assessed as two categories per one question. We also estimated and compared the diagnostic accuracy of the three groups. Statistical analyses were performed using SPSS Statistics 20.0 (IBM®, Armonk, NY, United States).

Table 1 Characteristics of colon polyps in the exam *n* (%)

	Number
Size	
⩽ 5 mm	14 (23.3)
5 < and ⩽ 10 mm	32 (53.4)
⩾ 10 mm	14 (23.3)
Type	
Ip	14 (23.3)
Isp	9 (15.0)
Is	19 (31.7)
Ⅱa	16 (26.6)
Ⅱb	0 (0.0)
Ⅱc	1 (1.7)
Ⅲ	1 (1.7)
Location	
Rectum	5 (8.3)
Sigmoid colon	24 (40.0)
Descending colon	5 (8.3)
Transverse colon	13 (21.7)
Ascending colon	11 (18.4)
Cecum	2 (3.3)
Histology	
TA with LGD	32 (53.3)
TA with HGD	3 (5.0)
VTA with LGD	2 (3.3)
VTA with HGD	1 (1.7)
Adenocarcinoma	4 (6.7)
Chronic colitis	13 (21.7)
Hyperplastic polyp	3 (5.0)
Inflammatory polyp	2 (3.3)

TA: Tubular adenoma; LGD: Low grade dysplasia; HGD: High grade dysplasia; VTA: Villotubular adenoma.

RESULTS

The colon polyp characteristics used in the exam are summarized in Table 1. Figure 2 illustrates the inter-observer agreement of the estimated polyp morphology. The overall Fleiss' kappa value of the inter-observer agreement was 0.510 in the pre-test, 0.618 in the post-test, and 0.580 in the follow-up test. In the expert group, the inter-observer agreement improved after education (0.533 in pre-test → 0.713 in post-test); however, after four months, the agreement decreased to a level similar to that of the pre-test (0.544 in the follow-up test). In the beginner group, the inter-

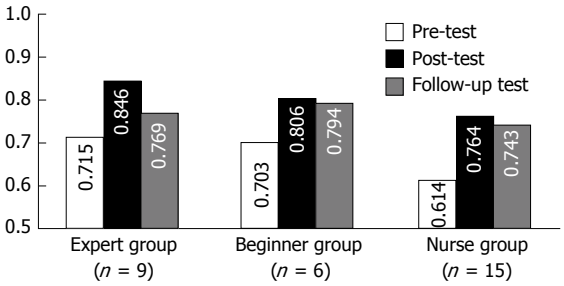


Figure 3 Diagnostic accuracy of the estimation for polyp morphology.

Table 2 Difference in assessment of polyp morphology per polyp *n* (%)

	Pre-test	Post-test	Follow-up test
Number of polyps			
One category per one question			
Ip	9 (15.0)	8 (13.3)	12 (20.0)
Isp	6 (10.0)	3 (5.0)	0 (0.0)
Is	0 (0.0)	6 (10.0)	3 (5.0)
Ⅱa	0 (0.0)	11 (18.3)	6 (10.0)
Total	15 (25.0)	28 (46.7)	21 (35.0)
Two categories per one question			
Ip-Isp	6 (10.0)	8 (13.3)	5 (8.3)
Isp-Is	7 (11.7)	7 (11.7)	8 (13.3)
Is-Ⅱa	24 (40.0)	12 (20.0)	20 (33.3)
Ⅱa-Ⅱc	0 (0.0)	1 (1.7)	1 (1.7)
Ⅱc-Ⅲ	1 (1.7)	1 (1.7)	1 (1.7)
Total	38 (63.3)	29 (48.3)	35 (58.3)
Three categories per one question			
Ip-Isp-Is	1 (1.7)	1 (1.7)	4 (6.7)
Isp-Is-Ⅱa	5 (8.3)	1 (1.7)	0 (0.0)
Is-Ⅱa-Ⅱc	1 (1.7)	0 (0.0)	0 (0.0)
Ⅱa-Ⅱb-Ⅱc	0 (0.0)	1 (1.7)	0 (0.0)
Total	7 (11.7)	3 (5.0)	4 (6.7)

observer agreement improved after education (0.514 in pre-test → 0.631 in post-test); after four months, the agreement slightly decreased to a value similar to that at the post-test (0.616 in the follow-up test). In the nurse group, the inter-observer agreement improved after education (0.508 in pre-test → 0.566 in post-test); after four months, the agreement was slightly improved to a value similar to that at the post-test (0.576 in follow-up test).

In Table 2, the difference in assessment of polyp morphology was shown for each test. The proportion of one category per one question was 25.0% in the pre-test, 46.7% in the post-test, and 35.0% in the follow-up test. The proportion of two categories per one question was 63.3% in the pre-test, 48.3% in the post-test, and 58.3% in the follow-up test. The proportion of three categories per one question was similar in each test.

The overall diagnostic accuracy of the estimation for polyp morphology in the pre-test, post-test, and follow-up tests was 0.662, 0.797, and 0.761, respectively (data not shown). Figure 3 shows the diagnostic accuracy of the estimation for polyp morphology of each group. In the expert group, the diagnostic accuracy was improved after education (0.715 in pre-test → 0.846 in post-test); however, after four months, the diagnostic accuracy decreased (0.769 in the follow-up test). In the beginner and nurse groups, diagnostic accuracy improved after education (beginner group, 0.703 in pre-test → 0.806 in post-test; nurse group, 0.614 in pre-test → 0.764 in post-test); after four months, the diagnostic accuracy was similar to the value at the post-test (beginner group, 0.794 in follow-up test; nurse group, 0.743 in follow-up test).

DISCUSSION

In this study, 15 endoscopists and 15 nurses participated in a total of three exams to evaluate the validity of the Paris classification in assessing colon polyp morphology. When analyzing exam results, we found that the education improved inter-observer agreement and the diagnostic accuracy of estimated polyp morphology. In addition, the effectiveness of education was sustained over time in the beginner and nurse groups, especially.

The Paris classification is a system that classifies the endoscopic appearance of superficial neoplastic lesions in the digestive tract and distinguishes polypoid and non-polypoid lesions[14]. Polypoid lesions are divided into pedunculated (Ip) or sessile (Is) types, and non-polypoid lesions are divided into superficial slightly elevated (Ⅱa), flat (Ⅱb), superficial depressed (Ⅱc), and excavated (Ⅲ) types[14]. The awareness of non-polypoid lesions and technical advances including chromoendoscopy, high-resolution endoscopy, and magnifying endoscopy have contributed to a higher detection rate of early colorectal carcinoma[19,20]. The detection rate of adenoma has been shown to be closely associated with the risk of interval CRC[21]. In a systematic review, the miss rate for adenomatous polyps was 13% for sizes of 5-10 mm and 26% for sizes of 1-5 mm, and the miss rate for non-adenomatous polyps was 22% with a size < 10 mm[22]. Also importantly, it holds true that we can see only what we already know[23]. Before the endoscopic findings of SSA/P were reported, the detection of SSA/P was rare, and even detected SSA/Ps were considered to be hyperplastic polyps[16,24]. Therefore, proper awareness of the Paris classification in assessing colon polyp morphology could be helpful in increasing polyp detection rates.

We assessed inter-observer agreement and diagnostic accuracy for colon polyp morphology in three exams, pre-, post-, and follow-up tests. After education for 20 min, inter-observer agreement and diagnostic accuracy improved. These results demonstrated that a short, didactic education program was effective in correcting individuals' misunderstandings when assessing the morphology of colon polyps according to the Paris classification. However, after four months,

the inter-observer agreement and diagnostic accuracy of expert groups were markedly decreased, whereas those of beginner and nurse groups were similar to the level of the post-test. Van Doorn *et al*[18] identified that the inter-observer agreement of seven Western experts was moderate and unchanged at three months post-training. In our study, test results for nine experts were similar to those of the Western experts; however, the test results of beginner and nurse groups were different from those of the experts. These results indicate that the effectiveness of education for polyp morphology is lasting for beginner and nurse groups, but not for the expert group. Members of the expert group had performed more than 1000 colonoscopies over an extended time and assessed the morphology of colon polyps according to their own understanding of the Paris classification. This might explain why the educational effect in this group did not last more than a few months. The beginner and nurse groups had little to no experience with colonoscopic examination and had utilized the Paris classification for a short time. For this reason, the education for polyp morphology in the beginner and nurse groups could more easily alter their existing knowledge, and the educational effect could last for several months. These findings suggest that appropriate education at the onset of learning endoscopy is effective in improving inter-observer agreement and diagnostic accuracy when assessing the morphology of colon polyps.

This study has some limitations. First, it was performed in a single medical center in Korea. Although we included a total of 30 participants, our results might not represent the practice of Eastern endoscopists as a whole. However, because data are limited regarding Eastern endoscopists' assessments of colon polyp morphology according to the Paris classification, our results could be helpful in assessing the validity and usefulness of the Paris classification in clinical practice. Second, we did not evaluate inter-observer agreement or diagnostic accuracy according to polyp size. Polyps used in the exam were collected in various sizes (Table 1), in order to minimize selection bias. Third, our study included a small number of type Ⅱb, type Ⅱc, and type Ⅲ polyps (3.4%). According to the results of Kudo *et al*[25] who classified the morphology of 9533 colon polyps, 57% were type I s, 39% were type Ⅱa or Ⅱb, 4% were type Ⅱc, and 0% were type Ⅲ. Therefore, it is difficult to fully assess the inter-observer variability for type IIb, type Ⅱc, and type Ⅲ polyps due to their rarity.

In conclusion, this study is the first to evaluate the validity of the Paris classification system in Eastern countries for evaluating the morphology of colon polyps. Our results indicate that an education program could be helpful in improving inter-observer agreement and diagnostic accuracy when determining colon polyp morphology, and such an educational program is expected to be especially effective upon first learning endoscopy. Based on these study results, we suggest that daily practice of morphology assessment of colon polyps and proper education in the Paris classification are essential in maintaining the quality of colonoscopic examination.

COMMENTS

Background

The Paris classification is an international classification system for describing gastrointestinal tract polyp morphology based on endoscopic appearances. However, there is little data regarding inter-observer variability and the impact of education about this classification on clinical practice. In this study, they aimed to determine the inter-observer variability for colon polyp morphology and to identify whether education can improve agreement among observers.

Research frontiers

This study presents that the education program used in this study can improve inter-observer agreement and diagnostic accuracy in assessing the morphology of colon polyps; it is especially effective when first learning endoscopy.

Innovations and breakthroughs

In this study, a total of 30 Korean endoscopists and nurses participated. This study included a pre-test, an educational program, a post-test, and a follow-up test. The authors assessed inter-observer agreement for all participants and compared expert, beginner, and nurse groups. The authors also estimated and compared the diagnostic accuracy of the three groups.

Applications

Daily practice of morphology assessment of colon polyps and the proper education of the Paris classification are essential in maintaining the quality of colonoscopic examination.

Terminology

Pre-test: a test without any education. Post-test: a test after a 20-min educational program. Follow-up test: a test after four months without an educational program

Peer-review

Based on these study results,a proper education in the Paris classification are important to maintaining the quality of colonoscopicexamination, especially in new learner in endoscopy.

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ORIGINAL ARTICLE

Retrospective Study

Body mass index does not affect the survival of pancreatic cancer patients

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Abstract

AIM

To evaluate the association of body mass index (BMI) with the overall survival of pancreatic ductal adenocarcinoma (PDAC) patients.

METHODS

A retrospective analysis of PDAC patients diagnosed in the National Cancer Center of China between January 1999 and December 2014 was performed. These patients were categorized into four BMI groups (< 18.5, 18.5-22.9, 23-27.4 and ⩾ 27.5 kg/m^2). χ^2 tests for comparison of the proportions of categorical variables, and Student's *t*-test or Mann-Whitney test for continuous variables were employed. Survival analysis was performed with the Kaplan-Meyer method. Their HRs of mortality and 95%CIs were estimated using the Cox proportional hazards model.

RESULTS

With a median age of 59.6 years (range: 22.5-84.6 years), in total 1783 PDAC patients were enrolled in this study. Their mean usual BMI was 24.19 ± 3.53 for the whole cohort. More than half of the patients (59.3%) experienced weight loss during the disease onset and progression. Compared with healthy-weight individuals, newly diagnosed patients who were overweight or obese had more severe weight loss during their disease onset and progression ($P < 0.001$). Individuals who were overweight or obese were associated with positive smoking history ($P < 0.001$). A significant difference in comorbidity of diabetes ($P = 0.044$) and coronary artery disease ($P < 0.001$) was identified between high BMI and normal-weight patients. After a median follow-up of 8 mo, the survival analysis showed no association between BMI and the overall survival ($P = 0.90$, $n = 1783$). When we stratified the whole cohort by pancreatic cancer stage, no statistically significant association between BMI and overall survival was found for resectable ($P = 0.99$, $n = 217$), unresectable locally advanced ($P = 0.90$, $n = 316$) and metastatic patients ($P = 0.88$, $n = 1250$), respectively. The results did not change when we used the BMI at diagnosis.

CONCLUSION

Our results showed no significance of BMI for the overall survival of PDAC patients.

Key words: Body mass index; Pancreatic cancer; Overweight; Smoking history; Survival analysis

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Core tip: It remains controversial whether body mass index (BMI) influences the prognosis of pancreatic cancer. The strengths of this study included a large quantity of patients and the accurate BMI categorization according the Asian criterion. To the best of our knowledge, it is the largest study in Asia to evaluate the prognostic role of BMI on the overall survival of pancreatic cancer patients. Our results showed no significant influence of BMI on the overall survival of pancreatic ductal adenocarcinoma patients and are consistent with those of many other studies.

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INTRODUCTION

Pancreatic cancer is a devastating malignant cancer, with a 5-year survival rate of < 6%[1]. The survival length of patients is greatly influenced by disease stage at diagnosis, but few other survival markers have been well characterized[2-4].

Excessive adipose can impair both humoral and cellular immunity, which may be important in the development of pancreatic cancer. Although many studies have demonstrated that a high body mass index (BMI), an indirect measure of adiposity, is associated with increased risk of developing pancreatic cancer, it remains controversial whether BMI influences the prognosis of pancreatic cancer. Several studies have reported that a higher BMI is associated with decreased survival of pancreatic cancer patients[5-10], whereas other results informed that BMI was not a statistically significant prognostic factor[11-15]. Tsai *et al*[16] even expressed improved long-term survival of pancreatic cancer patients with higher BMIs. As such, we conducted a single-center, large-scale retrospective study to determine the associations between BMI and overall survival of Chinese pancreatic ductal adenocarcinoma (PDAC) patients.

MATERIALS AND METHODS

Patients

The medical records of all patients diagnosed with PDAC from January 1999 to December 2014 in the Cancer Hospital, Chinese Academy of Medical Science and Peking Union Medical College were studied by retrospective chart review. All patients had histological diagnosis of PDAC confirmed by pathology. Clinical information was systematically abstracted from these patients' medical records using an established abstraction form by trained individuals with a medical background. For the whole cohort, the extracted information included age, sex, weight, height, smoking history, alcohol use history, previous history of cancer, and previous abdominal surgery. The patients' height and weight of 1 year prior to diagnosis and at diagnosis were all recorded. Comorbidities were also recorded, such as diabetes, coronary artery disease and hypertension. Clinical disease stage was defined on the basis of the patients' initial computed tomographic images and endoscopic ultrasound reports, as stated in the American Joint Committee on Cancer Staging Manual using the TNM staging system. Follow-up information was obtained through telephone-based interviews, as well as from the records of outpatient follow-up, if any. The primary outcome variable was overall survival, defined for patients as the period from date of diagnosis to date of death. Informed consent was obtained from all individual participants included in the study.

Statistical analysis

BMI was calculated as weight (kg) divided by the square of height (m^2), using self-reported usual adult height and weight. According to the Asian criterion, BMI was classified as underweight (< 18.5 kg/m^2),

Table 1 Demographics, comorbidity and stage of patients grouped according to body mass index

Characteristic	All patients	BMI category, kg/m^2				*P* value
		< 18.5	18.5-22.9	23-27.4	⩾ 27.5	
Patients	1783	98 (5.5)	588 (33.0)	791 (44.4)	306 (17.2)	
Sex						0.97
Male	1009 (56.6)	54 (3.0)	334 (18.7)	445 (25.0)	176 (9.9)	
Female	774 (43.4)	44 (2.5)	254 (14.2)	346 (19.4)	130 (7.3)	
Male/female ratio	1.30	1.23	1.31	1.29	1.35	
BMI as kg/m^2, mean ± SD	24.19 ± 3.53	17.60 ± 0.84	21.16 ± 1.23	25.07 ± 1.29	29.68 ± 2.11	
Age at diagnosis in yr						
Median	59.6	62.3	59.0	59.6	59.7	
Range	22.5-84.6	28.7-81.7	22.5-80.2	30.7-84.6	26.0-84.6	
Weight loss as % of usual adult weight						
None	725 (40.7)	61 (3.4)	333 (18.7)	299 (16.8)	32 (1.8)	
> 0, ⩽ 10%	652 (36.6)	23 (1.3)	190 (10.7)	321 (18.0)	118 (6.6)	
> 10%	406 (22.8)	14 (0.79)	65 (3.6)	171 (9.6)	156 (8.7)	< 0.0001
Any weight loss	1058 (59.3)	37 (2.1)	255 (14.3)	492 (27.6)	274 (15.4)	< 0.0001
Smoking						0.0003
Never	1243 (69.7)	72 (4.1)	408 (22.9)	568 (31.9)	195 (10.9)	
Ever	527 (29.6)	22 (1.2)	176 (9.9)	220 (12.3)	109 (6.1)	
Alcohol						0.42
Never	1377 (77.2)	76 (4.3)	465 (26.1)	614 (34.4)	222 (12.5)	
Ever	398 (22.3)	21 (1.2)	121 (6.8)	174 (9.8)	82 (4.6)	
Comorbidity						
Diabetes	398 (22.3)	13 (0.7)	125 (7.0)	196 (11.0)	64 (3.6)	0.044
Coronary artery disease	247 (13.9)	8 (0.4)	46 (2.6)	166 (9.3)	27 (1.5)	< 0.0001
Hypertension	267 (15.0)	9 (0.5)	88 (4.9)	111 (6.2)	59 (3.3)	0.055
Previous history of cancer	14 (0.8)	1 (0.06)	6 (0.34)	6 (0.34)	1 (0.06)	0.72
Previous abdominal surgery	87 (4.9)	7 (0.39)	34 (1.9)	34 (1.9)	12 (0.67)	0.35
Stage group						0.89
Resectable	217 (12.2)	15 (0.84)	73 (4.1)	93 (5.2)	36 (2.0)	
Unresectable locally advanced	316 (17.7)	17 (0.95)	108 (6.1)	143 (8.0)	48 (2.7)	
Metastatic	1250 (70.1)	66 (3.7)	407 (22.8)	555 (31.1)	222 (12.5)	

Data are *n* (%), unless otherwise indicated. BMI: Body mass index.

healthy weight (18.5-22.9 kg/m^2), overweight (23.0-27.4 kg/m^2), and obese (⩾ 27.5 kg/m^2). χ^2 tests for comparison of the proportions of categorical variables, and Student's *t*-test or Mann-Whitney test for continuous variables were employed. The unconditional logistic regression model was used to estimate the ORs and 95%CIs for the associations among BMI, clinical characteristics and overall survival of PDAC patients, and for the PDAC subtypes in different clinical stages. Survival analysis was performed by the Kaplan-Meyer method. The group with BMI 18.5-22.9 kg/m^2 was the reference group. All *P*-values presented were two-sided, and all analyses were carried out using SAS Software Version 9.2 (SAS Institute, Cary, NC, United States). Statistical significance was defined as a *P* value of < 0.05.

RESULTS

Clinical characteristics

Data were extracted from a total of 1783 subjects with PDAC who had complete medical history. Histological or cytological diagnosis was obtained from the primary pancreatic lesion or metastases in all cases. The pathology was confirmed by two pathologists. Baseline characteristics of 1783 patients with pancreatic cancer are listed in Table 1. Of those subjects, 1009 (56.6%) were male, with a median age of 59.6 years. Among the total, 29.6% patients had a positive smoking history, 22.3% had a drinking history, and 22.3%, 13.9% and 15.0% patients had diabetes, coronary artery disease and hypertension at diagnosis of pancreatic cancer as concomitant comorbidities, respectively.

Among those with known disease stage, 217 had potentially resectable tumors at the time of diagnosis, 316 had locally advanced disease, and 1250 had distant metastasis. Their mean baseline BMI was 24.19 kg/m^2, and 44.4% of the patients were classified as overweight and 17.2% as obese. More than half of the patients (59.3%) experienced weight loss during their disease onset progression. Compared with healthy-weight individuals, the newly diagnosed patients who were overweight or obese had more severe weight loss during the disease onset progression ($P < 0.001$). Individuals who were overweight or obese are associated with a positive smoking history ($P = 0.0003$). A significant difference in comorbidity of diabetes ($P = 0.044$) and coronary artery disease ($P < 0.001$) was identified between high BMI values and normal-weight patients.

Survival analysis

The median follow-up duration was 8 mo for all cases. Median overall survival for each BMI category (kg/m^2)

Table 2 Pancreatic cancer survival by usual adult body mass index category

Stage group	BMI category	*n*	Median overall survival, mo (95%CI)	HR	95%CI	*P* value	P_{trend} value
Overall	< 18.5	98	8.0 (5.8-11.3)	1.12	0.83-1.52	0.45	
	18.5-22.9	588	9.7 (8.7-11.1)	1.00	Ref.	Ref.	
	23-27.4	791	9.5 (8.6-10.5)	0.99	0.87-1.14	0.92	
	⩾ 27.5	306	9.8 (7.6-11.2)	1.05	0.88-1.25	0.61	0.90
Resectable	< 18.5	15	11.1 (3.3-19.3)	1.92	0.74-5.01	0.18	
	18.5-22.9	73	15.6 (10.1-21.3)	1.00	Ref.	Ref.	
	23-27.4	93	16.7 (11.9-27.4)	0.92	0.59-1.44	0.71	
	⩾ 27.5	36	16.2 (4.5-23.7)	1.13	0.61-2.08	0.70	0.99
Unresectable locally advanced	< 18.5	17	7.0 (3.4-26.1)	0.83	0.38-1.84	0.65	
	18.5-22.9	108	8.7 (7.3-11.7)	1.00	Ref.	Ref.	
	23-27.4	143	8.6 (6.8-11.5)	1.03	0.73-1.45	0.86	
	⩾ 27.5	48	8.6 (4.9-15.6)	0.95	0.59-1.54	0.84	0.90
Metastatic	< 18.5	66	4.9 (2.8-8.0)	1.07	0.76-1.51	0.70	
	18.5-22.9	407	5.5 (4.0-6.4)	1.00	Ref.	Ref.	
	23-27.4	555	5.7 (3.7-6.8)	0.99	0.84-1.16	0.89	
	⩾ 27.5	222	5.6 (3.3-7.7)	1.02	0.84-1.27	0.76	0.88

BMI: Body mass index.

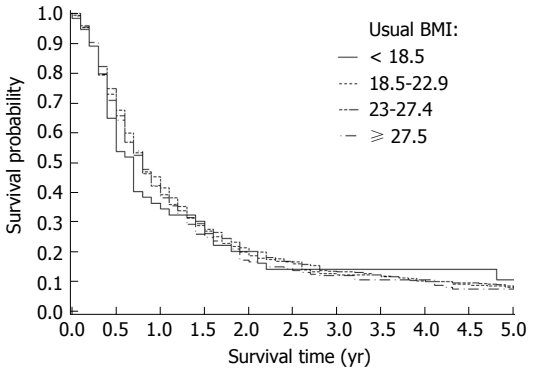


Figure 1 Unadjusted Kaplan-Meier analysis of overall survival among 1783 pancreatic cancer patients according to usual adult body mass index category. BMI: Body mass index.

was as follows: < 18.5: 8 mo; 18.5-22.9: 9.7 mo; 23.0-27.4: 9.5 mo; and ⩾ 27.5: 9.8 mo (Table 2). Kaplan-Meier survival comparisons (Figure 1) showed no association between BMI and the overall survival of pancreatic cancer patients ($P > 0.05$). When we stratified the whole cohort by pancreatic cancer stage, no statistically significant association between BMI and overall survival was found for resectable ($P = 0.99$, $n = 217$), unresectable locally advanced ($P = 0.90$, $n = 316$) and metastatic ($P = 0.88$, $n = 1250$) patients. The multivariate Cox regression survival analysis showed the same results (Figures 2 and 3). Considering the effect of cancer-related weight loss among patients, the results did not change when we used the BMI at diagnosis.

DISCUSSION

Overweight/obesity and its related morbidities represent a growing health problem, claiming 2.8 million lives annually according to the WHO 2010 Global Report[17]. Obesity has been found to be associated with a wide array of morbidities, including cardiovascular disease, chronic kidney disease, sleeping disorder, type 2 diabetes, and several forms of cancer. Obesity is considered to be an adverse prognostic factor in malignancies, such as breast and colon cancers[18,19]. In our study, a significant difference in comorbidity of diabetes and coronary artery disease was identified between high BMI values and normal-weight patients. But survival analysis showed that BMI did not affect the survival of pancreatic cancer patients.

Previous studies conferring the role of BMI were mainly carried out in cohorts of United States adults, which presented a higher percentage of obese (BMI ⩾ 30) and very obese (BMI ⩾ 35) patients and the BMI categorization as opposed to our Asian criterion. For a Mayo Clinic cohort of patients covering all stages of pancreatic cancer respectively, including early-stage patients who underwent surgery, McWilliams *et al*[6] reported that the BMI at diagnosis had a negative impact on survival. This was particularly pronounced in the very obese patients with a BMI of 35 to 39.99 kg/m^2 (HR = 1.32, 95%CI: 1.08-1.62) and > 40 kg/ m^2 (HR = 1.60, 95%CI: 1.26-2.04), respectively. In a case control study designed to assess the risk of developing pancreatic cancer in overweight persons, Li *et al*[5] described a shorter survival period for patients who were overweight or obese during the year prior to diagnosis. Another study from Olson *et al*[11] demonstrated that obesity was non-significantly associated with poorer survival of PDAC patients, particularly in the resected group (HR = 1.62, 95%CI: 0.76-3.44). The experience from Chen *et al*[20] showed that a higher BMI increases the risk for postoperative complications after pancreatectomy in the Chinese population. However, Tsai *et al*[16] reported that obese patients undergoing pancreaticoduodenectomy for pancreatic cancer had improved long-term survival, independent of known

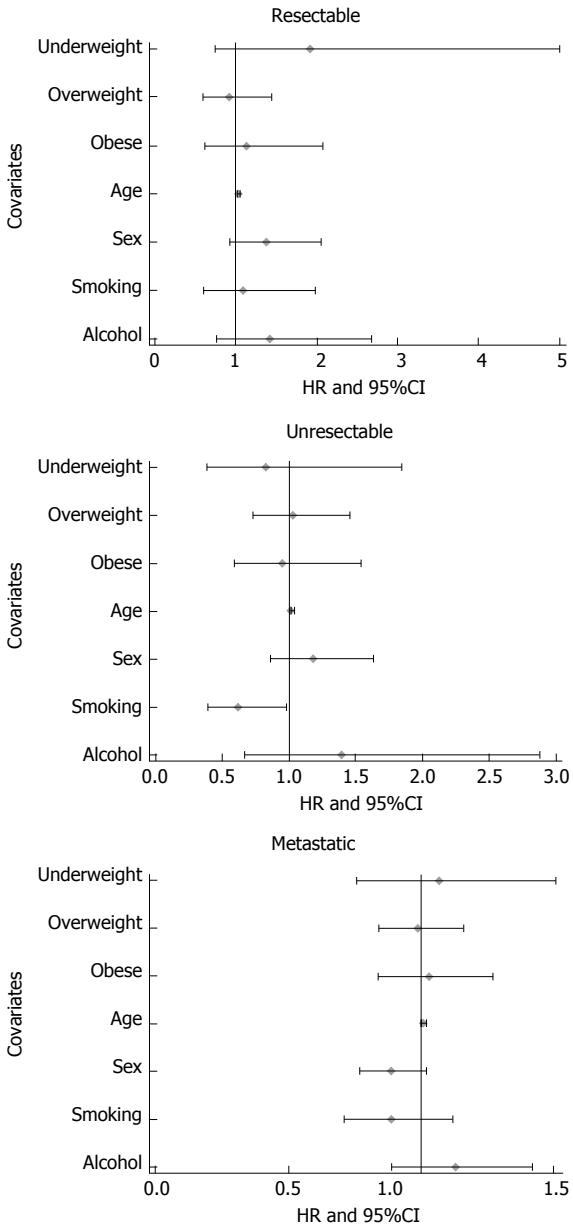


Figure 2 Body mass index and survival of pancreatic cancer patients by stage. Forest plots within stage groupings for resectable, locally advanced and metastatic patients at initial diagnosis of pancreatic cancer are shown.

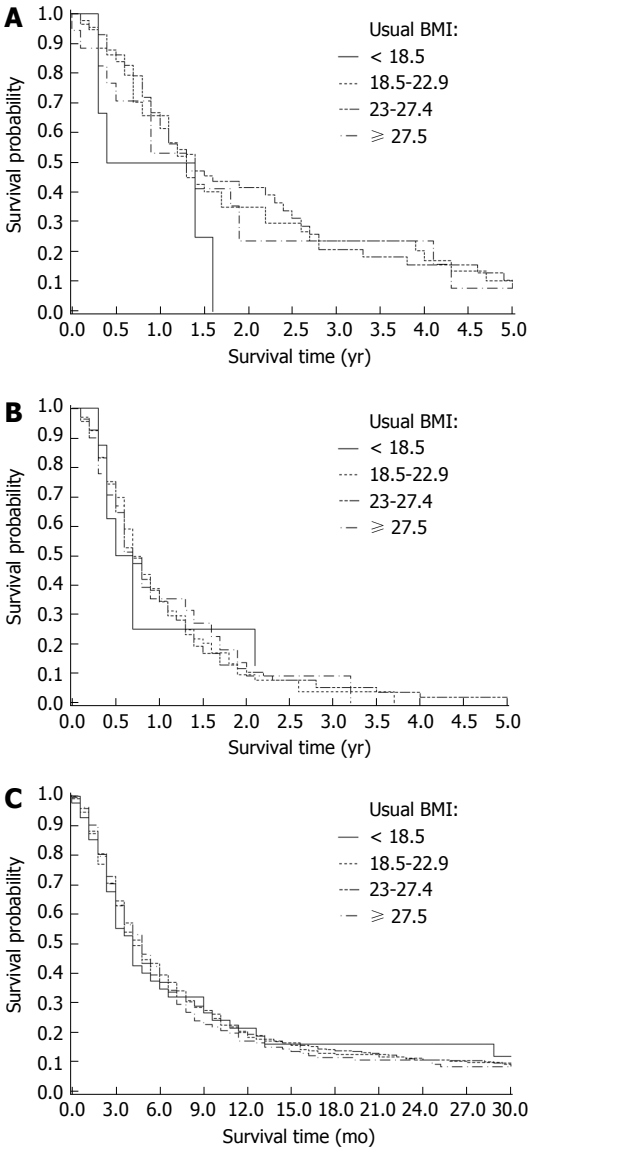


Figure 3 Kaplan-Meier analysis. A: Unadjusted Kaplan-Meier analysis among 217 pancreatic cancer patients undergoing radical resection according to BMI category; B: Unadjusted Kaplan-Meier analysis among 316 unresectable locally advanced pancreatic cancer patients according to BMI category; C: Unadjusted Kaplan-Meier analysis among 1250 pancreatic cancer patients with distant metastasis according to BMI category. BMI: Body mass index.

clinicopathologic factors. Big controversy has lasted about the real role of BMI on pancreatic cancer for years. The results in this study need to be replicated in more studies with larger sample sizes with a greater power.

Limited information was available on the possible effect of weight loss on the survival of PDAC patients. As we know, loss of weight is usual for pancreatic cancer patients either before or after diagnosis. In our study, more than half of the patients experienced weight loss during their disease progression, especially in the obese group, which might explain why the obesity-specific co-morbidities such as diabetes were not more prevalent in the obese group compared with the normal populations. Dalal *et al*[21] believed that obese patients experienced higher loss in weight, skeletal muscle and visceral adipose tissue, which may contribute to poorer survival of pancreatic cancer patients. So, we carried out the survival analysis using patients' BMIs at usual time as well as at diagnosis, but the results did not change.

The strengths of this study included a large quantity of patients and the accurate BMI categorization according the Asian criterion. To the best of our knowledge, it is the largest study in Asia to evaluate the prognostic role of BMI on the overall survival of pancreatic cancer patients. Our results showed no significant influence of BMI on the overall survival of PDAC patients and are consistent with those of many other studies[11-15]. We, however, must admit that

there are several limitations for the current study. The retrospective nature of this study can be associated with selection bias as well as increased risk of differential misclassification bias. Another shortcoming was the limited generalizability because the entire study population came from a single hospital.

In conclusion, BMI has not shown any clear relationship with the survival outcome of pancreatic cancer. More studies are needed to validate this finding and evaluate the mechanism behind the observation.

COMMENTS

Background

Excessive adipose can impair both humoral and cellular immunity, which may be important in the development of pancreatic cancer. Although many studies have demonstrated that a high body mass index (BMI), an indirect measure of adiposity, is associated with increased risk of developing pancreatic cancer, it remains controversial whether BMI influences the prognosis of pancreatic cancer. Several studies have reported that a higher BMI is associated with decreased survival of pancreatic cancer patients, whereas other results informed that BMI was not a statistically significant prognostic factor.

Research frontiers

The authors conducted a single-center, large-scale retrospective study to determine the associations between BMI and overall survival of Chinese pancreatic ductal adenocarcinoma (PDAC) patients.

Innovations and breakthroughs

To the best of our knowledge, this is the largest study in Asia to evaluate the prognostic role of BMI on the overall survival of pancreatic cancer patients.

Applications

In this study, BMI did not affect the survival of pancreatic cancer patients. The results provide more insight into the role of BMI on pancreatic cancer.

Peer-review

The present retrospective study demonstrated that BMI has no relationship with survival of PDAC in a large single center. The paper has high value for inclusion in the current literature on the topic.

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ORIGINAL ARTICLE

Observational Study

Access to biologicals in Crohn's disease in ten European countries

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Abstract

AIM

To analyze access (availability, affordability and acceptability) to biologicals for Crohn's disease (CD) in ten European countries and to explore the associations between these dimensions, the uptake of biologicals and economic development.

METHODS

A questionnaire-based survey combined with desk research was carried out in May 2016. Gastroenterologists from the Czech Republic, France, Germany, Hungary, Latvia, Poland, Romania, Slovakia, Spain and Sweden were invited to participate and provide data on the availability of biologicals/biosimilars, reimbursement criteria, clinical practice and prices, and use of biologicals. An availability score was developed to evaluate the restrictiveness of eligibility and administrative criteria applied in the countries. Affordability was defined as the annual cost of treatment as a share of gross domestic product (GDP) per capita. Correlations with the uptake of biologicals, dimensions of access and GDP per capita were calculated.

RESULTS

At the time of the survey, infliximab and adalimumab were reimbursed in all ten countries, and vedolizumab was reimbursed in five countries (France, Germany, Latvia, Slovakia, Sweden). Reimbursement criteria were the least strict in Sweden and Germany, and the strictest in Hungary, Poland and Slovakia. Between countries, the annual cost of different biological treatments differed 1.6-3.3-fold. Treatments were the most affordable in Sweden (13%-37% of the GDP per capita) and the least affordable in the Central and Eastern European countries, especially in Hungary (87%-124%) and Romania (141%-277%). Biosimilars made treatments more affordable by driving down the annual costs. The number of patients with CD on biologicals per 100000 population was strongly correlated with GDP per capita (0.91), although substantial differences were found in the uptake among countries with similar economic development. Correlation between the number of patients with CD on biologicals per 100000 population and the availability and affordability was also strong (-0.75, -0.69 respectively).

CONCLUSION

Substantial inequalities in access to biologicals were largely associated with GDP. To explain differences in access among countries with similar development needs further research on acceptance.

Key words: Crohn's disease; Biological therapy; Access; Inequality; Europe

Core tip: We carried out a questionnaire survey with gastroenterologists combined with desk research to analyze access to biologicals for Crohn's disease in ten European countries. Regarding availability, reimbursement criteria were the least restrictive in Sweden and Germany, and the most restrictive in Hungary, Poland and Slovakia. Between countries, the annual cost of biological treatments differed 1.6-3.3-fold. Treatments were the most affordable in Sweden and the least affordable in Hungary and Romania. The number of patients on biologicals per 100000 population was strongly correlated with gross domestic product, although substantial differences were found in the uptake among countries with similar economic development.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract that is characterized by diarrhea, abdominal pain, rectal bleeding, fever and fatigue. The prevalence of CD in Europe varies from 1.5 to 213 cases per 100000 persons[1,2]. Due to the early onset and chronic character of the disease, patients have to deal with a considerable impairment in health-related quality of life[3,4] and lower work capacity throughout their lifetime, leading to substantial economic burden both on patients and society[5].

Biological drugs have revolutionized the treatment of inflammatory bowel diseases, like CD. Infliximab was the first biological drug that received its marketing authorization in Europe by the European Medicines Agency (EMA) in 2001 for adult patients with moderately to severely active CD[6]. This was followed by adalimumab, registered in 2003, vedolizumab, registered in 2014 and ustekinumab registered in 2016. Recently, biosimilars of infliximab (2013, 2016) and of adalimumab (2017) have been registered as well. These drugs are monoclonal antibodies with different mechanisms of action (infliximab and adalimumab are anti-tumor necrosis agents, vedolizumab is an anti-integrin drug and ustekinumab is an interleukin-12 and -23 inhibitor) but with similar safety profile and comparable efficacy[7-9]. Clinical evidence confirmed the efficacy, safety and effectiveness of these drugs for the treatment of CD, as they substantially improve the ability to achieve disease remission, slow disease

progression, decrease the need of surgery and increase work participation and quality of life[6]. However, biologicals are more costly than standard treatments, annual cost of drug therapy is usually above €10000 per patient. Mainly due to the increase in the use of biological drugs, recent studies indicated that direct healthcare costs have shifted from hospitalization and surgery towards drug therapy[10-12].

Access to biologicals varies significantly between countries. This is largely driven by differences in budgetary constraints[3,13]. Due to the high price and budget impact of biologicals, most countries have regulated the access to reimbursed treatment. Differences in regulations lead to inequalities in access to biologicals even among European countries with a very similar economic situation[3,14]. Rencz *et al*[3] found an up to 96-fold difference in the uptake of biologicals for CD among nine selected countries. The reasons for this heterogeneity in access to biologicals among the Central and Eastern European countries (CEE countries) has not been clarified. However, according to the authors, it is not explained by differences in prevalence and incidence of inflammatory bowel diseases, prices of biologicals, total expenditure on health, geographical access, and cost-effectiveness.

The appearance of biosimilar drugs as potentially cost-effective alternatives is expected to lead to improvements in access to biological therapy. The first biosimilar infliximab drugs (brand names Remsima and Inflectra) were approved in 2013 by the EMA for the same indications as the original biological drug[10]. A couple prospective and a number of retrospective cohorts with biosimilar infliximab in inflammatory bowel diseases have confirmed that its efficacy and safety are comparable to those of the original biological product[3,10,14]. Biosimilars are substantially (20%-70%) cheaper than the originator. Budget impact analyses suggest that significant savings can be achieved with biosimilar infliximab in CD[14,15]. In Belgium, Germany, Italy, the Netherlands and the United Kingdom, the annual projected cost savings are estimated to be €11.95 million (10% price reduction) to €35.85 million (30% price reduction) in the case of CD patients[15]. Total cost savings achievable over three years in Bulgaria, the Czech Republic, Hungary, Poland, Romania and Slovakia are expected to be €8.0 million (switching not allowed) and €16.9 million (switching allowed in 80% of the patients)[16]. These savings can be used either to improve access to biological treatments (*e.g.*, increase the number of patients with access to biologicals), or can be allocated to other areas of care[17].

This study explores three different dimensions of access to biologicals (originators and biosimilars) for CD, namely availability, affordability and acceptability in ten selected European countries (the Czech Republic, France, Germany, Hungary, Latvia, Poland, Romania, Slovakia, Spain and Sweden).

These countries differ not only regarding their economic development but also regarding the organization and financing of their health care system, which might also influence the access to biological treatments[3,14,18]. While Spain and Sweden have a tax-based health care system, France and Germany follow the Bismarkian model with social health insurance. In the CEE countries (except for the Czech Republic) the share of public financing is usually lower than in the Western-European countries. The source of public resources is mainly tax revenue in Sweden and Spain, while it is social health insurance contribution in the rest of the countries (see further in details[19]).

Thus, in our study, we also aim to explore whether differences in availability and affordability of biologicals are associated with the uptake of biologicals (in terms of number of patients on biologicals per 100000 population) and the economic situation of the country or the financing of the health care system.

MATERIALS AND METHODS

Study design

A questionnaire was developed to collect information on access to biologicals and was sent in May 2016 to one expert (gastroenterologist) in each of the ten European countries included in the study, *i.e.*, the Czech Republic, France, Germany, Hungary, Latvia, Poland, Romania, Slovakia, Spain and Sweden. The questionnaire was developed based on questionnaires used in prior studies in rheumatoid arthritis of Putrik *et al*[18,20]. The country experts who were invited to fill in the questionnaire, were selected based on the principle of non-probability convenience sampling, which resulted in a sample drawn through the professional network of the researchers.

The questionnaire was sent to the 10 experts who accepted to take part in the survey (contributors of the paper). The returned questionnaires were checked and in case of missing or incomplete answers, the collaborating experts were contacted to clarify the information. Finally, a preliminary report including the results was sent to all collaborating experts for a review and data check.

The questionnaire-based survey was combined with desk research, where relevant indicators, such as countries' gross domestic product (GDP), population size and health care financing indicators were identified. The number of CD patients on biologicals was extracted from resources provided by the collaborating experts or was calculated from the total number of CD patients and the estimated share of CD patients on biologicals. Furthermore, drug prices and other data derived from the questionnaires, were also checked during the desk research.

Questionnaire

The questionnaire included questions on (1) the

availability, reimbursement status and prices of originator and biosimilar biologicals registered for CD at the time of the survey (Remicade, Remsima, Inflectra, Humira and Entyvio)[Stelara (ustekinumab) and Flexabi (biosimilar infliximab) were not in use at the time of the survey]; (2) the clinical and reimbursement guidelines and eligibility criteria for biological treatment of adults with luminal CD; (3) the number of adult CD patients in the given country and the use of biologicals; and (4) additional dimensions of access to biologicals. The collaborating experts were also asked to indicate the reference for the data they provided (*i.e.*, for drug prices, prevalence data and use of biologicals).

Analysis

The three dimensions of access - affordability, availability, acceptability were analyzed separately.

Availability: We identified the number of biologicals for CD registered and reimbursed in the ten countries based on data from the questionnaire and desk research. Based on these data, we also developed an availability score to assess the restrictiveness of clinical eligibility and administrative requirements to biologicals, based on the following items: (1) whether there is a required level of disease activity [such as Crohn's Disease Activity Index (CDAI)] or disease severity for initiation of biological treatment: not specified (0 point), CDAI > 220 (1 point), CDAI > 300 (2 points); (2) required failure of /intolerance to non-biological treatment before a patient is eligible for a biological: not required (0 point), steroids (1 point), immunosuppressive (1 point), steroids OR immunosuppressive (1 point), steroids AND immunosuppressive (2 points); (3) whether there are other administrative procedures required after the indication of the need of a biological is given: no other procedures (0 point), other requirements (*e.g.*, approval or authorization by the health insurance fund) (1 point); (4) whether only approved centers can administer biological treatment: no restriction to approved centers (0 point), restriction to approved centers (1 point); and (5) whether only specific specialists can indicate and prescribe biologicals for the treatment of CD in adults: gastroenterologist, immunologist and GP/other (0 point), gastroenterologist and immunologist only (1 point), gastroenterologist only (2 points).

For each country, the subscores were summed up to obtain the country availability score within the range 0-8. Higher score indicates more restrictive clinical eligibility criteria and administrative requirements.

Affordability: Based on data from the questionnaire and desk research, the annual drug cost per person (2016) was calculated for each drug available in a given country and was also presented as a percentage of the country GDP per capita, which was the affordability ratio in our study. The annual costs were calculated based on the prices provided by collaborating experts, which were verified in the desk-research. The sources of the acquisition cost of drugs were mainly state institutes for drug control, European drug databases, pharmaceutical companies and national health insurance funds. If prices found during the desk research were not matching with the answers given by the experts, clarification was asked from the respondents to identify the most accurate data. Information on drug dose and frequency of its administration for induction and maintenance therapy was taken from on EMA product information (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002782/WC500168528.pdf).

Acceptability: The questionnaire contained a question on barriers to access to biologicals. The collaborating experts were asked to indicate which of the following items they considered as a barrier to access to biologicals: limited availability of the drugs due to financial reasons; too strict reimbursement criteria; strict monitoring requirements; physicians' preferences; patients' co-payments; patients' preferences; limited access of patients to inflammatory bowel diseases centers (IBD centers); limited access of patients to health care in general. Experts were also asked to give an estimation on out of every 10 of their infliximab patients how many were treated with biosimilars.

We calculated Pearson's correlations between the affordability (defined as the annual cost of treatment as a percentage of GDP), the availability score (explained above), the number of CD patients on biologicals per 100000 population the GDP per capita, and the share of public expenditure in the total health expenditure and the share of governmental expenditure in the total public health expenditure. Significance level of 5% was used.

RESULTS

Availability

Registration, reimbursement: At the time of the survey (May 2016), five biologicals were approved by the European Medicine Agency (Remicade: 01/2001, Humira: 09/2003, Remsima, Inflectra: 09/2013, Entyvio: 05/2014.). Infliximab and adalimumab were reimbursed in all the ten countries. In Latvia and Hungary, only biosimilar infliximab was reimbursed for new patients. Entyvio was only reimbursed in France, Germany, Slovakia, Spain and Sweden. Furthermore, according to the experts, in Slovakia and Spain, Entyvio could not be used as a first line biological therapy, only as a second line after a failure of the first biological.

Eligibility criteria for biological treatment and administrative requirements: Eligibility criteria for

Table 1 Clinical and reimbursement guidelines

Country	Guideline	Source, organization, last update, web-link
The Czech Republic	Clinical	Bortlík *et al*[25] 2016 by IBD Working Group of the Czech Society of Gastroenterology
	Reimbursement	Reimbursement criteria of the SUKL[1]; Edited by: SUKL, www.sukl.cz
France	Clinical	ECCO guidelines, Crohn's Disease Guidelines (2010), Check list ANTI TNF, Check list VEDOLIZUMAB by GETAID[2] https://www.getaid.org/recommandations.html
	Reimbursement	No
Germany	Clinical	German Guidelines on Crohn's disease; DGVS German Society of Gastroenterology (2014)
	Reimbursement	No
Hungary	Clinical	Miheller *et al*[26] 2009
	Reimbursement	The diagnostic and treatment of Crohn's disease] by NHIFA[3] (2013) http://www.oep.hu/data/cms989735/0626_a_felnottkori_crohn_betegseg_diagnosztikajanak_es_kezelesenek_finanszirozasi_protokollja.pdf
Latvia	Clinical	No national guideline, but following the ECCO guideline
	Reimbursement	National Health Service of Latvia. No specific document, but part of the general regulations on medication reimbursement.(2016)
Poland	Clinical	[The treatment of Crohn's Disease (ICD-10 K 50)], National Health Fund, (2014) http://onkologia-online.pl/upload/obwieszczenie/2015.10.28/b/b.32.pdf
	Reimbursement	No
Romania	Clinical	National Insurance Fund Protocol (2013) http://www.cnas.ro/default/index/index/lang/EN
	Reimbursement	National Insurance Fund protocol (2013) http://www.cnas.ro/default/index/index/lang/EN
Slovakia	Clinical	No national guideline, but following the ECCO guideline
	Reimbursement	Protocol for starting and continuing the biological treatment. Date first approvals: infliximab 2005, adalimumab 2008, vedolizumab 2016; The Slovakian Gastroenterology Association and The Union of Health Insurance Companies.
Spain	Clinical	Guidelines for biologics by GETECCU[4] (2013) http://geteccu.org/formacion/guias-y-documentos-de-consenso; Cabriada *et al*[27] 2013
	Reimbursement	No
Sweden	Clinical	(1) National Guidelines for the treatment of Crohn's disease; The Swedish Society of Gastroenterology (2017) http://www.svenskgastroenterologi.se/sites/default/files/pagefiles/Riktlinjer_Lakemedelsbehandling_vid_Crohns_2012.pdf (2) The use of IFX biosimilar in patients with IBD; Swedish Society of Gastroenterology (2017) http://www.svenskgastroenterologi.se/sites/default/files/pagefiles/SGF_riktlinjer_Biosimilarer_150903.pdf (3) The Medical Product Agency: Drug treatment of IBD, novel recommendations by the Medical Product Agency, Sweden (2012) https://lakemedelsverket.se/upload/halso-och-sjukvard/behandlingsrekommendationer/L%C3%A4kemedelsbehandling%20vid%20inflammatorisk%20tarmsjukdom%20-%20ny%20rekommendation_bokm%C3%A4rken.pdf
	Reimbursement	No

[1]State Institute for Drug Control; [2]Groupe d'Étude Thérapeutique des Affections Inflammatoires du Tube Digestif; [3]National Health Insurance Fund Administration; [4]Grupo Español de Trabajo en Enfermedad de Crohn y Colitis Ulcerosa.

biological treatment were based on national clinical guidelines in France, Germany, Poland, Spain and Sweden (Table 1). In Latvia and Slovakia, the eligibility criteria were based on reimbursement guidelines since there are no national clinical guidelines available. In Hungary, Romania and the Czech Republic, both clinical and reimbursement guideline existed. While in the Czech Republic both clinical and reimbursement guidelines were followed, in Hungary and Romania clinical practice followed the reimbursement guideline when differences of clinical and reimbursement guidelines occur. The references for the guidelines for each country are shown in Table 1.

An overview of the clinical criteria for eligibility for the initiation of biological treatment and further administration requirements are presented in Table 2. According to the respondents, none of the countries had requirements on disease duration to initiate a biological treatment. In six countries CDAI scale was required to be used for the assessment the disease severity. In the Czech Republic, Romania and Slovakia, a CDAI score $\geqslant$ 220 was required to start biological treatment, while in France, Hungary and Poland, only patients with CDAI score > 300 were entitled for treatment. (There are some exemptions, for example the contributing experts from Poland and Slovakia also mentioned that patients with severe fistulising CD did not have to fulfill the CDAI score requirement.) In the Czech Republic, France, Germany, Romania, Spain and Sweden a failure of one non-biological treatment (steroid OR immunosuppressant) was required to start biological treatment, while in Hungary, Latvia, Poland and Slovakia patients had to fail both steroids and immunosuppressant treatment. In most of the countries there were no specific criteria to satisfy for maintaining biological therapy, but maintenance was based on the clinicians' judgement. Only in Hungary and in Sweden, it was recommended to evaluate maintenance on the CDAI scale.

In six countries (the Czech Republic, Hungary, Poland, Romania, Slovakia and Spain), only approved centers could use biologicals. In Latvia, the treatment could only be started in a center where three different gastroenterologists gave approval. In most countries,

Table 2 Clinical and administrative requirements of biological treatment (2016)

	Cz	Fr	D	Hu	Lv	Pl	Ro	Sk	Es	Se
Required level of disease activity (such as CDAI) or disease severity required for initiation of biological treatment										
Not specified (0 point)	x		x		x				x	x
CDAI > 220 (1 point)							x	x		
CDAI > 300 (2 points)		x		x		x				
Required failure of /intolerance to non-biological treatment before a patient is eligible for a biological										
Steroids (1 point)			x							
Immunosuppressive (1 point)							x			
Steroids OR immunosuppressive (1 point)	x	x							x	x
Steroids AND Immunosuppressive (2 points)				x	x	x		x		
Other procedures required after the indication of a biological treatment										
No other procedures (0 point)	x	x	x	x	x	x			x	x
Other requirements (*e.g.*, approval or authorization by the health insurance fund) (1 point)							x	x		
Approved centers necessary for a biological treatment										
No restriction to approved centers (0 point)		x	x							x
Restriction to approved centers (1 point)	x			x	x	x	x	x	x	
Specialists who may indicate and prescribe biologicals for the treatment of CD in adults										
Gastroenterologist, immunologist and GP/other (0 point)			x							x
Gastroenterologist and immunologist (1 point)		x								
Only gastroenterologist (2 points)	x			x	x	x	x	x	x	
Total availability score (min 0 to max 8)	4	4	1	7	5	7	6	7	4	1

Cz: The Czech Republic; Fr: France; D: Germany; Hu: Hungary; Lv: Latvia; Pl: Poland; Ro: Romania; Sk: Slovakia; Es: Spain; Se: Sweden.

treatment could be started immediately after indication. However, in Slovakia and Romania, the treatment could only start after the authorization process was completed, including a written application to the health insurance company, and/or the purchase and delivery of the medication by the company. In six countries, only gastroenterologists had the permission to indicate and prescribe biologicals to patients with CD. In Germany and France, immunologists could also indicate and prescribe biologicals. In Germany and Sweden, other specialties such as internists, surgeons or GPs were similarly entitled to prescribe and indicate biologicals.

Overall, Sweden and Germany had the lowest availability scores among the ten countries (1 out of 8), while Hungary, Poland and Slovakia had the highest scores (7 out of 8), indicating the most restrictive eligibility criteria and administration requirements (Table 2).

Regarding the availability of the biosimilars, Inflectra and/or Remsima were reimbursed in all of the ten countries at the time of the analysis. Three countries (the Czech Republic, Hungary, Spain) had specific criteria on switching to biosimilars. In Hungary, new infliximab patients had to be treated with a biosimilar, and in Poland, patients receiving the original biological drug were obliged to switch to the biosimilars of infliximab as maintenance therapy once the biological infliximab was used. In Spain switching was mandatory only in some hospitals/regions.

Affordability

The annual cost of treatment per patient ranged from €10638 (Poland) to €29081 Euro (Germany) for Remicade; from €9157 (Sweden) to €23915 (Germany) for Remsima; from €6841 (Sweden) to €22213 (Germany) for Inflectra; from €10625 (France) to €24402 (Germany) for Humira; and from €19243 (Sweden) to €30218 (Spain) for Entyvio. Between countries, the annual therapeutic cost of Remicade showed a 2.7-fold variation, while the difference for the biosimilar Inflectra and Remsima showed a 2.6 and 3.3-fold variation respectively. For Humira and Entyvio, these were 2.3 and 1.6 respectively. According to data provided by the respondents, the appearance of the two biosimilars led to a price reduction for Remicade in some countries, which resulted in the same annual cost of originator and biosimilar infliximab products in five countries (the Czech Republic, France, Latvia, Poland and Slovakia) (Table 3).

Large differences can be seen in the cost of treatment as a percentage of GDP per capita across countries (Table 3). Based on these indicators, treatments are most affordable is Sweden (13%-37% of the GDP) and least affordable in the CEE countries, especially in Hungary (87%-124%) and Romania (141%-277%). Biosimilars made infliximab treatment more affordable, as the cost of the cheapest biosimilar treatment was lower than the GDP per capita (except for Romania).

In half of the countries, all five biologicals were covered at 100% by the health insurance system. Although in two countries, patient co-payments existed. In Germany a 10% copayment was required, in Latvia this was 25%.

Acceptability

Different barriers were selected from a list by the respondents with regard to the access to biologicals, such as "limited availability of the drugs due to

Table 3 Affordability of biologicals - annual costs and annual costs (2016) as a percentage of gross domestic product per capita (2014)

	Cz	Fr	D	Hu	Lv	Pl	Ro	Sk	Es	Se
Annual total drug cost per patient (€)										
Remicade	11925	13439	29081	15204	11202	10638	15469	12020	16591	16169
Remsima	11925	13439	23915	13694	11202	10638	12375	12020	12443	9157
Inflectra	11925	13439	22213	10674	11201	10638	12375	12020	12443	6841
Humira	11131	10625	24402	12326	14050	14800	24360	13697	12209	15286
Entyvio	-	-	24651	-	-	-	22275	20207	30218	19243
Annual cost, % of GDP (Affordability ratio)										
Remicade	69%	36%	69%	124%	80%	84%	176%	74%	73%	31%
Remsima	69%	36%	57%	111%	80%	84%	141%	74%	55%	18%
Inflectra	69%	36%	53%	87%	80%	84%	141%	74%	55%	13%
Humira	65%	28%	58%	100%	101%	117%	277%	84%	54%	30%
Entyvio	-	-	59%	-	-	-	253%	124%	133%	37%
Average, without Entyvio	68%	34%	59%	106%	85%	92%	184%	77%	59%	23%
Average, all drugs	68%	34%	59%	106%	85%	92%	198%	86%	74%	26%

Annual total drug cost was calculated based on available list prices, and the EMA product information on the recommended drug dose and frequency. For infliximab we calculated with an average body weight of 75 kg. All costs were converted to Euros using the official exchange rate as of May 2016: 1 EUR= 27.025 CZK = 312.44 HUF = 4.3861 PLN = 9.2605 SEK. Cz: The Czech Republic; Fr: France; D: Germany; Hu: Hungary; Lv: Latvia; Pl: Poland; Ro: Romania; Sk: Slovakia; Es: Spain; Se: Sweden; GDP: Gross domestic product.

financial reasons" (the Czech Republic, Poland and Sweden) and "physicians' preferences" (Germany, Poland and Sweden) being the ones most frequently mentioned. Other barriers selected were "too strict reimbursement criteria" (the Czech Republic and Poland), "limited access to IBD centers" (Germany and Poland), "limited access to healthcare in general" (Poland and Romania), "patients' co-payments" (Latvia) and "patients' preferences" (Germany). Other barriers mentioned were time consuming and lengthy authorization process (Slovakia), non-referral of patients to specialist (Germany). According to the experts, after the introduction of biosimilars the access to biologicals became easier/much easier (the Czech Republic, Poland, Slovakia and Sweden) or stayed the same (Germany, Latvia and Romania). No clear opinion was reported for the other three countries.

Use of biologicals and its associations with the affordability availability and the economic development: The estimated number of CD patients, and the number of CD patients on biologicals (with references to the data sources) are presented in Table 4. The estimated number of CD patients treated with biologicals per 1000 patients showed a large variance between countries, ranging from 1.8 in Latvia to 312.6 in France. The number of patients on biologicals per 100000 in the population was the highest in Sweden (53.5), and the lowest in Latvia (0.2).

Experts gave an estimation on how many out of every 10 of their infliximab patients were treated with biosimilars. There were large differences between countries. At the time of the survey, in Romania, less than 1 out of 10 infliximab patients was treated with biosimilar, while in Latvia all infliximab patients were treated with biosimilars. The remaining countries had a treatment rate between 2/10 and 4/10, except for Poland with a treatment rate of 7/10.

In Table 5, we present the correlation matrix of the variables of interest. Correlation between the number of CD patients on biologicals per 100000 population and the availability score and affordability was strong (-0.75, -0.69 respectively). GDP per capita was strongly associated with both prevalence of biologicals in CD population (0.91), availability score (-0.88) and affordability measure (-0.75). According to the results, we found no significant correlations between health care financing indicators and access. Thus, we can conclude that it is rather the wealth of the country than the organization or financing of the health care system, which influences access. The association between prevalence, GDP and the measures of access are also presented as graphs (Figure 1).

DISCUSSION

In this study, we have analyzed three dimensions of access to biological therapy for CD, namely availability, affordability and acceptability in ten European countries. We have also explored the associations between these dimensions and the uptake of biologicals (in terms of number of patients on biologicals per 100000 population) as well as the economic development of the countries.

Regarding availability, there is a wide European consensus on clinical guidelines based on the best available evidence. For instance, the European Crohn's and Colitis Organization regularly publishes their recommendations on the management of CD. However, besides the uniformity in drug registration and international professional guidelines, we found that treatment practices and access to biological treatment are still highly diverse in Europe. We found the least restrictive eligibility criteria and

Table 4 Number of Crohn's disease patients and the use of biologicals

Country	Estimated number of CD patients/source		Number of patients on biologicals[1]/source		Patients on biologicals per 100000 population (calculated)	Patients on biologicals per 1000 patients (calculated)
Cz	8768	Rencz *et al*[3], 2015 (based on estimation)	990	Rencz *et al*[3], 2015	9.4	112.9
Fr	72522	Kirchgesner *et al*[28], 2017 (administrative database)	22671	Estimation based on Kirchgesner *et al*[28], 2017	34.0	312.6
D	180000	Estimate by the collaborating expert based on CD incidence and prevalence in two regional cohort studies from the 90ties.	27000	Estimation (based on the estimated % of patients on biologicals and the total number of CD patients)	32.9	150.0
Hu	9775	Rencz *et al*[3], 2015 (based on epidemiology study)	1870	Rencz *et al*[3], 2015	19.0	191.3
Lv	1695	Rencz *et al*[3], 2015 (based on estimation)	3	Rencz *et al*[3], 2015	0.2	1.8
Pl	32049	Rencz *et al*[3], 2015 (based on estimation)	888	Rencz *et al*[3], 2015	2.3	27.7
Ro	11000	Estimate for 2016 by the collaborating expert based on National database including 13 IBD centers	253	Rencz *et al*[3], 2015	1.3	23.0
Sk	3687	Rencz *et al*[3], 2015 (epidemiology study)	690	Rencz *et al*[3], 2015	12.7	187.1
Es	60000	Arin Letamendia *et al*[29], 2008 (prospective, population-based study)	15000	Estimation (based on the estimated % of patients on biologicals from the ENEIDA database[2] and the total number of CD patients)	32.3	250.0
Se	34318	SWIBREG[3] combined with the Swedish National Patient Register	5270	SWIBREG[3] combined with The Prescribed Drug Register	53.5	153.6

[1]The sources of the number of patients on biologicals in Rencz *et al*[3], 2015 are National gastroenterology societies, ministries of health, IMS data, personal communication; [2]ENEIDA is a large Spanish database (ENEIDA), promoted by the Spanish Working Group in Crohn's and Colitis (GETECCU) (partial, not population based study); [3]Swedish national quality registry for IBD. Cz: The Czech Republic; Fr: France; D: Germany; Hu: Hungary; Lv: Latvia; Pl: Poland; Ro: Romania; Sk: Slovakia; Es: Spain; Se: Sweden; GDP: Gross domestic product; CD: Crohn's disease.

Table 5 Correlation matrix

	No. of patients on biologicals per 100000 population	Availability score	Affordability ratio	GDP per capita	% of public health expenditure in the total health expenditure	% of general government expenditure in public health expenditure
No. of patients on biologicals per 100000 population	1.0000	-	-	-	-	-
Availability score	-0.7497 (P = 0.0125)	1.0000	-	-	-	-
Affordability ratio	-0.6920 (P = 0.0266)	0.5989 (P = 0.0673)	1.0000	-	-	-
GDP per capita	0.9077 (P = 0.0003)	-0.8810 (P = 0.0008)	-0.7464 (P = 0.0132)	1.0000	-	-
% of public health expenditure in the total health expenditure	0.3879 (P = 0.2680)	-0.5338 (P = 0.1120)	-0.1553 (P = 0.6683)	0.4907 (P = 0.149)	1.0000	-
% of general government expenditure in public health expenditure	0.6661 (P = 0.0713)	-0.4384 (P = 0.2772)	-0.3741 (P = 0.3612)	0.4233 (P = 0.296)	0.1547 (P = 0.7146)	1.0000

Sources: Population, GDP per capita (2014): The World Bank, % of public health expenditure in the total health expenditure (2015): The World Bank, European health for all database; % of general government expenditure in the total public health expenditure (2013) OECD. GDP: Gross domestic product.

administration requirements in Sweden and Germany, and the most restrictive criteria in the CEE countries, namely in Hungary, Poland and Slovakia. In most of the CEE countries examined, there are separate reimbursement guidelines followed in the clinical practice. According to these, the eligibility criterion of treatment is usually ⩾ 300 CDAI score, which is higher than the recommendations of national and international clinical guidelines (⩾ 220 CDAI score). Furthermore, in these CEE countries, biological therapy is recommended only if patients fail both corticosteroid and immunosuppressive therapy. Regarding other

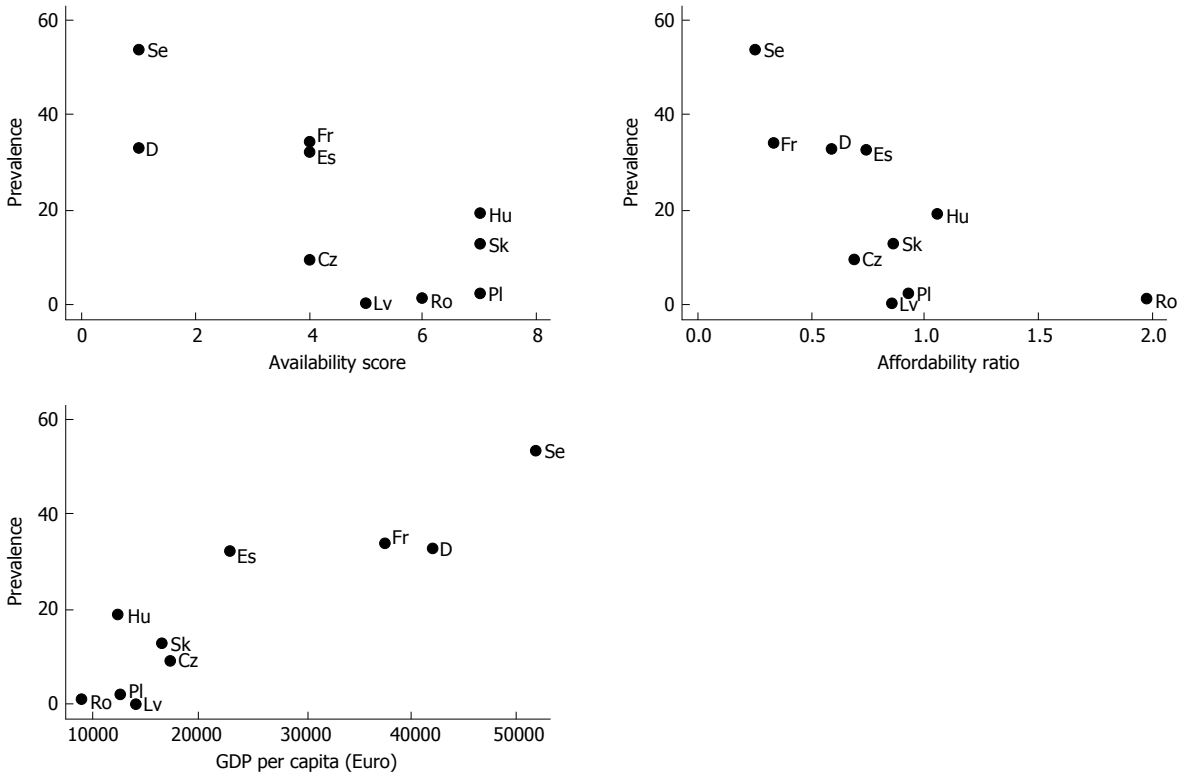


Figure 1 Associations between availability, affordability, gross domestic product per capita and the uptake of biosimilars. Cz: The Czech Republic; Fr: France; D: Germany; Hu: Hungary; Lv: Latvia; Pl: Poland; Ro: Romania; Sk: Slovakia; Es: Spain; Se: Sweden; Prevalence: Patients on biologicals per 100000 population. GDP: Gross domestic product.

requirements, in most of the countries, biologicals may only be indicated and prescribed by gastroenterologist and only approved centers may treat patients with biologicals.

We found large (1.6-3.3 times) differences regarding the annual cost of biological treatments across the countries. Treatments are most affordable in Sweden and Germany, and least affordable in the CEE countries, considering a higher economic burden of the biologicals in these countries. The annual cost of adalimumab treatment exceeds the GDP per capita in four CEE countries (Hungary, Latvia, Poland and Romania). The cheapest available infliximab treatment exceeds the GDP per capita only in Romania. Thus, biosimilars improve the affordability of biologicals. In countries where vedolizumab was available at the time of the survey, the yearly cost of treatment was lower than the GDP per capita only in Sweden and Germany.

The number of patients treated with biologicals per 100000 population varied greatly between the countries (0.2-53.5). The prevalence is the highest in Sweden, followed by Germany, France and Spain and the lowest in Poland, Romania and Latvia. We found that the uptake is strongly correlated with the GDP per capita of the country. However, we can also see large differences between countries with similar economic situation as it was also found by previous papers[3,14]. In the Czech Republic, Hungary and Slovakia, the CD prevalence (9.4-19) is much higher than in Poland and Latvia (2.3 and < 1 respectively). Reimbursement criteria do not necessarily explain the differences in the uptake of biologicals either, as availability scores are the same in Hungary, Poland and Slovakia. Furthermore, in Poland the treatments are slightly more affordable than in Hungary. In Poland, limited access to IBD centers and to healthcare in general were indicated by the collaborating expert as barriers to access in addition to strict reimbursement criteria. Furthermore, Poland is the only country where maximum duration of maintenance treatment is limited to 12 mo. In Latvia, substantial patient co-payments (25%) can also contribute to the low uptake of biologicals.

In a previous study on access to biologicals in CD, Rencz *et al*[3] found that access to biologicals in inflammatory bowel diseases varied greatly (up to 96-fold differences were found) even in some selected CEE countries. We found even higher inequalities in access among Western European and CEE countries. In rheumatology, many more patients are treated with biologicals than in inflammatory bowel diseases, at least in the CEE countries[21]. Similarly to our findings for CD, Putrik *et al*[18,20], Orlewska *et al*[22], and Hoebert *et al*[23] also found that macro-economic indicators (such as GDP or total health expenditure) largely explained the differences in access to biological treatment in rheumatoid arthritis. However, Gulácsi *et al*[14] highlighted that GDP cannot always explain the intercountry differences, which we also showed in our analysis.

We found that the number of patients treated with

biologicals per 100000 population also correlates with availability and affordability, but the correlations among these items are lower than with the GDP per capita. As mentioned before, even though Hungary, Poland and Slovakia have the same availability scores, the uptake of biologicals is much lower in Poland as pointed out by Gulácsi (2016) as well[14]. The same stands for Spain and Latvia. Nevertheless, we observed that the availability and affordability dimensions are also correlated. Thus, in countries where biological therapy is less affordable, reimbursement conditions (eligibility criteria and administrative requirements) are more restrictive.

Acceptability of biologicals, including attitudes of physicians and patients, appears to be an important determinant of the uptake of biologicals and most likely to explain differences among countries with similar economic development, availability and affordability. Thus, this factor needs further research.

We found that biosimilars improved the affordability of biologicals, and drove down the cost of infliximab treatment under the GDP per capita in most of the countries. The decrease in cost leads to budget saving in most of the countries, which could be reinvested to treat more patients and improve access to therapy. The use of biosimilars was the most frequent in Poland (7/10) due to a mandatory switch of all infliximab patients to biosimilar. Furthermore, recently in 2017, as an incentive, the maximum infliximab treatment was prolonged to 24 mo while adalimumab is still limited to 12 mo. In Latvia all infliximab patients received biosimilar. In Hungary, new infliximab patients had to be treated with biosimilar, and also in Spain switching was mandatory depending on the center. For biosimilars, the acceptability of these drugs by health care actors is even more crucial to realized budget savings. According to previous studies, a negative attitude was primarily due to the lack of efficacy and safety data in inflammatory bowel diseases[17]. In a study carried out in 2014 among 51 Hungarian gastroenterologists, 20% had no concerns and 65% some concerns about biosimilars to treat CD[17]. Nevertheless, in a discrete choice experiment, physicians were more willing to use biosimilars when some benefits regarding the access to treatment was offered for patients in return[17,24].

Finally, we also acknowledge some limitations of this study. Only 10 countries participated in the study, although the sample is diverse, as countries with different economic development were selected, which enabled a comparison between Western-European and CEE countries. Country-specific data were provided by a single gastroenterologist in each country. Nevertheless, these data were verified by the desk research. To calculate annual cost of treatments, publicly available official list prices were used as real prices are not known and can vary even within countries. There are uncertainties regarding the epidemiology data used for the analysis. For example, it is also difficult to provide reliable data on the total number of CD patients in the countries, as in most countries registers exist only for patients treated with biologicals. There are considerable differences in the prevalence of CD across the European countries. These differences can show real diversities across countries, but this can also be the result of different methodological approach or time of the epidemiological studies as well as of the different prevalence of undiagnosed CD patients. Thus, we used the number of patients on biologicals per 100000 population in the correlation analysis to disregard the differences in prevalence across the 10 countries. Nevertheless, in some cases we also had to rely on estimations of experts regarding the total number of patients on biologicals, which results in uncertainties in the number of patients on biologicals per 100000 population as well. Also, we carried out a macro level analysis, and did not consider the determinants of access at individual level, such as socio-demographic characteristics of patients. Finally, in this study, we did not aim to explore whether worse access to treatments impacts the patient's health status.

We found substantial inequalities in the access to biologicals for CD among European countries. Access was strongly determined by the economic development of the country. However we revealed large differences even among countries with a similar economic development. These differences cannot be entirely explained by the availability (eligibility criteria) or the affordability of biologicals, thus acceptance of and attitude to biologicals should be explored further.

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COMMENTS

Background

Access to biologicals in Crohn's disease (CD) varies significantly between countries, largely driven by differences in budgetary constraints. The aim of our study is to analyze access (availability, affordability and acceptability) to biologicals for CD in ten European countries and to explore the associations between these dimensions, the uptake of biologicals and economic development.

Research frontiers

Affordability of biological treatments greatly varies across countries. Due to the high price and budget impact of biologicals, most countries have regulated the access to reimbursed treatment. Differences in regulations lead to inequalities in access to biologicals even among European countries with a very similar economic situation. Nevertheless, the appearance of biosimilar drugs as potentially cost-effective alternatives is expected to lead to improvements in access to biological therapy.

Innovations and breakthroughs

The authors compared access to biologicals for CD among ten European countries, and we found substantial inequalities. Reimbursement criteria were the least strict in Sweden and Germany, and the strictest in Hungary, Poland and Slovakia. Treatments were the most affordable in Sweden (13%-37% of the GDP per capita) and the least affordable in the Central and Eastern European countries, especially in Hungary (87%-124%) and Romania (141%-277%).

Applications

The authors concluded that access was strongly determined by the economic development of the country. However large differences were revealed even among countries with a similar economic development, which cannot be entirely explained by the availability (eligibility criteria) or the affordability of biologicals. Thus, other factors such as acceptability and attitudes, also strongly determine the use of biologicals in a given country.

Terminology

CD is a chronic inflammatory disorder of the gastrointestinal tract that is characterized by diarrhea, abdominal pain, rectal bleeding, fever and fatigue. Biological drugs have revolutionized the treatment of inflammatory bowel diseases. These drugs are monoclonal antibodies with different mechanisms of action (infliximab and adalimumab are anti-tumor necrosis agents, vedolizumab is an anti-integrin drug and ustekinumab is an interleukin-12 and -23 inhibitor) but with similar safety profile and comparable efficacy. Clinical evidence confirmed the efficacy, safety and effectiveness of these drugs for the treatment of CD, as they substantially improve the ability to achieve disease remission, slow disease progression, decrease the need of surgery and increase work participation and quality of life.

Peer-review

The authors present that the difference of accessibility to biologicals, namely availability, affordability, and acceptability, for CD in ten selected European countries and the associations between these dimensions with the uptake of biologicals and economic development. Because limited data exist describing the accessibility to biologics according to economic development, I regard this to be an important study. The results are thoroughly analyzed and discussed. The manuscript is well-written.

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ORIGINAL ARTICLE

Observational Study

Temporal trends in the misdiagnosis rates between Crohn's disease and intestinal tuberculosis

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Abstract

AIM

To investigate the temporal trends in the misdiagnosis rate between Crohn's disease (CD) and intestinal tuberculosis (ITB) in South Korea.

METHODS

We retrospectively reviewed the medical records of patients managed for CD or ITB at Asan Medical Center, a tertiary referral hospital, Seoul, Korea between 1996 and 2014. The temporal trends in the misdiagnosis rates between the two diseases were analyzed. The demographic and clinical characteristics were compared between CD patients who were initially misdiagnosed as ITB (final CD group) and vice versa (final ITB group). Final diagnostic criteria for ITB and medication for CD before definite diagnosis of TB were also analyzed in final ITB group.

RESULTS

In total, 2760 patients were managed for CD and 772 patients for ITB between 1996 and 2014. As well, 494 of the 2760 CD patients (17.9%) were initially misdiagnosed as ITB and 83 of the 772 ITB patients (10.8%) as CD. The temporal trend in misdiagnosing CD as ITB showed a decrease (OR = 0.89, 95%CI: 0.87-0.91, $P < 0.001$), whereas the temporal trend in misdiagnosing ITB as CD showed an increase (OR = 1.06, 95%CI: 1.01-1.11, $P = 0.013$). Age at diagnosis, presenting symptoms, and proportion of patients with active/past perianal fistula and active/inactive pulmonary tuberculosis (TB) were significantly different between final CD group and final ITB group. Forty patients (48.2%) in final ITB group were diagnosed by favorable response to empirical anti-TB treatment. Seventeen patients (20.5%) in final ITB group had inappropriately received corticosteroids and/or thiopurines due to misdiagnosis as CD. However, there were no mortalities in both groups.

CONCLUSION

Cases of CD misdiagnosed as ITB have been decreasing, whereas cases of ITB misdiagnosed as CD have been increasing over the past two decades.

Key words: Crohn's disease; Intestinal tuberculosis; Misdiagnosis

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Core tip: In South Korea, tuberculosis (TB) is still prevalent, but its incidence has been decreasing. On the other hand, the incidence and prevalence of Crohn's disease (CD) has been increasing as in many Asian countries. In this paper, we demonstrated inverse trends in the misdiagnosis rates between CD and intestinal TB (ITB) over the last two decades. That is, cases of CD misdiagnosed initially as ITB have been decreasing, whereas cases of ITB misdiagnosed initially as CD has been increasing. More attention is needed for the correct diagnosis of CD or ITB, which could improve patients' outcomes with proper management.

Seo H, Lee S, So H, Kim D, Kim SO, Soh JS, Bae JH, Lee SH, Hwang SW, Park SH, Yang DH, Kim KJ, Byeon JS, Myung SJ, Yang SK, Ye BD. Temporal trends in the misdiagnosis rates between Crohn's disease and intestinal tuberculosis. *World J Gastroenterol* 2017; 23(34): 6306-6314 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6306.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6306

INTRODUCTION

Crohn's disease (CD) and intestinal tuberculosis (ITB) frequently show similar clinical and endoscopic features and it is difficult to differentiate between the two diseases, especially in areas with a high prevalence of tuberculosis (TB), such as Asia and Africa[1-5]. In South Korea, the incidence of TB has been decreasing[6], but it is still higher than that of Western countries. According to the Global TB Report 2013 by the World Health Organization, Korea had an incidence of 108 TB cases per 10^5 person-year, as well as a prevalence of 146 TB cases per 10^5 population in 2012[7]. Meanwhile, in the past, CD was considered a rare disease in Asian countries compared to Western countries[8-10]. Due to the low incidence of CD and the clinical inexperience in cases of CD, a significant proportion of patients with CD have been misdiagnosed as ITB, and anti-TB drugs have been administered inappropriately. However, with the increasing incidence and prevalence of CD in Asian countries including South Korea[9-11], and with the increasing attention of Asian physicians to inflammatory bowel diseases (IBD), the proportion of CD cases misdiagnosed as ITB is expected to decrease gradually. On the other hand, ITB cases initially misdiagnosed as CD could be increasing in Asian countries.

It is important to differentiate between CD and ITB because the treatments and prognoses are quite different between the two diseases[3,12,13]. In most cases, ITB can be cured with proper anti-TB drugs

with a favorable outcome, while CD is a chronic disease needing lifelong management. Moreover, the misdiagnosis of ITB as CD can lead to worse outcomes, because immunosuppressive drugs, such as corticosteroids, thiopurines, and anti-tumor necrosis factor agents can aggravate ITB[14]. Conversely, the misdiagnosis of CD as ITB can render the CD patients exposed to the potential toxicity of anti-TB drugs and delay the proper treatment of CD. Therefore, making an accurate differential diagnosis between CD and ITB at the earliest possible stage is very critical[4]. Although previous researchers have suggested clinical, endoscopic, and pathologic clues that could be helpful in a differential diagnosis between these two diseases[3,12,15-25], it is still not easy to tell them apart.

In this study, we aimed to analyze the temporal trends in the misdiagnosis rates between CD and ITB in Korea. In addition, we analyzed the clinical characteristics of ITB cases initially misdiagnosed as CD and CD cases initially misdiagnosed as ITB.

MATERIALS AND METHODS

Study population

Between January 1996 and December 2014, 2723 and 772 patients were managed for CD and ITB, respectively, at the Asan Medical Center, a tertiary university hospital in Seoul, South Korea, and they were included in this study. The diagnosis of ITB was based on Korean diagnostic guideline of ITB[26] and was considered established when at least one of the following criteria was met[16,27,28]: (1) histological demonstration of caseating granulomas; (2) identification of acid-fast bacilli in a histological specimen; (3) positive culture of *Mycobacterium tuberculosis* from a biopsy specimen; (4) clinical, colonoscopic, radiologic, and/or operative evidence of ITB associated with proven TB elsewhere; and (5) response to anti-TB therapy without subsequent recurrence in patients with clinical, colonoscopic, radiologic, and/or operative evidence of ITB. Colonoscopic findings which favor a diagnosis of ITB were as follows; involvement of fewer than four segments, anorectal lesions, patulous ileocecal valve, and scars or pseudopolyps, as described previously[16]. Some of the ITB patients in this study were described previously[27]. CD was diagnosed based on conventional clinical, radiologic, endoscopic, and histopathologic criteria[9,16,29].

Study design

For the characterization of study subjects, we retrospectively reviewed the medical records and the IBD registry of the Asan Medical Center. The IBD registry of the Asan Medical Center and the characteristics of this hospital-based cohort of CD patients has been described previously[30]. To evaluate the initial clinical presentation, we also reviewed the medical records and endoscopic images provided by referring physicians. Through the reviewing process, the following characteristics were abstracted and compared between CD patients misdiagnosed as ITB (final CD group) and ITB patients misdiagnosed as CD (final ITB group): gender, age at diagnosis, interval from misdiagnosis to definite diagnosis, presenting symptoms, active and/or past perianal fistula, active pulmonary TB (PTB), radiologic evidence of old PTB, and institutions where misdiagnosis was made. For the final ITB group, medication for CD before definite diagnosis of ITB was also abstracted. We also analyzed the final diagnostic criteria for ITB in the final ITB group. The study protocol was approved by the Institutional Review Board of the Asan Medical Center (IRB No. 2010-0882).

Temporal trends in the misdiagnosis rates

To evaluate the temporal trends in the misdiagnosis rates between CD and ITB, the number of patients diagnosed with CD at the initial evaluation but finally diagnosed with ITB and vice versa were verified every year between 1996 and 2014. The annual rate of misdiagnosing CD as ITB was defined by the proportion of patients with a preceding misdiagnosis as ITB (final CD group) among the patients diagnosed definitely with CD at that year. The rate of misdiagnosing ITB as CD was calculated by the opposite method.

Statistical analysis

Continuous variables were expressed as the mean with SD or the median with ranges. Discrete data were expressed as numbers and percentages. The clinical characteristics of the final CD group and the final ITB group were compared using the Student's *t*-test or the Mann-Whitney *U* test for continuous variables and the χ^2 test or the Fisher's exact test for categorical variables, where appropriate. The temporal trends in the misdiagnosis rates of CD and ITB were analyzed by the logistic regression analysis. We also calculated the intervals from misdiagnosis to correct diagnosis in each patient and values were expressed as median with interquartile range (IQR). The intervals from initial misdiagnosis to correct diagnosis in periods divided into 6 (1996-1999, 2000-2002, 2003-2005, 2006-2008, 2009-2011, and 2012-2014) were compared using the Kruskal-Wallis test.

A statistical analysis was carried out using IBM SPSS statistics, ver. 21.0 (IBM Co., Armonk, NY, United States) and SAS version 9.3 (SAS Institute Inc., Cary, NC). A P value < 0.05 was considered statistically significant.

RESULTS

During the 19-year study period, 494 of the 2760 patients with CD (17.9%) were initially misdiagnosed as ITB (final CD group). Conversely, 83 of the 772 patients with ITB (10.8%) were initially misdiagnosed as CD (final ITB group). Among 494 patients in final

Table 1 Demographic and clinical characteristics of Crohn's disease patients misdiagnosed as intestinal tuberculosis (final Crohn's disease group) and intestinal tuberculosis patients misdiagnosed as Crohn's disease (final intestinal tuberculosis group) *n* (%)

	Final CD group (*n* = 494)	Final ITB group (*n* = 83)	*P* value
Male	342 (69.2)	49 (59.0)	0.066[1]
Age at definite diagnosis[4] (yr)	23.4 (19.5-30.3)	35.8 (27.3-46.0)	< 0.001[2]
Interval from misdiagnosis to definite diagnosis[4] (mo)	5.2 (2.3-12.0)	4.0 (1.7-13.2)	0.082[2]
Presenting symptoms			
Abdominal pain	318 (64.4)	46 (55.4)	0.118[1]
Diarrhea	293 (59.3)	22 (26.5)	< 0.001[1]
Weight loss	104 (21.1)	12 (14.5)	0.165[1]
Hematochezia/melena	84 (17.0)	6 (7.2)	0.023[1]
Constipation	1 (0.2)	2 (2.4)	0.056[3]
Asymptomatic (incidentally diagnosed at routine health check-up)	8 (1.6)	10 (12.0)	< 0.001[3]
Fever	28 (5.7)	1 (1.2)	0.103[3]
Active and/or past perianal fistula	192 (38.9)	7 (8.4)	< 0.001[1]
Active pulmonary TB	0 (0.0)[5]	10 (12.7)[6]	< 0.001[3]
Old pulmonary TB in chest X-ray	5/469 (1.1)[7]	16/66 (24.2)[7]	< 0.001[3]
Medical institutions where misdiagnosis was made			0.015[1]
Primary clinic	44 (8.9)	12 (14.5)	
Secondary referral hospital	118 (23.9)	30 (36.1)	
Tertiary referral hospital	314 (63.6)[8]	41 (49.4)[9]	
Unknown	18 (3.6)		

[1]The *P* value by the χ^2 test; [2]The *P* value by the Mann-Whitney *U* test; [3]The *P* value by the Fisher's exact test; [4]The Median (Interquartile range); [5]Not evaluated in one patient; [6]Not evaluated in four patients; [7]Patients who had active pulmonary TB and patients whose chest X-ray film was unavailable were excluded; [8]Misdiagnoses were made at our institution in 68 patients (13.8%); [9]Misdiagnoses were made at our institution in 7 patients (8.4%). CD: Crohn's disease; ITB: Intestinal tuberculosis; TB: Tuberculosis.

CD group, misdiagnoses were made at primary clinics in 44 patients (8.9%), at secondary referral hospitals in 118 patients (23.9%), at the tertiary referral hospitals other than our institution in 246 patients (49.8%), and at our institution in 68 patients (13.8%). In 18 cases (3.6%), information on the institution where misdiagnoses were made was unclear. Among 83 patients in final ITB group, misdiagnoses were made at primary clinic in 12 patients (14.5%), at secondary referral hospital in 30 patients (36.1%), at the tertiary referral hospital other than our institution in 34 patients (41.0%), and at our institution in 7 patients (8.4%, Table 1).

The yearly trends in the misdiagnosis rates between CD and ITB are plotted in Figure 1. Although the misdiagnosis rates showed variability every year, the rate of misdiagnosing CD as ITB showed a decreasing tendency and the rate of misdiagnosing ITB as CD showed an increasing tendency over time. In 1996, a total of 34.3% of patients in final CD group were misdiagnosed initially as ITB, but the proportion of misdiagnosed cases was decreased to 8.1% in 2014. During the initial 5 years of observation period (1996-2000), 33.2% of final CD group have been misdiagnosed as ITB, but the rate of misdiagnosing CD as ITB went down to 9.9% during the last 5 years (2010-2014) of study period. The temporal trend in the rate of misdiagnosing CD as ITB showed a significant decrease over the 19 years in the logistic regression model (OR = 0.89; 95%CI = 0.87-0.91 per year increase in the study period; $P < 0.001$). In the cases with ITB, no patient was misdiagnosed initially as CD in 1996, but 15.6% were misdiagnosed initially as CD in 2014. During the initial 5 years of observation period (1996-2000), only 3.0% of final ITB group have been misdiagnosed as CD, whereas the rate of misdiagnosing ITB as CD increased to 13.1% during the last 5 years (2010-2014). Therefore, the trend in the rate of misdiagnosing ITB as CD showed a significant increase (OR = 1.06; 95%CI: 1.01-1.11 per year increase in the study period; $P = 0.013$).

We analyzed the temporal trends in the intervals from initial misdiagnosis to correct diagnosis (Figure 2). The median intervals from misdiagnosis to correct diagnosis were 5.2 mo (IQR: 2.3-12.2) in final CD group and 4.0 mo (IQR: 1.6-13.4) in final ITB group. During the study period, the intervals from initial misdiagnosis to final diagnosis were not changed significantly in both final CD group ($P = 0.394$ by Kruskal-Wallis test) and final ITB group ($P = 0.748$ by Kruskal-Wallis test) during study period (Figure 2).

Table 1 shows the demographic and clinical characteristics of the final CD group and of the final ITB group, respectively. The age at definite diagnosis was younger in the final CD group than in the final ITB group (median 23.4 years *vs* 35.8 years, $P < 0.001$). The most frequent symptom of both groups was abdominal pain. However, diarrhea and hematochezia/melena were more common in the final CD group than in the final ITB group ($P < 0.001$ and $P = 0.023$, respectively). Alternatively, an incidental diagnosis without any related symptom was more common in

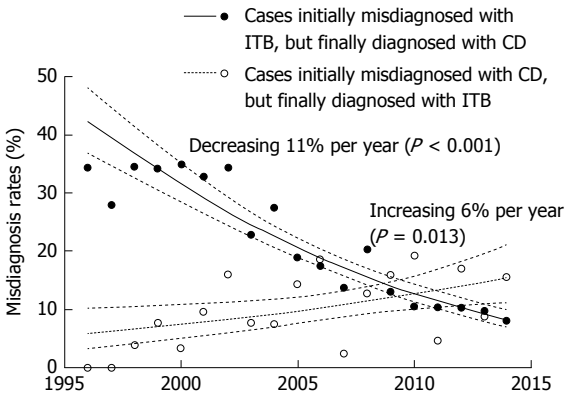


Figure 1 Temporal trends in the misdiagnosis rates between Crohn's disease and intestinal tuberculosis during the study period. Dots represent the observed misdiagnosis rates; lines represent the expected misdiagnosis rates and 95%CI according to the logistic regression analysis. The misdiagnosing rate of Crohn's disease (CD) as intestinal tuberculosis (ITB) decreased to 0.89 (95%CI: 0.87-0.91) fold per year ($P < 0.001$); The misdiagnosing rate of ITB as CD increased to 1.06 (95%CI: 1.01-1.11) fold per year ($P = 0.013$).

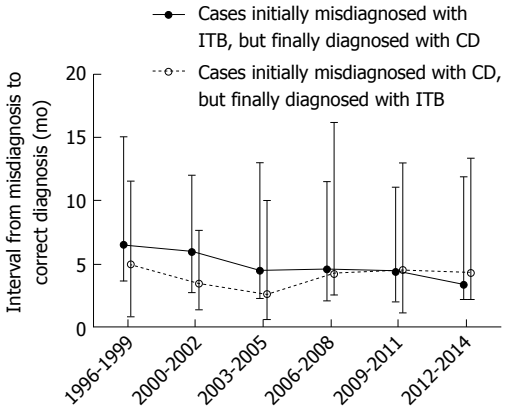


Figure 2 Temporal trends in the interval from misdiagnosis to correct diagnosis in cases of Crohn's disease initially misdiagnosed as intestinal tuberculosis (Final Crohn's disease group) and cases of intestinal tuberculosis initially misdiagnosed as Crohn's disease (Final intestinal tuberculosis group). The data are presented with the median and interquartile range. The interval from initial misdiagnosis to final diagnosis were not changed significantly in final Crohn's disease (CD) group ($P = 0.394$ by Kruskal-Wallis test) and final intestinal tuberculosis (ITB) group ($P = 0.748$ by Kruskal-Wallis test) during the study period.

the final ITB group than in the final CD group ($P < 0.001$). Only seven patients (8.4%) had an active and/or past perianal fistula in the final ITB group, whereas 192 (38.9%) patients in the final CD group had an active and/or past perianal fistula ($P < 0.001$). None of patients in final CD group had active PTB, but 10 patients (12.7%) in final ITB group had active PTB ($P < 0.001$). Inactive PTB in the radiologic evaluation were also more commonly observed in final ITB group (38.9% *vs* 8.4%, $P < 0.001$). Table 2 shows the final diagnostic criteria for ITB and history of inappropriate medication for CD in the final ITB group. In 40 patients (48.2%), favorable response to empirical tuberculosis treatment was applied as diagnostic criteria of ITB. In 17 patients (20.5%), corticosteroids and/or thiopurines were inappropriately given due to misdiagnosis as CD (Table 2). There were no mortalities in two groups.

Table 2 Characteristics of patients finally diagnosed with intestinal tuberculosis who were initially misdiagnosed as Crohn's disease; final diagnostic criteria for intestinal tuberculosis and medication for Crohn's disease before correct diagnosis *n* (%)

	Final ITB group[1] (*n* = 83)
Diagnostic criteria of ITB	
Positive culture of *M. Tb* alone	40 (48.2)
Positive AFB smear alone	1 (1.2)
Positive culture of *M. Tb* + Positive AFB smear	1 (1.2)
Caseating granuloma alone	1 (1.2)
Improvement after empirical anti-tuberculosis treatment in patients without the above findings	40 (48.2)
Medication for CD before definite diagnosis of TB	
No medication	21 (25.3)
5-ASA alone	42 (50.6)
5-ASA + Corticosteroids	12 (14.5)
5-ASA + Thiopurines	1 (1.2)
5-ASA + Corticosteroids + Thiopurines	4 (4.8)
Medication given, but unavailable records	3 (3.6)

[1]Final ITB group: Intestinal tuberculosis patients initially misdiagnosed as Crohn's disease. 5-ASA: 5-amino salicylic acids; AFB: Acid-Fast Bacilli; CD: Crohn's disease; ITB: Intestinal tuberculosis; *M. Tb*: *Mycobacterium tuberculosis*; TB: Tuberculosis.

DISCUSSION

In the present study, we demonstrated the inverse trends in the misdiagnosis rates between CD and ITB over the last two decades in South Korea. Comparing the final CD group and the final ITB group, different characteristics, such as age at diagnosis, presenting symptoms, presence of perianal fistula, and concurrent PTB could be identified.

In this study, we included all patients managed for CD or ITB in our institution and enrolled patients who were finally diagnosed as CD or ITB before referral to our institution to minimize referral bias. In addition, by thoroughly reviewing the IBD registry of our hospital, which has been maintained since 1997[30], as well as medical records and colonoscopic images from referring physicians, we could gather valid information on our study subjects to ensure diagnostic accuracy for study participants.

The incidence of active PTB in Koreans was as high as 393/10^5 person-year between 1988 and 1990, and 202/10^5 person-year between 1992 and 1994[31,32]. However, it appears to be decreasing with time, showing 119.3/10^5 person-year between 2006 and 2008[6]. Although the exact incidence of ITB is not well known, a previous Korean study reported that 32 of 59 patients (54.2%) with active PTB, but without any gastrointestinal symptoms were diagnosed with ITB by total colonoscopy, thereby suggesting a high concurrence of ITB among PTB patients[33]. Although

the actual incidence of ITB is uncertain, the incidence of ITB is also expected to have been decreasing together with PTB. On the other hand, the incidence of CD, which was once thought to be very rare compared to Western countries, has been increasing recently in South Korea[9,34]. There have been several studies showing similar trends in TB epidemiology in other endemic areas, such as South Asia, India, Saudi Arabia, and South Africa[35-37]. For CD, as with Korea, the increasing incidence could be also observed in other Asian and Middle Eastern countries, such as Japan[38], Hong Kong[39], Taiwan[40], and Saudi Arabia[36].

With the changing epidemiology of TB and CD, general practitioners and gastrointestinal specialists in Asian areas could be becoming more concerned with CD than with ITB. In addition, with the decreasing incidence of TB, ITB cases that could have been correctly diagnosed may be misdiagnosed as CD. Actually, for the 19-year period in this study, the misdiagnosis rate of ITB as CD showed a statistically significant increase, and vice versa, although they were relatively stable since 2009. The rate of misdiagnosing ITB as CD overcame the rate of misdiagnosing CD as ITB in 2010. The misdiagnosis between CD and ITB and inappropriate treatments based on the misdiagnosis could cause serious harm to patients, especially for ITB cases. In our study, corticosteroids and/or thiopurines were inappropriately given to 20.5% of ITB patients because of misdiagnosis as CD, which could have led to disseminated TB disease. Therefore, our study results are calling for more attention to the physicians managing patients with inflammatory disorders of the bowel. In the absence of a confirmatory test to differentiate ITB from CD, the current Asia-Pacific consensus recommend 8-12 wk of empirical anti-TB treatment for patients with diagnostic uncertainty[41], and one thing we should remember is that about a half of ITB cases (48.2%) with previous misdiagnosis as CD in our study could have been correctly diagnosed with ITB after anti-TB treatment. The finding that the other half of ITB patients (49.4%) were correctly diagnosed based on positive culture of *M. Tb* emphasizes the importance of *M. Tb* culture study when performing colonoscopy for cases having ileocolonic inflammation which is difficult to make a differential diagnosis.

Unfortunately, there were no significant changes in the lag time from initial misdiagnosis to correct diagnosis during the study period. This is probably because most of the misdiagnosed cases have been referred to the more specialized centers or have been re-evaluated after a few months of unresponsiveness to treatment, which emphasizes the importance of the initial correct differentiation between two diseases. Therefore, we suggest the strict following of guidelines or consensus[26,41] and the application of the appropriate scoring system, diagnostic algorithms, or prediction models according to the regional epidemiology of two diseases[3,16,18-25].

In daily clinical practice, relatively simple clinical information could be helpful in the differential diagnosis between CD and ITB. In the current study, the younger age at diagnosis was observed in CD patients than in ITB patients, which is consistent with the results of previous studies[42,43]. The frequency of initial presenting symptoms was different between the two groups in our study. Diarrhea, hematochezia/melena, active and/or past perianal fistula were common in final CD group, whereas active or inactive pulmonary TB was observed more often in final ITB group. It was in line with a recently published large meta-analysis including 38 studies comprising 2117 CD and 1589 ITB patients, which has shown that diarrhea, hematochezia, presence of perianal disease, and extraintestinal manifestations significantly favored CD, whereas fever, night sweats, lung involvement and ascites significantly favored ITB[25]. However, symptoms are not always helpful for a differential diagnosis, because they are subjective and conflicting results on the frequency of symptoms in CD and ITB patients have been reported, depending on the study population[3,44-47].

According to the previous report from our institution, 43.2% of CD patients showed an active and/or past perianal fistula at the time of CD diagnosis[48]. In line with that study, among the final CD group, 38.9% of subjects showed an active and/or past perianal fistula at initial presentation, which was significantly higher than the figure for the final ITB group. Although a perianal fistula and anorectal lesion could also be manifestations of ITB[49,50], a concurrent perianal fistula could strongly suggest the probability of CD rather than ITB[3].

There has been a report that the features of old or active PTB were found in 35% of Indian patients with ITB[51]. Similarly, in South Korea, active PTB was accompanied in 28%-49% of ITB patients, and evidence of old PTB was shown in 14%-24.8% of ITB patients[27,52,53]. However, in our final ITB group, active PTB was accompanied only in 12.7%, although it was significantly more frequent than in final CD group. Because only cases initially misdiagnosed as CD were enrolled in the final ITB group, a lower proportion of patients might have concurrent active PTB compared with previous studies[27,52,53]. Nonetheless, a simple chest X-ray is always needed for a correct differential diagnosis, because findings of active PTB could be a clue to the diagnosis of ITB.

There are a few limitations to this study. First, the study design was retrospective. However, we made every effort to gather reliable data from electronic medical records, the IBD registry, endoscopic images, and information from referring doctors to avoid information bias. Regardless of our effort, some important variables which would affect the risk of ITB and CD, such as socioeconomic characteristics, could not be analyzed in this study. Second, this study was based on a single referral center and the real-life situations of primary and secondary facilities could be somewhat

different, maybe showing higher rates of misdiagnosis than in this study. However, because about one third of the final CD group and about a half of the final ITB group were initially misdiagnosed at the primary clinics or secondary hospitals, and because misdiagnosed cases in tertiary hospitals were also referred from primary or secondary facilities because of diagnostic uncertainty, our results could represent a general phenomenon in our country.

In conclusion, cases of ITB misdiagnosed as CD have been increasing in Korea over the past two decades. Conversely, CD cases misdiagnosed as ITB have been decreasing. More attention is needed for the correct diagnosis of CD or ITB, which could improve patients' outcome with proper management.

COMMENTS

Background

Differential diagnosis between intestinal tuberculosis (ITB) and Crohn's disease (CD) is a challenging issue in areas with a high prevalence of tuberculosis (TB) and an increasing incidence of CD. However, rates of misdiagnosing CD and ITB as each other disease and their temporal trends are not well known.

Research frontiers

The incidence rate of TB is still high in Asia, but it is declining gradually with socioeconomic development. Meanwhile, the increasing incidence rate of CD is a widely observed phenomenon in most Asian countries. Most recent studies have focused on identifying clinical, endoscopic, radiologic, and histopathologic features which can be helpful to distinguish between two diseases and on suggesting predictive models or scoring systems. However, only a few studies have addressed how many patients are misdiagnosed as each other disease and none of studies have demonstrated how the misdiagnosis rates between CD and ITB have altered with changing epidemiology of two diseases.

Innovations and breakthroughs

This was the first study which has demonstrated inverse trends in misdiagnosis rates between CD and ITB over nearly two decades. That is, cases of CD misdiagnosed initially as ITB have been decreasing, whereas cases of ITB misdiagnosed initially as CD has been increasing.

Applications

The data in this article suggest that clinicians have been becoming more concerned about CD with increasing incidence of CD, leading to improved diagnostic accuracy for CD in recent years. At the same time, however, the rate of misdiagnosing ITB as CD increased and overcame the rate of misdiagnosing CD as ITB and that raises the alarm for physicians so as not to expose patients with ITB to potentially harmful immunosuppressive therapy.

Terminology

ITB is tuberculosis involving intestinal tract, which is one of the forms of extrapulmonary tuberculosis.

Peer-review

In the present original paper, the authors showed temporal trends of the misdiagnosis rates between CD and ITB from a patient cohort managed at a specialized center for inflammatory bowel diseases. As a result, the rates of misdiagnosis as ITB in cases of actual CD has been decreasing and the opposite has been increasing. Overall, findings of this study are novel and this is a well-written and interesting paper.

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ORIGINAL ARTICLE

Observational Study

Detection of metastatic cancer cells in mesentery of colorectal cancer patients

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Author contributions: Luo XL and Xie DX contributed equally to this work; Gong JP conceived and designed the study; Luo XL, Wu JX, Wu AD and Ge ZQ contributed to data acquisition, data analysis and interpretation; Luo XL and Xie DX wrote the article; Li HJ, Hu JB and Cao ZX revised and reviewed the article; all authors have reviewed and approved the final manuscript.

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Informed consent statement: Informed consent was obtained from all participants included in the study.

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Abstract

AIM

To detect the existence of isolated cancer cells in the mesentery of colorectum (named as Metastasis V), and investigate its clinical significance in colorectal cancer (CRC) patients.

METHODS

Sixty-three CRC patients who received radical excision between January 2012 and September 2015 were included. All the patients underwent laparoscopy-assisted radical colorectomy or proctectomy [with complete mesocolic excision (CME) or total mesorectal excision (TME)] with R0 dissections at the Department of Gastrointestinal Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The location and size of the primary lesions were recorded immediately after the tumor was removed, with the surrounding mesenterium completely separated along the intestinal wall. Each dissected mesentery sample was analyzed for hematoxylin-eosin staining and immunohistochemistry using cytokeratin 19 antibody. Image Pro Plus Software 6.0 (Media Cybernetics, CA, United States) was used

to semi-quantitatively measure the concentration of the cytokeratin 19 immunohistochemistry. The correlation between metastasis found in mesentery and clinicopathological characteristics was examined. The prognosis of patients was also evaluated by preoperative serum CEA level.

RESULTS

Metastasis V was detected in 14 of 63 (22.2%) CRC patients who underwent laparoscopy-assisted radical colorectomy or proctectomy (with CME or TME) with R0 dissection in our hospital between January 2012 and September 2015. There was no significant difference in age, gender, tumor size, and tumor location in patients with Metastasis V ($P > 0.05$). Metastasis V was more likely to occur in poorly differentiated tumor (5/11; 45.5%) than moderately (8/46; 17.4%) and well-differentiated one (1/6; 16.7%). The Metastasis V in N2 stage (9/14; 64.3%) was more frequent that in the N0 stage (3/35; 8.6%) or N1 stages (2/14; 14.3%). In addition, Metastasis V was positively related to the tumor invasive depth (T1:0/1, 0%; T2:1/12, 8.3%; T3:7/39, 17.9%; T4:6/11, 54.5%). Furthermore, pre-operative serum CEA level in Metastasis V-positive patients was significantly higher than in Metastasis V-negative patients (4.27 ng/mL *vs* 3.00 ng/mL).

CONCLUSION

Metastasis V might be associated with a poor prognosis of CRC patients.

Key words: Colorectal cancer; Colorectal mesentery; Serum CEA level; Radical surgery; Metastasis V

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Core tip: Local-regional recurrence of colorectal cancer (CRC) is common in patients who received R0 resection. Our previous study proposed a novel type of metastasis designated as "Metastasis V" in gastric cancer. Metastasis V is defined as the appearance of cancer cells in the mesentery in broad sense, and may be a risk factor for the poor prognosis after radical surgery. In this study, Metastasis V was also detected in the mesentery of colorectum, and it might be associated with the poor prognosis of CRC patients.

Luo XL, Xie DX, Wu JX, Wu AD, Ge ZQ, Li HJ, Hu JB, Cao ZX, Gong JP. Detection of metastatic cancer cells in mesentery of colorectal cancer patients. *World J Gastroenterol* 2017; 23(34): 6315-6320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6315.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6315

INTRODUCTION

Three decades ago, RJ Heald introduced the total mesorectal excision (TME) in the treatment of rectal cancer[1]. Subsequently, complete mesocolic excision (CME) was popularized by W Hoehenbuerg to reduce tumor relapse and improve prognosis in colon cancer patients[2]. These new approaches greatly contributed to the complete mesentery excision and improved lymph node harvest. Furthermore, with the development of surgical technology, neo-adjuvant chemotherapy and efficient perioperative management, the prognosis of patients with colorectal cancer (CRC) has been improved. However, local-regional recurrence of CRC is still often noted in the patients who received radical R0-resection with no lymphatic metastasis[3].

Direct invasion, lymphatic drainage, hematogenous spread and peritoneal dissemination are the four classical routes for local-regional recurrence or distant implantion of cancer cells. In addition to these pathways, the existence of metastatic cancer cells in the mesentery of colorectum has also been reported, and this type of metastasis was named as "extra-capsular spreads" or "extra-nodal metastasis"[4-7]. In our previous study, we proposed a novel type of tumor metastasis designated as Metastasis V in gastric cancer. Metastasis V is defined as the appearance of cancer cells in the mesogastrium with perigastric adipose tissue, and it may be a risk factor for patient survival after radical surgery[8]. Here, in this study, we further determined whether Metastasis V can be detected in the mesocolorectum, and examined its clinical significance.

MATERIALS AND METHODS

Patients, samples, and sectional tissue analysis

Sixty-three patients who underwent surgery for CRC at the Department of Gastrointestinal Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology between January 2012 and September 2015 were included in this study. The patients consisted of 25 female patients and 38 male patients. All of these patients received laparoscopy-assisted radical colorectomy or proctectomy (with CME or TME) with R0 dissection. All participants signed informed written consents in this research. This study was approved by the Tongji Hospital Ethics Committee.

Clinical samples of CRC were obtained from the resected colorectal tissue. The location and size of the primary lesions were recorded immediately after the tumor was removed, and then the mesenterium was completely separated from the intestinal wall along with the anterior edge. All specimens were fixed in 10% formaldehyde before processing. The lymph nodes were obtained from the mesenterium. Next, the mesentery was cut into strip-shape with a width of 2 cm in an outward-to-inward manner, vertical to the intestinal wall. The strip-shaped samples were cut into cube samples with a mean length of 2 cm.

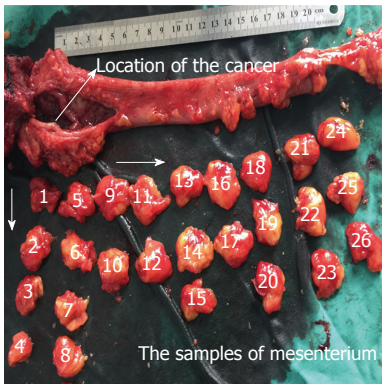


Figure 1 Samples of mesenterium from colorectal cancer patients. Analysis of large cross sectional tissue samples of mesentery of colorectum from surgically resected specimens.

Table 1 Data in 63 colorectal cancer patients

Parameters	Results
Gender	
Female	25
Male	38
Age (yr, mean, range)	55.5 (28-86)
Location of cancer	
Colon	11
Rectum	52
Differentiated grade	
Well	6
Moderately	46
Poorly	11
Affected lymph nodes	
N0	35
N1	14
N2	14
Invasive depth	
T1	1
T2	12
T3	39
T4	11

All of the small samples were numbered from inside (proximal end of the cancer) to outside (Figure 1). These samples were embedded in 10% formaldehyde. Demographic characteristics of the 63 patients are described in Table 1. The clinicopathological stage was determined by the 7th edition of the AJCC Cancer Staging Manual.

Immunohistochemical analysis of Metastasis V

Immunohistochemistry was performed using the cytokeratin 19 antibody. Cytokeratin 19 is specifically expressed in intestine epithelia cells and CRC cells[9]. Hence, it has been proven to be a highly sensitive marker for CRCs.

Immunostaining was performed by the standard streptavidin-biotin method. First, the slices were de-paraffinized and rehydrated. To retrieve antigenicity, the slices were boiled in 0.01 mol/L, pH 6.0 sodium citrate buffer for approximately 15 min. Endogenous peroxidase was restrained with 0.3% hydrogen peroxide for roughly 25 min. After blocking with 3% BSA for 30 min, the anti-cytokeratin 19 monoclonal antibody was arranged in blocking buffers and the slices were incubated at 4 ℃ overnight. After being washed in pH 7.4 PBS 3 times (5 min each time), the corresponding secondary antibody, retained at room temperature for approximately 50 min and marked by HRP was added. After one more wash with pH 7.4 PBS for 15 min, the chromogenic reagent DAB was added to the colorate. The slices were counterstained with Harris hematoxylin, dehydrated in a graded alcohol series (75% alcohol for 6 min, 85% alcohol for 6 min, absolute ethyl alcohol I for 6 min, and absolute ethyl alcohol II for 6 min), cleared in xylene for 5 min, and mounted. Individual tumor cells in mesenteries, separated from primary lesion and lymph nodes, were identified to be Metastasis V.

Immunohistochemistry image analysis

Image Pro Plus Software 6.0 (Media Cybernetics, CA, United States) was used to semi-quantitatively measure the concentration of the cytokeratin 19 immunohistochemistry. This procedure, was split into six steps: (1) discovering and surveying the section of interest; (2) adjusting the optical densities; (3) obtaining, transforming and preserving images; (4) amending the background and background staining; (5) configuration of the section of interest to determine the optical density; and (6) examining optical densities.

Statistical analysis

The Fisher's exact test, χ^2 test, and Mann-Whitney *U* test were used to inspect the significance of the differences between the covariates. We considered a *P* value of less than 0.05 statistically significant. Standard statistical analyses were performed by SPSS version 20.0.

RESULTS

Detection of Metastasis V in mesenterium of CRC patients

Metastasis V was detected in 14 of the 63 (22.2%) patients by immunohistochemistry staining. These isolated cancer cells separated from the primary lesion and lymph nodes were observed in the same slice (Figure 2). In terms of "T" stage, only one case showed limited muscular layer invasion, seven cases had sub-serosal invasion, and the rest had serosal invasion. In these patients, there were three lymph node negative patients with different invasive depths (Table 2).

Correlation between Metastasis V and clinical factors

The demographic characteristics of patients and the pathologic features of tumors with Metastasis V are shown in Table 3. There was no significant difference

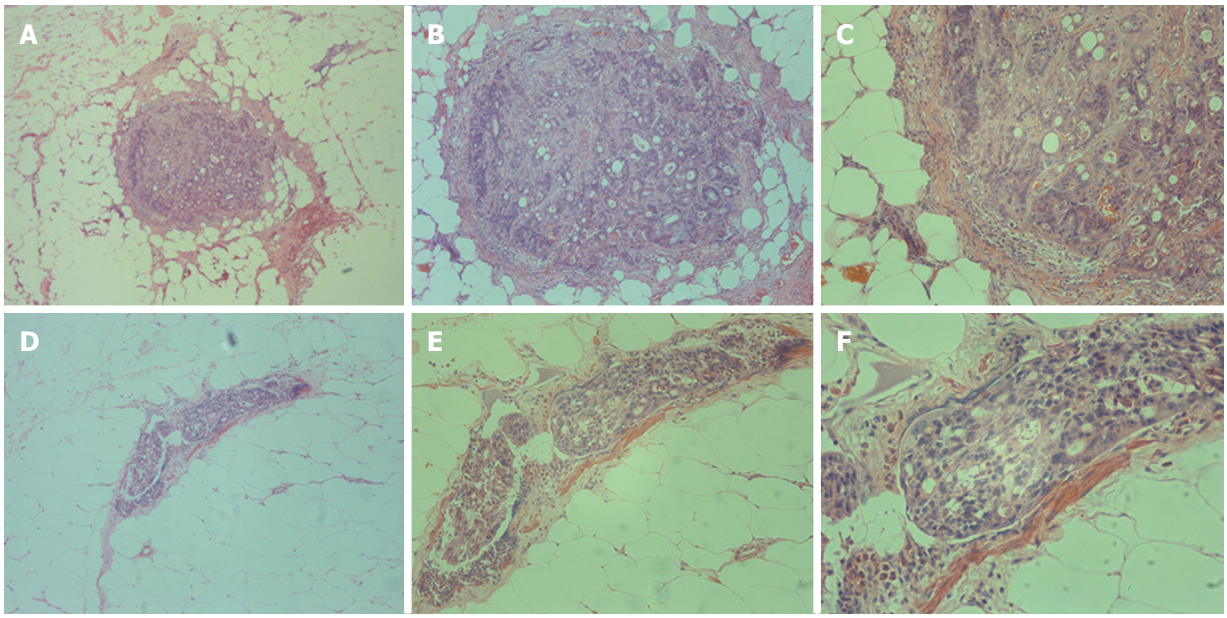


Figure 2 Detection of Metastasis V in colorectal cancer patients. Isolated cancer cells were detected in the mesentery of colorectum by HE staining. A (× 10), B (× 20) and C (× 40) represent the same slide; D (× 10), E (× 20) and F (× 40) represent the same slide.

Table 2 Data in Metastasis V-positive patients

Patients	Sex	Age (yr)	Cancer size (cm)	Cancer location	Differentiated grade	Invasive depth	Affected lymph nodes	TNM
1	F	37	2.5	Rectum	Poorly	Serosal	8/15	pT4N2M0
2	F	42	3	Rectum	Moderately	Subserosal	5/17	pT3N2M0
3	M	33	2	Rectum	Moderately	Serosal	5/15	pT4N2M0
4	M	48	6	Sigmoid	Well	Serosal	12/21	pT4N2M0
5	F	63	3	Rectum	Poorly	Muscularis	0/15	pT2N2M0
6	F	45	3	Rectum	Moderately	Serosal	5/28	pT4N2M0
7	M	58	3	Rectum	Poorly	Subserosal	8/12	pT3N2M0
8	M	64	4	Rectum	Poorly	Subserosal	7/15	pT3N2M1
9	M	54	6	Rectum	Moderately	Subserosal	2/13	pT3N1M0
10	F	60	2	Descending colon	Moderately	Subserosal	0/17	pT3NxM0
11	M	54	3	Rectum	Moderately	Serosal	5/12	pT4N2M0
12	M	58	3	Rectum	Moderately	Subserosal	0/16	pT3N0M0
13	F	57	3	Rectum	Poorly	Serosal	2/21	pT4N1M1
14	M	47	4	Rectum	Moderately	Subserosal	24/32	pT3N2M0

in age, gender, tumor size, and tumor location (P > 0.05). Metastasis V was more likely to occur in poorly differentiated tumor (5/11; 45.5%) than moderately (8/46; 17.4%) and well-differentiated one (1/6; 16.7%). The Metastasis V in N2 stage (9/14; 64.3%) was more frequent than that in the N0 stage (3/35; 8.6%) or N1 stages (2/14; 14.3%). In addition, Metastasis V was positively related to the tumor invasive depth (T1: 0/1, 0%; T2: 1/12, 8.3%; T3: 7/39, 17.9%; T4: 6/11, 54.5%).

Correlation between Metastasis V and preoperative CEA levels

Previous studies have demonstrated the correlation between increased preoperative CEA and the poor prognosis of rectal cancer patients[10,11]. Here, we further determined the correlation between Metastasis V and preoperative CEA levels. Totally 42 CRC patients who underwent operations in our department were retrospectively studied. Metastasis V was detected in nine patients according to the pathological reports. Six patients were excluded because their preoperative CEA data was missed. The CEA levels were divided into two grades. The CEA levels more than 3 times of normal range (15.00 ng/mL) were classified as high grade, while those less than that were designated as low grade. Our data indicated that preoperative serum CEA levels in Metastasis V-positive patients were significantly higher than in Metastasis V-negative patients (4.27 ng/mL *vs* 3.00 ng/mL) (Table 4).

DISCUSSION

In this study, we have shown that metastatic cancer

Table 3 Comparison between Metastasis V-positive patients and Metastasis V- negative patients

Parameters	Metastasis V		*P* value
	Negative	Positive	
Age (yr)			NS
< 45	8	3	
⩾ 45 and ⩽ 59	19	8	
⩾ 60	22	3	
Cancer size (cm)			NS
⩽ 3	24	10	
> 3 and < 5	14	2	
⩾ 5	11	2	
Gender			NS
Female	19	6	
Male	30	8	
Cancer location			NS
Colon	9	2	
Rectum	40	12	
Differentiated grade			NS
Well	5	1	
Moderately	38	8	
Poorly	6	5	
Affected lymph nodes			< 0.05
N0	32	3	
N1	12	2	
N2	5	9	
Invasive depth			< 0.05
T1	1	0	
T2	11	1	
T3	32	7	
T4	5	6	

Table 4 Comparison of preoperative CEA levels between Metastasis V-positive and Metastasis V-negative patients

Parameters	Metastasis V		*P* value
	Negative	Positive	
Median (ng/mL)	3	4.27	< 0.05
Grade			< 0.05
High (> 15.00 ng/mL)	5	5	
Low (⩽ 15.00 ng/mL)	73	16	

cells can reside in the mesentery of the colorectum of CRC patients. These tumor cells showed no direct attachment to the primary lesions, with no involvement of vessel-lymphatic drainage system, and are confined to the fatty tissues enveloped by the fascia proper (Figure 1). In our previous study, we proposed a novel type of tumor metastasis designated as Metastasis V in gastric cancer patients[8]. Thus, we believe that Metastasis V could be detected in the mesenteries of both gastric and CRC patients. Similar to our previous studies, the incidence of Metastasis V was closely related to the tumor invasion of CRC patients, which was mainly detected in the T3 and T4 tumors (Table 2). Metastasis V was also correlated with lymphatic metastasis. Lymphatic nodes staging N1-N3 had larger percentages of Metastasis V than N0 (Table 2). Therefore, immunohistochemical analysis of Metastasis V in these patients is strongly recommended in clinical practice.

More interestingly, the preoperative serum CEA levels in Metastasis V-positive patients were significantly higher than in Metastasis V-negative patients (4.27 ng/mL *vs* 3.00 ng/mL) (Table 4). These data indicated that the prognosis of the patients with Metastasis V-positive tumors was worse than that of patients with Metastasis V-negative tumors. Due to the limited number of patients in the current study, a larger sample of cohort study should be enrolled for further analysis.

Little is known about the exact route or mechanism of Metastasis V. It was hypothesized that primary tumor lesions penetrates the intestinal wall, and then the cancer cells could probably scatter into the fatty tissues enveloped by proper fascia. In this way, Metastasis V occurs not only in CRC and gastric cancers, but also in oral head-neck, pancreas and papilla of Vater tumors[12-20].

It has been well known in practice that radical surgery for cancers should include *en bloc* resections of the primary tumor and neighboring tissues. However, it is difficult to determine the exact boundaries of *en bloc* resection within gastrointestinal tumors. "TME" or "CME" can reduce the tumor recurrence and increase patient survival, however, the possible reason is still unknown. In our opinion, the mesenteries of colorectum enveloped by the proper fascia not only propose a precise boundary for resection, but also function as pathologic barriers of Metastasis V spreading. Thus, our findings are clinically significant because TME or CME should be a useful choice for surgical excision of Metastasis V. Further studies on the efficiency of Metastasis V excision *via* TME or CME will be investigated.

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COMMENTS

Background

Local-regional recurrence of colorectal cancer (CRC) is still often noted in the patients who received radical R0-resection with no lymph-node metastasis.

Research frontiers

In addition to the classical four metastatic patterns, the existence of metastatic cancer cells in the mesentery of colorectum has also been reported, and this type of metastasis was named as "extra-capsular spreads" or "extra-nodal metastasis". Examination of the clinical and oncological significance of isolated tumor cells found in mesentery of colorectum in CRC patients is one of the main frontiers in surgical oncology.

Innovations and breakthroughs

Metastasis V is defined as the appearance of cancer cells in the mesogastrium, and may be a risk factor for the poor prognosis after radical surgery. This study demonstrated that Metastasis V was also detected in the mesentery of colorectum, and it might be associated with the poor prognosis of CRC.

Applications

It is clinically useful to be assisted in evaluation of prognosis of CRC patients.

Peer-review

In this paper, the authors well described an interesting study about isolated cancer cells in the mesentery of colorectum (named as Metastasis V). This is an interesting point, and the issue of metastasis in patients with CRC is of main importance.

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ORIGINAL ARTICLE

Observational Study

Natural history of covert hepatic encephalopathy: An observational study of 366 cirrhotic patients

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Author contributions: Wang AJ and Zhu X designed the research; Wang AJ, Li BM, Gan N, Pei L, Hong JB, Xiao HY and Zhong JW followed up with the patients and performed the research; Zheng XL analyzed the data; Wang AJ, Peng AP and Zhu X wrote the paper.

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Abstract

AIM

To explore the natural history of covert hepatic encephalopathy (CHE) in absence of medication intervention.

METHODS

Consecutive outpatient cirrhotic patients in a Chinese tertiary care hospital were enrolled and evaluated for CHE diagnosis. They were followed up for a mean of 11.2 ± 1.3 mo. Time to the first cirrhosis-related complications requiring hospitalization, including overt HE (OHE), resolution of CHE and death/transplantation, were compared between CHE and no-CHE patients. Predictors for complication(s) and death/transplantation were also analyzed.

RESULTS

A total of 366 patients (age: 47.2 ± 8.6 years, male: 73.0%) were enrolled. CHE was identified in 131 patients (35.8%). CHE patients had higher rates of death and incidence of complications requiring hospitalization, including OHE, compared to unimpaired patients. Moreover, 17.6% of CHE patients developed OHE, 42.0% suffered persistent CHE, and 19.8% of CHE spontaneously resolved. In CHE patients, serum albumin < 30 g/L (HR = 5.22, P = 0.03) was the sole predictor for developing OHE, and blood creatinine > 133 μmol/L (HR = 4.75, P = 0.036) predicted mortality. Child-Pugh B/C (HR = 0.084, P < 0.001) and OHE history (HR = 0.15, P = 0.014) were predictors of spontaneous resolution of CHE.

CONCLUSION

CHE exacerbates, persists or resolves without medication intervention in clinically stable cirrhosis. Triage of patients based on these predictors will allow for more cost-effect management of CHE.

Key words: Covert hepatic encephalopathy; Overt hepatic encephalopathy; Natural history; Liver cirrhosis

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Core tip: Covert hepatic encephalopathy (CHE) was prevalent in clinically-stable cirrhosis in the tertiary care hospital. A history of overt hepatic encephalopathy (OHE) was the only risk factor for CHE in patients with OHE history, while a high model for end-stage liver disease score was the only risk factor for those without OHE history. Natural history of CHE in the absence of medication intervention included development of complications, persistence or spontaneous resolution of CHE. Triage of patients based on predictors for exacerbation and resolution, rather than the degree of impairment in daily work productivity or quality of life, could make clinical management of CHE more cost-effective.

Wang AJ, Peng AP, Li BM, Gan N, Pei L, Zheng XL, Hong JB, Xiao HY, Zhong JW, Zhu X. Natural history of covert hepatic encephalopathy: An observational study of 366 cirrhotic patients. *World J Gastroenterol* 2017; 23(34): 6321-6329 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6321.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6321

INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome that is most commonly associated with decompensated liver cirrhosis and incorporates a spectrum of manifestations that range from mild cognitive impairment to coma. Generally, HE is subdivided into covert HE (CHE, including minimal HE and West-Haven grade Ⅰ HE) and overt HE (OHE, West-Haven grades Ⅱ-Ⅳ HE)[1,2]. CHE is regarded as the pre-clinical stage of OHE and occurs in 20%-80% of patients with cirrhosis. CHE is associated with increased progression to OHE[3-5], poor health-related quality of life (HRQOL)[6,7], and high risk for traffic violations and accidents[8]. It is also an independent predictor for death, liver-related complications and hospitalizations[9-11]. It is more detrimental for patients in manual labor professions (which involve complex occupational operation and driving of machinery) than those with desk jobs (which involve verbal and intellectual skills). Thus, CHE could endanger patients and their coworkers[12]. The decision to treat CHE depends on the degree of impairment in daily work productivity or quality of life of patients[1,2].

Several studies have investigated the natural history of CHE[3-5,9-11,13-18]. However, in these studies, treatment of other cirrhosis complications [such as antibiotics for infection or gastroesophageal variceal bleeding (GVB), lactulose for constipation, or albumin infusion for ascites or hepatorenal syndrome] was not restricted. The medication used to treat these complications during follow-up could hinder the occurrence of CHE and underestimate its disadvantages. Furthermore, perhaps not all CHE patients need to be treated. Thus, we hypothesized that CHE can persist or resolve spontaneously without medication intervention in some clinically-stable cirrhotic patients. Therefore, the aims of the present study were: (1) to observe the real natural history of CHE without medication intervention before development of cirrhosis related complications; and (2) to determine predictors for exacerbation and spontaneous resolution (SR) of CHE, in order to explore the therapeutic indications for CHE, based on natural course without medication intervention. Our findings will help CHE patient triage and lead to more precise and cost-effective clinical management.

MATERIALS AND METHODS

Subjects, study design and ethics

This was an observational study performed between August, 2014 and November, 2016 at The First Affiliated Hospital of Nanchang University, which is a tertiary care level hospital in China. Ethics approval was obtained from the Ethics Committee of the hospital, and all participants provided written informed consent in accordance with the Helsinki Declaration.

Consecutive cirrhotic patients in the outpatient department of the hospital were screened and included

in the study if they were > 18 and < 65 years of age and had a clinical, radiological or histological diagnosis of cirrhosis. Exclusion criteria included the following: presence or history of malignancy, including liver cancer; history of transjugular intrahepatic porto-systemic stent shunt or shunt surgery; presence of systemic disease, significant head injury, neurological or psychiatric diseases; alcohol or psychoactive medication intake within 6 mo; antibiotics, lactulose, probiotics or L-ornithine-L-aspartate intake or albumin infusion within 6 wk; illiteracy or inability to perform neuropsychometric tests; or refusal to participate in the study. Patients with history of OHE within 6 mo of inclusion were also excluded to ensure total recovery of last OHE occurrence. As patients with resolved OHE may be at a risk for developing CHE, those patients with history of OHE 6 mo before inclusion were included.

OHE history was assessed based on a patient's medical documents of hospitalization before inclusion. Patients were followed prospectively at intervals of 3 mo in the outpatient department of the hospital. Serial psychometric hepatic encephalopathy score (PHES) along with regular clinical and laboratory evaluations were measured at each visit until death, liver transplantation, or a follow-up of 12 mo after initial screening. If the patients did not show up for the 3-mo follow-up, they were tracked by telephone calls from the study staff. The time to death/liver transplantation and the occurrence of the first cirrhosis-related complications requiring hospitalization were recorded. Any medications recommended to treat CHE by established guidelines[1,2], including antibacterial agents, lactulose, probiotics, L-ornithine-L-aspartate and albumin infusion, had not been administered to patients unless necessary. However, these medications were not restricted if patients developed any complications and physicians thought these medications were necessary. In such cases, time to complications were documented. The Child-Pugh classification (CPC) and model for end-stage liver disease (MELD) scoring system were used to evaluate the severity of liver damage. All clinical data and assessment of complications were obtained from interviews or inpatient/outpatient medical documents.

Clinical data documentation and definition of complications

Qualified physicians performed routine physical examinations and documented biochemical blood tests (*i.e.*, complete blood count, alanine aminotransferase, total bilirubin, albumin, creatinine, prothrombin time, international normalization ratio), venous ammonia, virological blood tests [*i.e.*, hepatitis B virus (HBV) DNA and hepatitis C virus RNA levels], and diagnostic imaging (ultrasonography, computed tomography, or magnetic resonance imaging). The etiology of cirrhosis, history of medication use, OHE and severity of ascites were documented.

The complications, which included OHE, gastrointestinal (GI) bleeding, hepatorenal syndrome, infections, primary hepatic carcinoma (PHC) and severe ascites requiring albumin infusion, were documented in mo. OHE was diagnosed clinically on the basis of impaired mental status, as defined by the West Haven Criteria, and impaired neuromotor function (*i.e.*, hyperreflexia, rigidity, myoclonus and asterixis) that required initiation of HE-related therapy with or without hospitalization, and had corroboration from a caregiver[10,19]. GI bleeding was evidenced by melena, hematemesis or hematochezia. Infections were defined as bacterial infections, including pneumonia, sepsis with or without identified infectious focus, urinary tract infection, biliary tract infection, central nervous system infection (*i.e.*, meningitis or brain abscess), and spontaneous bacterial peritonitis. PHC diagnosis was based on imaging findings or liver biopsy.

Definition of CHE and of its persistence and resolution

PHES tests were used to define CHE. All patients underwent PHES tests in one morning session after breakfast in a quiet room without distracting noises. A specially trained medical doctor assisted the enrolled subjects in finishing these tests. Based on the nomograms of healthy volunteers, the PHES tests were described as abnormal if the score was < -4[10,20]. Persistence of CHE was defined if CHE patients survived and continued having abnormal PHES results until completion of follow up, in the absence of any of above-mentioned complication(s) or liver transplantation. As CHE runs a fluctuating course, CHE SR was defined if patients had normal PHES results for at least 6 mo at regular follow-up visits, in the absence of above-mentioned medications, complications or liver transplantation. Alternate forms of PHES tests (three parallel versions for each test in our study) were used at 3-mo interval visits to eliminate practice learning effects[21].

Sample size determination and statistical analysis

Based on a previous study, about 38% of CHE patients developed first OHE episode while about 17% of cirrhotic patients without CHE developed OHE[10]. Based on sample size calculations according to established formulas[22], the minimum sample size necessary for the current study was 78 patients in each group. It is reported that the prevalence of CHE in Chinese cirrhotic patients is about 40%. Thus, a minimum of 195 cirrhotic patients were enrolled to statistically compare the incidence of OHE between the CHE group and unimpaired group.

Continuous variables were expressed as mean ± SD. Categorical variables were described as the number and proportion of each category. Rate differences of OHE development, development of the first cirrhosis-related complications requiring hospitalization and death, between patients with and without CHE, were estimated with Kaplan-Meier analysis and log-rank test. If patients did not develop OHE or other complications

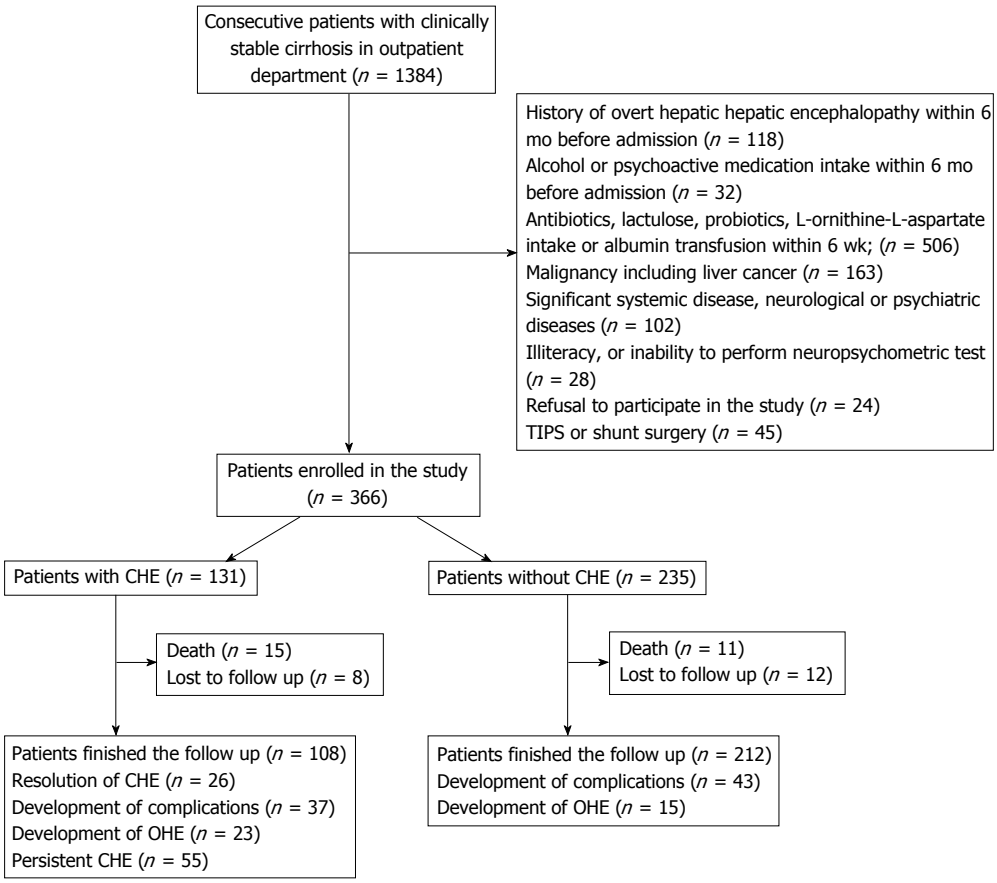


Figure 1 Flow diagram of cirrhotic patients included in the study. CHE: Covert hepatic encephalopathy; OHE: Overt hepatic encephalopathy; TIPS: Transjugular intrahepatic portosystemic stent.

and survived, "truncated" was described in the Kaplan-Meier curve. In order to determine risk factors for CHE and predictors for the resolution of CHE, potential parameters were included and first analyzed in the univariate analysis. All tested variables with *P* values < 0.05 were entered into a logistic regression after adjusting for age, sex and level of education. The χ^2 or Fisher's exact test was used for categorical variables. The analysis of variance was performed as appropriate to determine associations for continuous data. All tested variables with *P* values < 0.05 were entered into a logistic regression after adjusting for age and sex. In order to determine predictors for developing OHE, the first cirrhosis-related complications requiring hospitalization or mortality in CHE patients, potential parameters were analyzed by univariate analysis. All tested variables with *P* values < 0.05 were entered into a Cox regression after adjusting for age and sex. All statistical analyses were two-tailed at the 5% level. We used the Statistical Package for Social Science (SPSS Inc.; Chicago, IL, United States) to analyze our data.

RESULTS

Clinical characteristics and prevalence of CHE in cirrhotic patients

A total of 1384 consecutive outpatient cirrhotic patients were screened, and 366 patients (age: 47.2 ± 8.6 years, male: 73.0%, CPC-A: 43.2%, CPC-B: 50.5%, CPC-C: 6.3%, CPC score: 6.9 ± 1.6, MELD score: 15.4 ± 4.4) fulfilled the criteria and were enrolled in the study (Figure 1). Patients were followed for a mean of 11.2 ± 1.3 mo. CHE was identified in 131 (35.8%) patients. The clinical characteristics of CHE and the unimpaired group are shown in Table 1.

Risk factors of CHE in cirrhotic patients

Compared with unimpaired patients, CHE patients were older and had a more frequent OHE history. After adjusting for age, sex and level of education, logistic analysis indicated that a history of OHE (β: 2.72, OR = 7.18, 95%CI: 3.45-14.92, *P* < 0.001) was the only risk factor for CHE in patients with OHE history, while a MELD score > 20 (β: 1.71, OR = 5.53, 95%CI: 1.33-22.89, *P* = 0.02) was the only risk factor for patients without OHE history.

Complications and mortality in cirrhotic patients

Among the 366 patients included in this study, 323 (88.3%) completed the follow-up and survived. Twenty-three patients (unimpaired: 18.3% *vs* CHE: 28.2%) died, and 20 patients (unimpaired: 5.1% *vs* CHE: 6.1%) were lost to follow-up. None of the patients developed PHC or underwent liver transplantation during follow-

Table 1 Comparison of baseline characteristics between cirrhotic patients with and without covert hepatic encephalopathy (unimpaired)

	Unimpaired, *n* = 235	CHE, *n* = 131	*P* value
Sex, female/male	67/168	32/99	0.40
Age in yr	46.3 ± 8.9	48.8 ± 7.8	0.009
Cause			
HBV-associated	180 (76.7)	94 (71.8)	0.31
HCV-associated	8 (3.4)	4 (3.1)	0.86
Alcohol-associated	25 (10.6)	19 (14.5)	0.28
Others	22 (9.4)	14 (10.7)	0.68
Level of education			
Primary school	34 (14.5)	45 (34.4)	< 0.001
Junior high school	99 (42.1)	52 (39.7)	0.65
Senior high school	70 (29.8)	33 (25.2)	0.35
College degree or above	32 (13.6)	1 (0.8)	< 0.001
Creatinine	69.0 ± 16.3	71.3 ± 28.0	0.32
Venous ammonia	44.3 ± 4.2	57.9 ± 8.9	< 0.001
Albumin	33.5 ± 7.7	26.2 ± 6.1	< 0.001
High total bilirubin	17.3 ± 12.3	17.0 ± 9.1	0.76
Platelets, 10^9/L	102.6 ± 99.7	98.5 ± 96.7	0.70
International normalization ratio	1.14 ± 0.12	1.14 ± 0.15	0.99
Ascites, none/mild/severe	138/65/32	19/30/82	< 0.001
History of OHE	12 (5.1)	54 (41.2)	< 0.001
CPC, N, A/B/C	118/111/6	40/74/17	< 0.001
MELD score	14.6 ± 2.9	16.9 ± 6.0	0.02

Data are presented as *n*, *n* (%) or mean ± SD. CHE: Covert hepatic encephalopathy; CPC: Child-Pugh classification; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Model for end-stage liver disease; OHE: Overt hepatic encephalopathy.

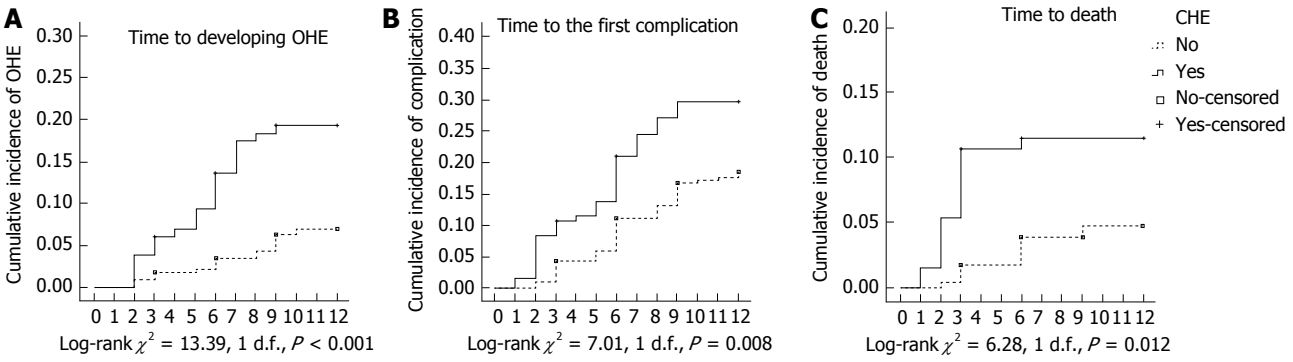


Figure 2 Time from inclusion to development of the first cirrhosis-related complications requiring hospitalization and death for patients with and without covert hepatic encephalopathy. A: Time from inclusion to development of OHE; B: Time from inclusion to development of the first cirrhosis-related complications requiring hospitalization; C: Time from inclusion to death. The Kaplan-Meier curve for estimated cumulative incidence in the Y axis and months from initial entry in the X axis. Log-rank statistics were used to compare groups. CHE: Covert hepatic encephalopathy; OHE: Overt hepatic encephalopathy.

up. The prevalence of cirrhosis-related complications requiring hospitalization and mortality were 28.2% and 11.5% in the CHE group *vs* 18.3% and 4.7% in the unimpaired group, respectively. Patients in the CHE group had higher rates of mortality (P = 0.012) and incidence of first cirrhosis-related complications requiring hospitalization (P = 0.008), including OHE (P < 0.001), compared to patients in the unimpaired group (Figure 2).

Natural history of CHE

After 11.2 ± 1.3 mo of follow-up for 131 CHE patients, 108 patients (82.4%) had completed the follow-up and survived. Thirty-seven patients (28.2%) developed cirrhosis-related complications requiring hospitalization, including 23 patients that developed OHE (17.6%). Fifty-five patients (42.0%) suffered persistent CHE, while 26 patients (19.8%) had CHE SR. The comparisons of clinical characteristics between CHE patients with and without OHE development, between those with and without development of the cirrhosis-related complications requiring hospitalization, between those with and without CHE SR, and between survivors *vs* non-survivors during follow-up are shown in Table 2.

Cox analysis of all the potential parameters (with P values < 0.05 from the univariate analysis shown in Table 2) indicated that only serum albumin < 30 g/L was a predictor for developing OHE. Serum creatinine > 133 μmol/L, MELD score > 20 and blood platelet < 80

Table 2 Comparison of clinical characteristics between 131 covert hepatic encephalopathy patients with and without overt hepatic encephalopathy development, between those with and without development of the first cirrhosis-related complications requiring hospitalization, between those with and without resolution of covert hepatic encephalopathy, and between survivors and non-survivors during follow-up

	Development of OHE			Complication(s)			Death			Resolution of CHE		
	Yes, *n* = 23	No, *n* = 108	*P* value	Yes, *n* = 37	No, *n* = 94	*P* value	Yes, *n* = 15	No, *n* = 116	*P* value	Yes, *n* = 26	No, *n* = 105	*P* value
Male sex	19 (82.6)	80 (74.1)	0.39	33 (89.2)	66 (70.2)	0.02	14 (93.3)	85 (73.3)	0.09	14 (53.8)	85 (81)	0.004
Age in yr	46.4 ± 7.9	47.3 ± 8.7	0.56	47.3 ± 8.3	47.2 ± 8.7	0.90	44.7 ± 7.8	47.4 ± 8.6	0.13	47.8 ± 8.2	49.0 ± 7.8	0.48
HBV-associated	17 (73.9)	77 (71.3)	0.80	27 (73.0)	67 (71.3)	0.85	9 (60)	85 (73.3)	0.28	17 (65.4)	77 (73.3)	0.42
Creatinine	80.5 ± 42.7	68.6 ± 16.8	0.001	76.5 ± 33.2	68.0 ± 16.1	0.001	95.3 ± 46.4	67.9 ± 16.6	< 0.001	72.6 ± 23.7	71.0 ± 29.1	0.80
Albumin in g/L	27.2 ± 8.1	31.3 ± 7.8	0.003	29.9 ± 7.9	31.1 ± 8.0	0.23	29.0 ± 8.4	31.0 ± 7.9	0.21	27.8 ± 5.3	25.8 ± 6.2	0.13
Total bilirubin in μmol/L	23.3 ± 16.7	16.5 ± 10.2	< 0.001	22.5 ± 16.3	15.7 ± 8.9	< 0.001	29.5 ± 24.0	16.3 ± 9.0	< 0.001	15.8 ± 10.5	17.2 ± 8.8	0.47
Platelets, 10^9/L	62.0 ± 37.2	105.7 ± 102.4	0.009	81.8 ± 105.8	106.6 ± 95.9	0.046	81.3 ± 59.2	102.7 ± 100.8	0.29	131.1 ± 109.2	90.35 ± 92.1	0.05
INR	1.2 ± 0.2	1.1 ± 0.1	< 0.001	1.2 ± 0.1	1.1 ± 0.1	< 0.001	1.3 ± 0.2	1.1 ± 0.1	< 0.001	1.1 ± 0.1	1.1 ± 0.2	0.49
Ascites, none/mild/severe	2/14/2007	12/28/68	0.03	8/21/2008	11/22/61	0.35	1/14/2000	19/29/68	0.03	9/13/2004	15/21/69	0.25
History of OHE	13 (56.5)	41 (38.0)	0.10	17 (45.9)	37 (39.4)	0.49	12 (80)	42 (36.2)	0.001	3 (11.5)	51 (48.6)	0.001
CPC, A/B/C	3/18/2	37/56/15	0.06	6/23/8	34/51/9	0.04	7/8/2000	40/67/9	< 0.001	3/2/2021	19/71/15	< 0.001
MELD score	19.6 ± 4.7	14.9 ± 4.1	< 0.001	19.2 ± 4.2	14.3 ± 3.8	< 0.001	23.3 ± 5.0	14.8 ± 3.7	< 0.001	13.7 ± 4.0	17.7 ± 6.2	0.002
Complications	23 (100)	14 (13.0)	< 0.001	37 (100)	0	< 0.001	10 (66.7)	27 (23.3)	< 0.001	0	37 (35.2)	< 0.001
OHE	23 (100)	0	< 0.001	23 (62.2)	0	< 0.001	7 (46.7)	16 (13.8)	0.002	0	23 (21.9)	< 0.001
Mortality	7 (30.4)	8 (7.4)	0.002	10 (27.0)	5 (5.3)	< 0.001	15 (100)	0	< 0.001	0	15 (14.3)	< 0.001

Data are presented as *n*, *n* (%) or mean ± SD. CHE: Covert hepatic encephalopathy; CPC: Child-Pugh classification; HBV: Hepatitis B virus; INR: International normalization ratio; MELD: Model for end-stage liver disease; OHE: Overt hepatic encephalopathy.

× 10^9/L were predictors for development of the cirrhosis-related complications requiring hospitalization; serum creatinine > 133 μmol/L was the only predictor for mortality of CHE patients (Table 3). After adjusting for age and sex, multivariate logistic analysis showed that CPC-B/C (β: -2.48, HR = 0.084, 95%CI: 0.026-0.264, P < 0.001) and OHE history (β: -1.91, HR = 0.15, 95%CI: 0.03-0.67, P = 0.014) were predictors for CHE SR.

DISCUSSION

Until now, no study has explored the real natural history of CHE in the absence of medication intervention. Our study shows that CHE can worsen, persist, or even spontaneously resolve without medication intervention over a 1-year period. Patients with an OHE history, low serum albumin, high serum creatinine, decreased blood platelet count, or poor liver function should be treated to prevent complications and reduce mortality. Patients with CPC-A and without OHE history may be monitored with caution without treatment. It is our speculation that triage of CHE patients based on predictors for exacerbation and resolution, rather than on the degree of impairment in daily work productivity or quality of life, will make clinical management more precise and cost-effective.

There are many testing strategies focusing on paper-pencil abnormalities, computerized or neurophysiological tests, such as PHES, repeatable battery for the assessment of neuropsychological status (commonly known as RBANS), inhibitory control test (ICT), EncephalApp Stroop smartphone App, critical flicker frequency (CFF), and electroencephalography (EEG). According to the guidelines, the diagnosis of CHE in multi-center studies should utilize at least two of the current validated testing strategies, consisting of PHES and one of the following: computerized (continuous reaction time, ICT, scan reaction time, or Stroop) or neurophysiological (CFF or EEG).

In single-center or clinical routine studies, investigators should use tests with which they are familiar for assessing the severity of HE, provided that normative reference data are available and the tests have been validated for use in the patient population[1,2]. In this single-center study, we used PHES, which has been previously validated in China[20], to define CHE diagnosis, persistence and resolution. In our study, 35.8% of consecutive clinically stable cirrhotic patients were diagnosed with

Table 3 Cox analysis of predictors for developing overt hepatic encephalopathy, the first cirrhosis-related complications requiring hospitalization, and mortality in 131 covert hepatic encephalopathy patients

	Predictor(s)	β	HR	95%CI	*P* value
For developing OHE	Albumin < 30 g/L	1.65	5.22	1.22-22.32	0.03
For developing the first	Cr > 133 μmol/L	1.24	3.46	1.46-8.20	0.005
cirrhosis-related complications	MELD score > 20	2.31	10.12	4.14-24.74	< 0.001
	PLT < 80 × 10^9/L	2.52	2.44	1.69-6.60	0.013
For mortality	Cr > 133 μmol/L	1.51	4.75	1.10-20.44	0.036

Cr: Creatinine; MELD: Model for end-stage liver disease; OHE: Overt hepatic encephalopathy; PLT: Platelet.

CHE based on PHES tests, in agreement with previous Chinese studies[7,20].

Only a few studies have discussed risk factors of CHE in cirrhotic patients[23,24]. However, these studies excluded patients with OHE history. As patients with resolved OHE may be at risk for developing CHE, risk factors for patients with OHE history also need to be explored. Yoshimura *et al*[25] enrolled patients with and without OHE history and revealed that OHE history was strongly associated with CHE, while cirrhosis-related symptom scores were associated with CHE for patients without OHE history, which is similar to our findings.

Several studies have focused on searching for predictors of OHE in cirrhotic patients. A recent study reported that CHE and elevated blood NH_3 levels contributed to OHE development in cirrhosis[3]. Another study indicated that OHE history, presence of CHE and an albumin level < 3.5 g/dL were associated with OHE development in cirrhosis[5]. Based on our findings, it is more cost-effective to screen and identify predictors of OHE development in CHE patients rather than in all cirrhotic patients. Our results showed that only serum albumin level < 30 g/L is the predictor for developing OHE in CHE patients. We propose that CHE patients with a low level of serum albumin should be treated rather than being monitored to reduce hospitalizations and mortality.

Several studies have investigated the natural history of CHE[3-5,9-11,13-18]. Recently, five studies showed that CHE is associated with occurrence of OHE and reduced survival rate[9-11,17,18]. Thomsen *et al*[9] enrolled 106 clinically-stable cirrhotic patients with no previous history of OHE and followed them for 230 ± 95 d. They found that 13.3% of CHE patients (defined by CFF, EEG or PHES) developed OHE, and the survival rate was 86.7%. In a multicenter study by Patidar *et al*[10], a total of 170 cirrhotic patients were followed for a mean of 13 mo. They found that 30% of cirrhotic patients developed at least one OHE episode, and that CHE, which was defined by PHES only, increased their risk of developing OHE, hospitalization and death/transplant despite controlling for MELD.

A study by Ampuero *et al*[11] demonstrated that patients with CHE, as defined by CFF, had a reduced 5-year survival rate compared to those without CHE (57.4%-68.6% *vs* 70%-82%). It also predicted the occurrence of OHE (37.1%-38.9% in patients with CHE *vs* 18.3%-24.6% in those without CHE). In a multi-center study by Allampati *et al*[17], a total of 437 cirrhotic patients and 308 controls were followed for 11 mo. The authors reported that 13% of patients developed an episode of OHE, which was predicted by CHE defined by the EncephalApp based on adjusted United States population norms, ICT or PHES.

However, none of the above studies stopped medications used for the treatment of other complications (*i.e*., antibiotics for infection or significant GVB, albumin transfusion for ascites, lactulose for constipation) during follow-up, which can play important roles in preventing occurrence of OHE and help CHE resolution. Persistence and SR of CHE were also not explored in these studies. Therefore, the real natural course of CHE without medication intervention was not evaluated, and therefore the risk of developing complications and mortality in CHE patients could be underestimated due to the medications used during follow-up. Furthermore, though CHE was reported to be associated with a higher risk of developing complications and reduced survival rate in these studies, it remains to be determined if all CHE patients need to be treated.

Our study revealed that CHE can resolve after 3 mo following the initial diagnosis, or can last as long as 1 year in the absence of medication intervention. Predictors for CHE SR were also explored in our study for the first time. Our data indicated that patients with predictors for SR could be monitored without resorting to medications, and receive appropriate medical support if predictors for development of complications and mortality appeared. Our findings also suggested that CHE patients need to be repeatedly evaluated at appropriate time intervals, as CHE can fluctuate over time.

There are some limitations in our study. Firstly, we conducted a single-center observational study with a relatively short follow-up period. However, we are the first to explore the natural history of CHE without medication intervention with a sample size large enough and time period long enough to identify predictors for exacerbation and SR of CHE. In addition, as cirrhotic patients developed complications over time, fewer patients were free of medications at a longer period of follow-up. Then it was of little significance to explore the natural course of CHE due to the limited number of patients. Secondly, a 3-mo interval between

visits was arbitrarily decided by the researchers. As CHE and OHE fluctuate and cognitive changes may be missed by this frequency, shorter intervals of observation would have been more convincing. However, it is our opinion that a 3-mo interval is the minimal time interval to eliminate learning effects for PHES. Thirdly, only some of the medications for treating cirrhosis, including antibacterial agents, lactulose, probiotics, L-ornithine-L-aspartate and albumin, were restricted unless necessary. However, some other medications, such as proton pump inhibitors, diuretics, non-selective beta-blocker and anti-HBV agents, were not restricted during our follow-up, as the potential impact of these agents on CHE is still in debate.

In summary, CHE is prevalent in clinically-stable cirrhotic patients and is associated with an increased risk of complications, hospitalizations and mortality. CHE can evolve into OHE, persist or spontaneously resolve without medication intervention within a short period. Although data are lacking regarding the standard management of CHE in clinical practice, we believe triage of patients based on predictors of exacerbation and improvement of CHE in natural history could make clinical management of CHE more precise and cost-effective.

COMMENTS

Background

Covert hepatic encephalopathy (CHE) is regarded as the pre-clinical stage of hepatic encephalopathy (HE). The natural history of CHE in cirrhotic patients in the absence of medication intervention has not yet been studied. The aim of this study was to explore the prevalence and natural history of CHE in clinically-stable cirrhosis in a tertiary care hospital, and to identify predictors for exacerbation and resolution of CHE without medication intervention.

Research frontiers

Several studies have investigated the natural history of CHE. Their findings indicate that CHE is prevalent in clinically-stable cirrhotic patients and is associated with an increased risk of complications, hospitalizations and mortality. However, medications treating other cirrhosis complications are not restricted in CHE patients. These agents used during follow-up would treat CHE, hinder the occurrence of other complications, and underestimate the disadvantages of CHE.

Innovations and breakthroughs

The present study indicated that CHE could worsen, persist or even spontaneously resolve without medication intervention over a 1-year period. Serum albumin < 30 g/L was the sole predictor for overt HE (OHE) and blood creatinine > 133 μmol/L predicted mortality. Child-Pugh A classification and absence of OHE history can predict spontaneous resolution of CHE.

Applications

To make the clinical management of CHE more precise and cost-effective, patients with an OHE history, low serum albumin, high serum creatinine, decreased blood platelet count or poor liver function should be treated to prevent complications and reduce mortality. Patients with Child-Pugh A classification and those without OHE history may be monitored with caution without treatment. Triage of CHE patients may be based on predictors for exacerbation and resolution rather than on the degree of impairment in daily work productivity or quality of life.

Peer-review

The authors conducted a well-written observational study. The case enrollment and variable choices are appropriate. Despite the fact that this study is a single-center observational study with a relatively short-period follow up, it provides some new insights.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Circular RNA hsa_circ_0000745 may serve as a diagnostic marker for gastric cancer

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Abstract

AIM

To determine whether circular RNAs (circRNAs) are involved in pathological processes of gastric cancer (GC).

METHODS

Three circRNAs with differential expression in GC and colorectal cancer were randomly selected for validation by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), using 20 pairs of gastric tissues and normal tissues. Based on the predicted circRNA-miRNA network, we then focused on hsa_circ_0000745, which was found to be down-regulated in 20 GC tissues compared with normal tissues. The hsa_circ_0000745

levels were further analyzed by qRT-PCR in 60 GC tissues and paired adjacent non-tumor tissues, as well as 60 plasma samples from GC patients and 60 plasma samples from healthy controls. The associations between the levels of hsa_circ_0000745 and the clinicopathological features of GC patients were statistically assessed. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of hsa_circ_0000745 in GC.

RESULTS

Hsa_circ_0000745 was down-regulated in GC tissues *vs* non-tumorous tissues ($P < 0.001$) and in plasma samples from patients with GC *vs* healthy controls ($P < 0.001$). The expression level of hsa_circ_0000745 in GC tissues correlated with tumor differentiation, while the expression level in plasma correlated with tumor-node-metastasis stage. The area under the ROC curve (AUC) of hsa_circ_0000745 in plasma was 0.683, suggesting good diagnostic value. Plasma hsa_circ_0000745 level combined with carcinoembryogenic antigen (CEA) level increased the AUC to 0.775.

CONCLUSION

Hsa_circ_0000745 plays an important role in GC and its expression level in plasma in combination with CEA level is a promising diagnostic marker for this malignancy.

Key words: Circular RNA; circ_0000745; Gastric cancer; Diagnosis; Noncoding RNA

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Core tip: This study demonstrates that expression of hsa_circ_0000745 is down-regulated in human gastric cancer (GC), with significantly lower levels detected in human GC tissues compared to paired adjacent non-tumor tissues, and in plasma of GC patients compared to plasma from normal healthy controls. Detection of this stable circular RNA in plasma may represent a clinically useful and convenient marker for GC diagnosis, with higher sensitivity and specificity when analyzed in combination with carcinoembryogenic antigen.

Huang M, He YR, Liang LC, Huang Q, Zhu ZQ. Circular RNA hsa_circ_0000745 may serve as a diagnostic marker for gastric cancer. *World J Gastroenterol* 2017; 23(34): 6330-6338 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6330.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6330

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide, and is the most frequently diagnosed cancer among East Asian populations[1-3]. Despite extensive research attention, the mortality rate of GC remains high due to the fact that it is usually diagnosed at late stages. To improve early diagnosis and reduce the high mortality rate of GC[4-6], it is critical to continue elucidating the molecular mechanisms of GC, which may help identify markers for earlier diagnosis and novel therapeutic targets.

Endogenous noncoding RNAs, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and the recently identified circular RNAs (circRNAs), play key regulatory roles in various cellular physiological processes and may contribute to pathological processes such as cancer formation and progression[7,8]. Unlike linear RNAs which terminate with 5' caps and 3' tails, circRNAs form covalently closed loop structures with neither 5' to 3' polarity nor a polyadenylated tail. They are not simply by-products of mis-spliced RNAs or splicing errors, but rather they are the products of regulated back-spliced RNAs with distinct sets of *cis*-elements and/or *trans*-factors[9]. Moreover, the formation of circRNAs involves the occurrence of back-splicing by the canonical spliceosome[10].

Recent research has demonstrated that circRNAs are widely involved in myriad physiological and pathological processes, including neuronal differentiation, synaptogenesis, neurological disorders, angiogenesis, prion disease, and cancer[11-16]. Likewise, circRNAs have been described as a class of biomarkers related to aging in Drosophila[17] and as putative disease biomarkers in human saliva[18]. Li *et al*[19] reported that hsa_circ_002059, a typical circRNA, was significantly down-regulated in GC tissues compared with paired adjacent non-tumorous tissues, suggesting its potential as a novel and stable biomarker for the diagnosis of GC.

The collective findings from the research on circRNAs indicate that they may play crucial roles in the molecular pathways of cancer. However, only a few publications have reported on the relationship between circRNAs and cancer. Whether and how circRNAs expression is dysregulated in GC remains unknown.

In this study, we randomly selected three differentially expressed circRNAs (hsa_circ_0000745, hsa_circ_0085616, and chr16:30740286-30740893|+) from two circRNA databases: CircBase (http://circbase.org/) and circ2Traits (http://gyanxet-beta.com/circdb/), which had been characterized using RNA-Seq analysis of ribosomal RNA-depleted total RNA from three GC or colorectal cancerous tissues and paired normal tissues[20,21] (Table 1). We then focused our attention on circRNA hsa_circ_0000745, which is encoded by the sperm antigen with calponin homology and coiled-coil domains 1 gene (*SPECC1*) that is located at chr17:20107654-20109225. We selected hsa_circ_0000745 for investigation based on preliminary broad sequencing analysis and prediction of its binding to various miRNAs with known relations

Table 1 The three differently expressed circRNAs in human gastric cancer and colorectal cancer samples

CircRNA	Chrom	TxStart	TxEnd	circBaseID	Best_transcript	Gene name	Catalog
Chr16: 30740287-30740893 | +	16	30740286	30740893	266	NM_006662	SRCAP	Exonic
Hsa_circ_0000745	17	20107645	20109225	790	NM_152904	SPECC1	Exonic
Hsa_circ_0085616	8	131370262	131374017	127	NM_001247996	ASAP1	Exonic

Chrom: Chromosome; Tx: Transcription.

to GC. By expanding the numbers of human GC and control samples for investigation, we were able to identify a statistically significant down-regulation of hsa_circ_0000745 expression in both GC tissues and plasma samples from patients with GC. Moreover, we were able to determine that the down-regulated expression correlated with tumor differentiation. Overall, the data identifies hsa_circ_0000745 as a novel potential biomarker for diagnosing GC.

MATERIALS AND METHODS

Patient samples

This study was approved by the Institutional Ethics Review Board of Anhui Provincial Hospital Affiliated to Anhui Medical University (China) and was conducted according to the Ethical Guidelines for Human Genome/Gene Research issued by the Chinese Government. Human samples of GC tissues and paired adjacent non-tumor tissues were prospectively collected from 60 patients at the Anhui Provincial Hospital from January 2016 to January 2017. Upon removal, the respective tissues were immediately submerged in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) and stored at -80 ℃ until analysis. None of the patients had undergone chemotherapy or radiotherapy prior to the operation (resection).

The GC diagnosis was confirmed by histopathology. Tumor stages were determined according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging criteria.

Before any treatment was applied, each study participant's peripheral blood (2-3 mL) was collected in an ethylenediaminetetraacetic acid tube, centrifuged at 3000 × *g* for 10 min to obtain plasma, and stored at -80 ℃. Following the age- and sex-matching criteria of the study design, fresh normal plasma samples were collected from 60 healthy volunteers.

All research subjects provided written informed consent prior to study enrollment.

RNA extraction and quality control

Total RNA was isolated from three samples of gastric tumor and paired adjacent non-tumor (normal) tissues, as well as from plasma samples, using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. RNA integrity was assessed using standard denaturing agarose gel electrophoresis. Total RNA from each specimen was quantified and quality assurance was provided using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, United States).

Transcriptome high-throughput sequencing and subsequent bioinformatics analysis

Transcriptome high-throughput sequencing and subsequent bioinformatics analysis were performed by Cloud-Seq Biotech (Shanghai, China). The paired-end reads were harvested on a HiSeq 4000 System (Illumina, San Diego, CA, United States). The high-quality reads were aligned to the reference genome/transcriptome with STAR software[22], and circRNAs were detected and identified with DCC software[23]. EdgeR software[24] was used to normalize the data and perform analysis to determine differential expression among the circRNAs.

Quantitative reverse transcription-polymerase chain reaction validation assay

Total RNA samples were reverse-transcribed into cDNA with a random primer using SuperScript™ Ⅲ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

The expression of circRNAs was measured using quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix (Takara, Tokyo, Japan) in a ViiA 7 Real-time PCR System (Applied Biosystems Inc, Foster City, CA, United States). The sequences of the divergent primers for the detection of hsa_circ_0000745 by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were 5′-GTTGAAAGTAGCCCGAGCAG-3′ and 5′-ACGTG GCACAGACCTCTCTC-3′. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as a control) were 5′-CTCGCTTCGGCAGCACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′. These primers were synthesized by Invitrogen (Shanghai, China). The reaction conditions were as follows: 95 ℃ for 10 min, and 40 cycles of 95 ℃ for 10 s and 60 ℃ for 60 s. The RNA levels were normalized to human GAPDH. The expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method.

All of the quantitative PCR reactions were conducted in triplicate. The appearance of a single-peak in the melt-curve suggested the specificity of the PCR products.

Statistical analysis

All experimental data were analyzed using SPSS

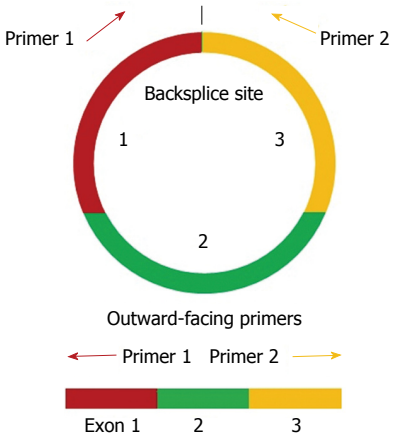


Figure 1 Schematic diagram of the polymerase chain reaction primers used to specifically detect circular transcripts. Divergent polymerase chain reaction primers, rather than the more commonly used convergent primers, were designed for the circRNAs.

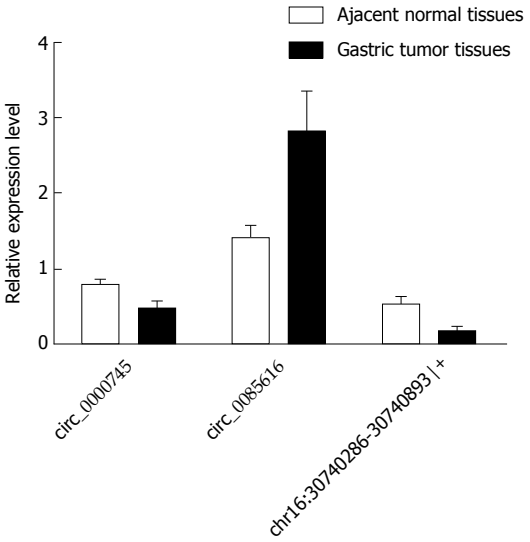


Figure 2 Validation of the expression levels of the three circRNAs using quantitative reverse transcription-polymerase chain reaction analysis. The three differently expressed circRNAs were validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 20 gastric tumor tissues and paired adjacent non-tumor tissues. The qRT-PCR results are presented as mean and SE of the mean.

software (version 22.0; IBM, Armonk, NY, United States) and GraghPad Prism 5.0 (GraphPad Software, La Jolla, CA, United States). The expression level of each circRNA was represented as fold-change using the $2^{-\Delta\Delta Ct}$ method. Differences of hsa_circ_0000745 levels between GC tissues and adjacent non-tumor tissues, and between plasma samples from patients with GC and healthy controls were assessed using *t*-test. The correlation between hsa_circ_0000745 levels and clinicopathological factors was further analyzed by one-way analysis of variance. The receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value. The cut-off value of hsa_circ_0000745 was analyzed with SPSS software. A

Table 2 Primers used for quantitative reverse transcription-polymerase chain reaction analysis of circular RNA and mRNA levels

Target ID	Primer sequence, 5'-3'	Product size in bp
Circ_0000745	F: GTTGAAAGTAGCCCGAGCAG	204
	R: ACGTGGCACAGACCTCTCTC	
Circ_0085616	F: GACTACAACTCGCCCACCAC	200
	R: TCCATTTCTGGGCCATAATC	
Chr16:3074028 6-30740893|+	F: CCTTTGCACCGTATTGTGTG	199
	R: GGCAGGGTACAGAAATCCAG	
GAPDH	F: CTCGCTTCGGCAGCACA	122
	R: AACGCTTCACGAATTTGCGT	

bp: Base pairs; F: Forward; R: Reverse.

P value < 0.05 was considered statistically significant.

RESULTS

Validation of differentially expressed circRNAs by qRT-PCR

Among the three differentially expressed circRNAs examined (hsa_circ_0000745, hsa_circ_0085616, and chr16:30740286-30740893|+), hsa_circ_0085616 was reported to be up-regulated in colorectal tissues compared to that in normal tissues[20], while hsa_circ_0000745 and chr16:30740286-30740893|+ were found to be down-regulated in GC tissues compared to paired adjacent non-tumor tissues[21]. The observed changes in expression levels were validated by qRT-PCR analysis using 20 sets of gastric tissues and paired adjacent non-tumor tissues.

Divergent primers, rather than the more commonly used convergent primers, were designed for quantitative amplification of the circRNAs (Figure 1 and Table 2). The qRT-PCR results showed that circ_0085616 was up-regulated, while circ_0000745 and chr16:30740286-30740893|+ were down-regulated in the GC tissues compared to the paired adjacent non-tumor tissues (Figure 2), suggesting that the general expression patterns of the three circRNAs in GC were consistent with those in colorectal cancer.

CircRNA binding miRNA prediction by sequence analysis

Given that miRNAs play important roles in the progression of GC, some circRNAs could be involved in GC, likely *via* interacting with miRNAs. We analyzed the potential binding miRNAs for the three circRNAs using sequence analysis. The association of miRNAs with GC indicated that circRNAs may have a regulatory role in GC. A tree diagram of circRNAs and their potential binding miRNAs is shown in Figure 3. Based on these potential circRNA/miRNA interactions, hsa_circ_0000745 was predicted to be able to bind a spectrum of miRNAs, with the known functions

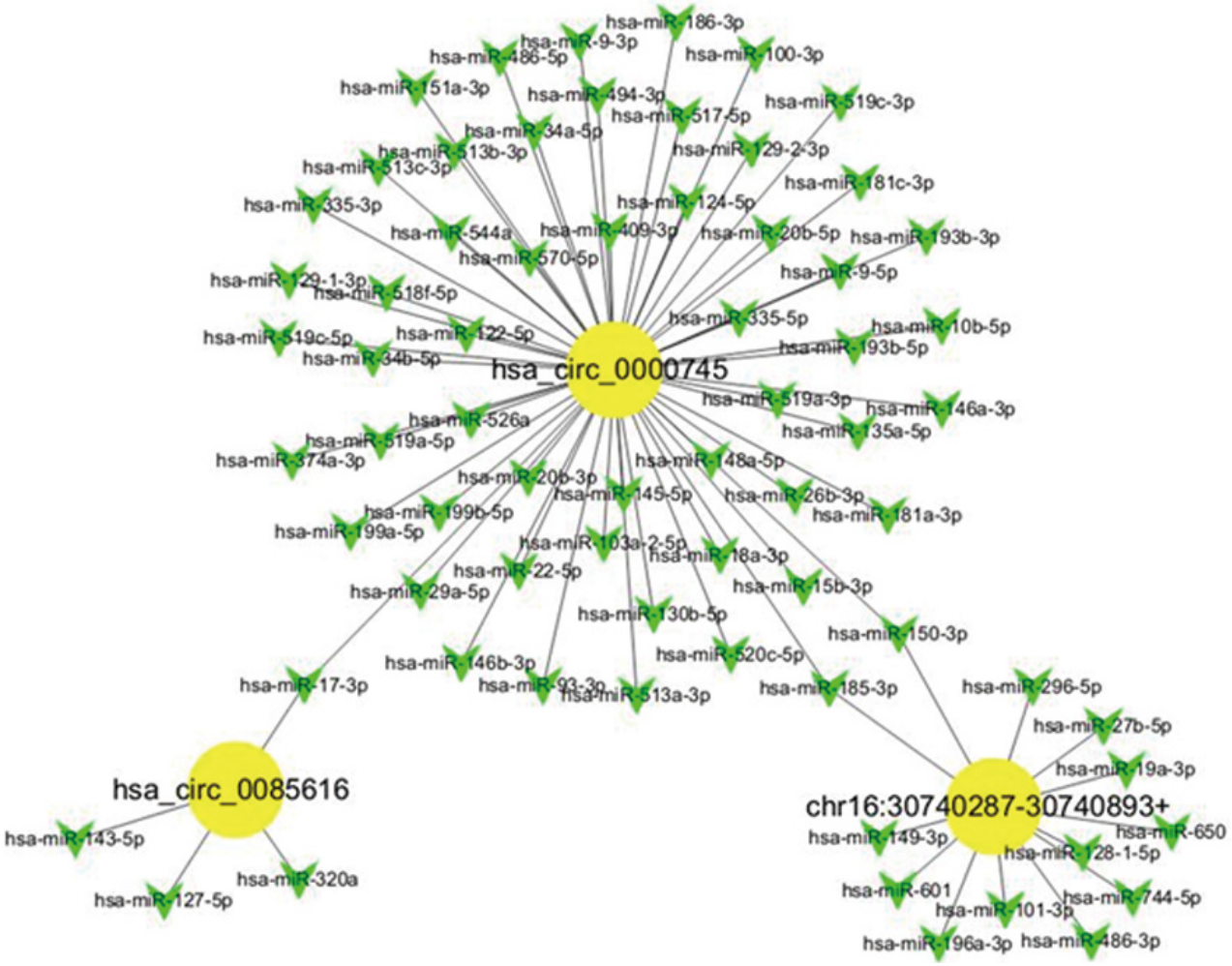


Figure 3 Network of circular RNAs and the predicted binding miRNAs.

suggesting its potential role in the regulation of GC growth.

Hsa_circ_0000745 expression is down-regulated in GC tissues and plasma samples from patients with GC

We tested hsa_circ_0000745 levels in GC tissues and plasma samples from patients with GC. As shown in Figure 4A, hsa_circ_0000745 expression was found to be down-regulated in the GC tissues ($P < 0.001$). Furthermore, hsa_circ_0000745 levels in the plasma samples of patients with GC were lower than those in plasma samples from healthy controls ($P < 0.001$; Figure 4B).

Potential diagnostic values of hsa_circ_0000745 in GC

Since we found that hsa_circ_0000745 expression levels were lower in GC tissues and patients' plasma samples, we further analyzed their association with clinicopathological features of patients with GC. As shown in Table 3, hsa_circ_0000745 levels in the GC tissues were significantly related to tumor differentiation ($P = 0.012$). However, we found no association between its levels with other clinicopathological features, such as age, sex, tumor diameters, lymphatic metastasis, TNM stage and carcinoembryogenic antigen (CEA) levels.

Meanwhile, hsa_circ_0000745 levels in plasma from GC patients were significantly related to TNM stage ($P = 0.046$). Nevertheless, we did not find any association between hsa_circ_0000745 and the other clinicopathological features.

To evaluate the potential diagnostic value, a ROC curve was generated for hsa_circ_0000745 levels in plasma. We found that the area under the ROC curve (AUC) was 0.683 (Figure 5). The sensitivity and specificity were 0.855 and 0.45, respectively. When the expression level of plasma hsa_circ_0000745 was combined with CEA level, the AUC increased to 0.775, while the sensitivity and specificity changed to 0.800 and 0.633, respectively.

DISCUSSION

Gastric carcinogenesis is a complicated biological process that involves a wide array of molecular abnormalities. In recent decades, the molecular

Table 3 Relationship of hsa_circ_0000190 expression levels ($2^{-\Delta\Delta Ct}$) in gastric cancer tissues with clinicopathological factors of patients with gastric cancer

Clinicopathological factor	*n*	Tissue hsa_circ_0000745		Plasma hsa_circ_0000745	
		Mean ± SD	*P* value	mean ± SD	*P* value
Age (yr)			0.757		0.195
< 60	33	0.77 ± 0.83		0.90 ± 0.96	
⩾ 60	27	0.58 ± 0.60		0.69 ± 0.53	
Sex			0.203		0.252
Male	43	0.59 ± 0.67		0.68 ± 0.60	
Female	17	0.92 ± 0.85		1.13 ± 1.12	
Diameter in cm			0.168		0.127
⩾ 5	26	0.76 ± 0.77		0.87 ± 0.76	
< 5	34	0.63 ± 0.71		0.76 ± 0.84	
Differentiation			0.012[a]		0.087
Well	2	0.96 ± 1.10		0.89 ± 0.44	
Moderate	25	0.65 ± 0.71		0.70 ± 0.66	
Poor	33	0.70 ± 0.77		0.88 ± 0.92	
Lymphatic metastasis			0.064		0.087
Present	44	0.71 ± 0.71		0.85 ± 0.78	
Absent	16	0.61 ± 0.81		0.69 ± 0.88	
TNM stage			0.056		0.046[a]
Ⅰ-Ⅱ	14	0.63 ± 0.81		0.60 ± 0.53	
Ⅲ-Ⅳ	46	0.70 ± 0.72		0.87 ± 0.87	
CEA in ng/mL			0.077		0.130
⩾ 5	10	0.79 ± 0.72		0.65 ± 0.59	
< 5	50	0.66 ± 0.74		0.85 ± 0.78	

[a]indicated statistical significance. CEA: Carcinoembryogenic antigen.

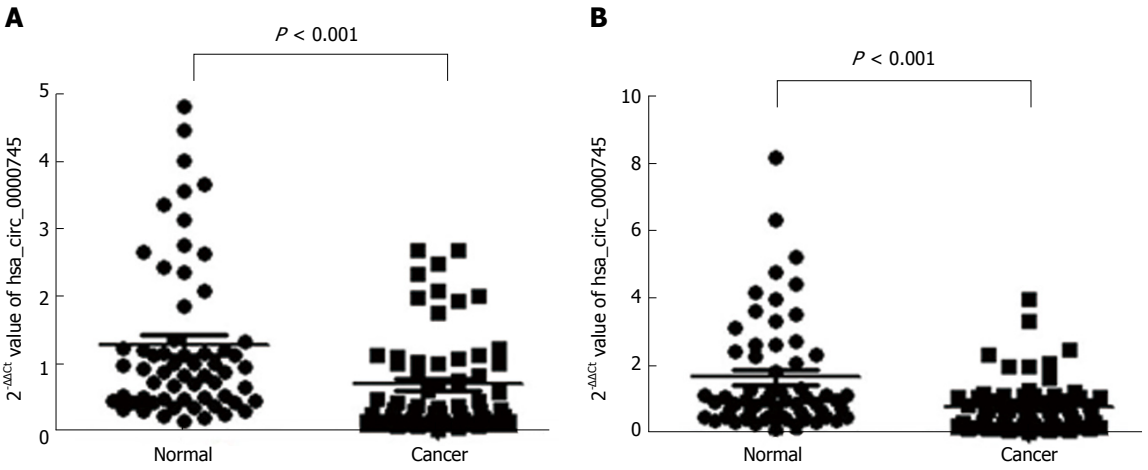


Figure 4 Hsa_circ_0000745 expression levels. A: Hsa_circ_0000745 expression levels in gastric cancer tissues and paired adjacent non-tumor tissues; B: Hsa_circ_0000745 expression levels in plasma samples from patients with gastric cancer and healthy volunteers. $P < 0.001$, $n = 60$. The expression is shown as the $2^{-\Delta\Delta Ct}$ value.

mechanisms of GC have been extensively investigated. Expression arrays for genes and noncoding RNAs, such as miRNAs and lncRNAs, have been applied and recognized as feasible and useful approaches to profiling the molecular signatures of GC[25,26].

Today, circRNAs are the newest type of noncoding RNAs, and their discovery expanded our knowledge of the complexity of noncoding RNAs. The circRNAs were originally thought to be by-products of splicing errors or the mRNA process[27]. They represent a stable, diverse and conserved class of RNA molecules[11,28]. Ongoing research is continuing to provide evidence that circRNAs are involved in the development and progression of diseases, especially cancer[29].

Hsa_circ_002059 was first found to be significantly down-regulated in GC and proposed as a potential novel and stable biomarker for the diagnosis of this particular cancer type[19]. According to other reports, expression of the circular RNA-ITCH is typically down-regulated in esophageal squamous cell carcinoma (ESCC) and colorectal carcinoma, and may exert an inhibitory effect on ESCC and colorectal cancer by suppressing the Wnt/β-catenin pathway[30,31]. Collectively, these findings have suggested that circRNAs play a crucial role in cancers, and that they may serve as the basis for developing new strategies

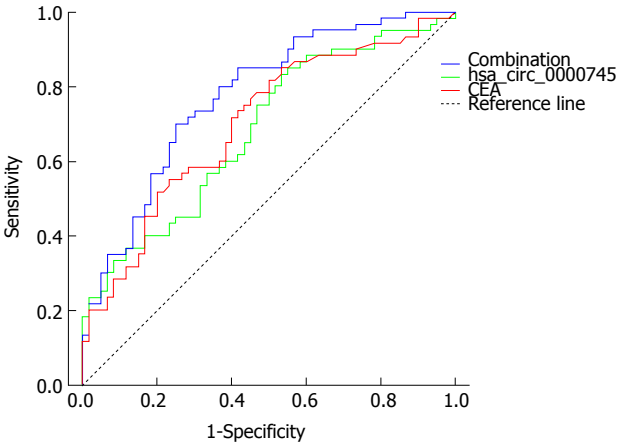


Figure 5 Receiver operating characteristic curve of plasma hsa_circ_0000745 and serum carcinoembryogenic antigen levels. CEA: Carcinoembryogenic antigen.

for diagnosis and therapy. However, the tumorigenesis mechanisms of GC are far from being completely elucidated and this idea is still in its infancy.

In this study, we randomly selected three differently expressed circRNAs, which we characterized using RNA-Seq analysis of ribosomal RNA-depleted total RNA from three GC or colorectal cancer tumor tissues and paired normal tissues. Circ_0085616 was up-regulated in colorectal cancer tissues compared with non-cancerous tissues, while circ_0000745 and chr16:30740286-30740893|+ were down-regulated in GC tissues compared with normal tissues. We further confirmed the RNA-Seq results of three circRNAs in 20 paired normal and cancerous gastric tissues by qRT-PCR analysis; circ_0085616 up-regulation and circ_0000745 and chr16:30740286-30740893|+ down-regulation in the GC tissues suggested that the circRNA sequencing results of GC are consistent with the expression patterns observed in colorectal cancer.

Recent studies have demonstrated that circRNAs can function as miRNA sponges or potent competitive endogenous RNA molecules[27,32-35]. Given that miRNAs play important roles in the progression of GC, it is possible and even likely that some circRNAs are involved in GC *via* interacting with miRNAs. We analyzed the potential binding miRNAs of the three circRNAs by sequence analysis. The association of miRNAs with GC indicated that circRNAs may have a regulatory role in GC. Based on the potential circRNA/miRNA interactions identified, hsa_circ_0000745 is potentially able to bind various miRNAs that may play critical roles in regulating GC growth and migration. However, additional studies are required to clarify whether these circRNAs are truly involved in GC, and whether circRNAs acting as miRNA sponges are a general phenomenon.

In this study, we found that hsa_circ_0000745 was down-regulated in GC tissues as well as in the plasma samples from patients with GC. It was suggested that hsa_circ_0000745 may be involved in other diseases besides GC, including various cancers (*i.e.*, breast cancer, colorectal cancer, hepatocellular carcinoma, lung cancer, ovarian cancer, and rhabdomyosarcoma) and cerebellar neurodegeneration, Waldenstrom macroglobulinemia, cardiac hypertrophy, cardiomyopathy, coronary artery disease, heart failure, myeloproliferative disorder, myocardial infarction, retinitis pigmentosa, and schizophrenia, according to the previous analysis (http://gyanxet-beta.com/circdb/). That is the main reason why we choose hsa_circ_0000745 for further study.

We found that expression levels of hsa_circ_0000745 in GC tissues were associated with tumor differentiation. However, its expression did not show any statistically significant relationship with tumor size, lymphatic metastasis or TNM stage (Table 3). Moreover, we determined its potential diagnostic value, reported herein for the first time. When compared with serum CEA levels, hsa_circ_0000745 had a higher sensitivity and specificity in the screening of GC (Figure 5). The combination of plasma hsa_circ_0000745 and serum CEA levels, however, increased the diagnostic value. In summary, our study revealed that the circular RNA hsa_circ_0000745 may serve as a diagnostic marker for GC.

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COMMENTS

Background

Circular RNA (circRNA) is a recently identified type of noncoding RNA that plays an important role in the biological processes of tumor development and progression. The stability of circRNA makes it an attractive candidate as a serological diagnostic marker for cancer. However, the circRNA expression profiles in gastric cancer (GC) have not been elucidated.

Research frontiers

Recent studies have shown that different tumors have distinctive differential expression profiles of various circRNAs, such as hsa_circ_002059 in GC and hsa_circ_0004277 in acute myeloid leukemia.

Innovations and breakthroughs

This is the first study to demonstrate that hsa_circ_0000745 is differentially expressed in plasma samples of GC patients and normal healthy controls, as well as in gastric tumor tissues and paired adjacent non-tumor tissues. Moreover, the data suggest the possibility that hsa_circ_0000745 may serve as a novel diagnostic marker for GC.

Applications

Expression of hsa_circ_0000745 was found to correlate with tumor differentiation and tumor-node-metastasis stage. When plasma hsa_circ_0000745 expression was combined with serum level of carcinoembryogenic antigen, the diagnostic

ability (especially specificity) for GC was improved.

Terminology

Circular RNA is a type of RNA which, unlike the better known linear RNA, forms a covalently closed continuous loop. GC is also known as stomach cancer, developing from the lining of the stomach. Diagnostic biomarkers are found in blood, urine or tissues and are abnormally expressed, in a trackable manner, in the presence of diseases.

Peer-review

This is the first study to show the promising diagnostic value of measuring circulating hsa_circ_0000745 levels in GC.

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ORIGINAL ARTICLE

Randomized Controlled Trial

P2Y1R is involved in visceral hypersensitivity in rats with experimental irritable bowel syndrome

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Abstract

AIM

To evaluate the role of P2Y1R in visceral hypersensitivity in rats with experimental irritable bowel syndrome.

METHODS

A rat model of irritable bowel syndrome was generated by intra-colonic administration of acetic acid (AA) and assessed by histology and myeloperoxidase (MPO) activity assay. Then P2Y1R expression in the colonic tissue was detected by Western blot. In order to explore the regulatory role of P2Y1R in visceral hypersensitivity, an agonist (MRS2365) and an antagonist (MRS2179) of P2Y1R were intra-colonically administered and effects were tested through a colorectal distension test. The abdominal withdrawal reflex and abdominal electromyography were tested during the course.

RESULTS

Model assessment tests showed an obvious inflammatory

reaction that appeared on the 2nd d after the AA injection, and the inflammatory reaction gradually recovered and almost disappeared on the 7th d. The model finished on day 8 and showed a clear feature of IBS that had no organic lesion. The average expression of P2Y1R was significantly higher in the AA group than in the naïve group (0.319 ± 0.02 *vs* 0.094 ± 0.016, $P < 0.001$). MRS2365 could effectively raise the colonic hypersensitivity status at intervention doses of 10 (AUC value from 0.30 ± 0.089 to 1.973 ± 0.127 mv·s, $P < 0.01$) and 100 μmol/L (AUC value from 0.290 ± 0.079 to 1.983 ± 0.195 mv·s, $P < 0.01$); MRS2179 could effectively reduce the hypersensitivity status at intervention dose of 100 μmol/L (from a mean baseline AUC value of 1.587 ± 0.099 mv·s to 0.140 ± 0.089 mv·s, $P < 0.0001$). Differences between the MRS2179 group (1.88 ± 1.45) and either the MRS2365 group (3.96 ± 0.19) or the combined treatment (MRS2179 and MRS2365) group (3.28 ± 0.11) were significant ($P < 0.01$).

CONCLUSION

P2Y1R plays a regulatory role in visceral hypersensitivity in rats with experimental IBS. Specific antagonists of P2Y1R may have potential therapeutic value in treating abdominal pain in IBS.

Key words: Irritable bowel syndrome; P2Y1 receptor; Regulation; Therapy; Visceral hypersensitivity

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Core tip: P2Y1R is believed to be part of a high-sensitivity response to mechanical stimulation but whether it is involved in visceral hypersensitivity of irritable bowel syndrome is discussing. We detected its expression in the colon of a rat model of irritable bowel syndrome, and explored its regulatory role in visceral hypersensitivity with a specific agonist and an antagonist. The abdominal withdrawal reflex and electromyography tests showed effective changes after intervention. It was interesting to note that in case of combined treatment of the two drugs, the antagonist showed higher effects, which indicated that other P2 receptors might be involved in the hypersensitivity of irritable bowel syndrome.

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INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by hypersensitivity and pain before defecation in the relative absence of colon inflammation or structural changes[1]. Studies have reported that visceral hypersensitivity could be mediated by purinergic signaling pathways, which participate in pain transmission[2-4] and possibly induce the pain and discomfort experienced by patients with IBS[5,6].

Nucleotides contribute to the sensation of pain in somatic and visceral organs[5-7]. Cells release adenosine triphosphate (ATP) and other nucleotides in response to a variety of stimuli *via* the mediation of a class of receptors termed P2[6]. P2 includes the ionotropic (P2XRs) and metabotropic (P2YRs) subtypes. P2XRs have been identified to contribute to pain transmission and be involved in many organic and functional gastrointestinal disorders including IBS[7,8]. The mammalian P2Y1R cloned thus far is widely expressed in animal tissues[6]. Accumulating evidence has shown that P2Y1R can mediate nociceptive signaling[9] and studies[10-12] have suggested that P2Y1R may provide a high-sensitivity system to mechanical stimulation in functional gastrointestinal disorders. However, whether P2Y1R participates in the generation and regulation of visceral hypersensitivity in IBS remains unknown.

In this study, we produced a rat model of post-infectious IBS by intra-colonic injection of acetic acid (AA), and measured the changes of P2Y1R expression in the colon of this model. Moreover, to further explore the role of P2Y1R in the development of visceral hypersensitivity, a specific agonist (MRS2365) and an antagonist (MRS2179) of P2Y1R were used to alter the functional status of P2Y1R, and the abdominal responses were detected after this alteration.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 180-250 g were used. Breeding, maintenance and euthanization of the animals followed the ethical guidelines for the investigations of experimental pain in conscious animals[13], and the study was approved by the Ethical Committee of Xi'an Jiaotong University. All animals were obtained from the standard animal laboratory at the Medical College of Xi'an Jiaotong University and were maintained at a consistent temperature of 20 ± 2 ℃ under a 12 h light/dark cycle with free access to food and water.

Model preparation

A rat model of post-infectious IBS (AA group) was produced in this study. After an overnight fast, the rats were anesthetized with 10% chloral hydrate by intraperitoneal injection at a dose of 1 mL/kg. On day 1, colitis was induced by intracolonic injection of 1 mL of 4% AA at the site 8 cm proximal to the anus for 60 s. Then the rats were allowed to recover for 7 d from the colitis and the modeling finished on day 8. A PBS group was processed in the same manner except that 1 mL of saline was injected instead of 4% AA. A naïve

control group comprised rats that were untreated.

Model assessment

The myeloperoxidase (MPO) activity assay was performed and histological sections of the distal colon were stained to determine the inflammatory condition and ensure the recovery from colitis on the 7th d. Then, abdominal responses and stool features of model rats were detected to assess the hypersensitivity and abdominal features.

Colorectal distension procedure and detection of visceral hypersensitivity: Colorectal distension (CRD) was conducted to assess the visceral perception thresholds in the gut[10,14], which was reported to be the best method to elicit visceral hypersensitivity[15]. Conscious rats of the AA, PBS and naïve groups (n = 8) were studied on the 8th d of modeling. They were fixed in Bollman cages (steel tubes; length, 18 cm; diameter, 6 cm) where they could not move freely, turn around or escape. After 2 d of adaptation, a disposable silicon balloon-urethral catheter (8F, B. Braun Medical Industries, Malaysia) lubricated with liquid paraffin oil was inserted intrarectally 8 cm from the anus and was then fixed to the tail with adhesive tape. The balloon was distended with pre-warmed (37 ℃) water. Ascending-limit phasic distension (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL) was applied for 30 s. Each volume was repeated three times at intervals of 5 min to obtain an accurate estimate.

During the CRD, abdominal hypersensitivity was measured by the abdominal withdrawal reflex (AWR) and electromyography (EMG). AWR is a semi-quantitative scored test for IBS models to assess hypersensitivity[16]. The score was assigned as follows: 0 = no behavioral response to distension; 1 = brief head movements followed by immobility; 2 = contraction of abdominal muscle without lifting of abdomen; 3 = lifting of abdomen; and 4 = body arching and lifting of pelvic structure.

EMG was also used to quantitatively measure visceral motor responses of conscious rats[17] during the CRD. The rats were first briefly anesthetized by diethyl ether inhalation and then fixed in the Bollman cages. A prepared bipolar silver electrode (diameter = 0.8 mm; SBT Co., Ltd., China) was used to link the rats to the biology function laboratory system. One end of the electrode was sunk into the external oblique muscle 0.5 cm above the inguinal ligament. The other end was connected to a BL-420F biology function laboratory system (Chengdu Taimeng Technology Co., Ltd., China) such that activation of the muscle could be recorded and analyzed on a computer.

Total stool weight: Stools were collected every day after the rats were placed into the Bollman cage. Total stool weight (weight of fresh defecation) and dry weight (weight of feces air-dried for 24 h) were measured. Water content of the stool was calculated by the equation: Water content = (dry weight/net weight) × 100%.

MPO activity assay: The MPO activity assay was performed to quantify the inflammation in the distal colon according to the model preparation procedure described previously[17]. Six rats from each group (the AA group, PBS group and naïve group) were randomly chosen to harvest distal colonic tissues on the 2nd and 7th d (3 rats per day for each group) after the injection of 4% AA to produce the model. These two time points were selected according to a previous study[1] to represent the inflammatory phase and the subsiding phase, respectively. Briefly, an 8-cm segment of distal colon was collected *via* laparotomy, minced in 1 mL of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 14 mmol/L hexadecyltrimethylammonium bromide, homogenized and sonicated. The lysates were frozen and thawed three times and then centrifuged for 2 min at 4 ℃ at 15000 rpm. Aliquots of the supernatants were mixed with potassium phosphate buffer containing o-dianisidine-HCL (Sigma-Aldrich, St. Louis, MO, United States) and 0.0005% H_2O_2. The change in absorbance at 460 nm was spectrophotometrically determined. The MPO activity was expressed as units/g of wet tissue. An enzyme unit was defined as the conversion rate of 1 μmol of H_2O_2 per min at 25 ℃.

Histological examination of colonic inflammation: To examine the extent of colonic inflammation, histological tissues were collected at the selected time points (the 2nd and 7th d as mentioned above). The tissues were fixed in formalin for 12 h, and pathological sections were sliced at a thickness of 5 μm and processed for hematoxylin-eosin staining. The slides were observed under an optical microscope, and inflammatory cells were observed by a pathologist blinded to the treatments and time points.

P2Y1R expression in colonic tissue of rats

Protein expression of P2Y1R in the distal colon of rats (n = 8) in the AA, PBS and naïve groups were examined by Western blot. Distal colonic tissue was collected under anesthesia by intraperitoneal injection of 10% chloral hydrate. The tissues were either used immediately or stored at -80 ℃ for later use. P2Y1R antibody was purchased from Santa Cruz Biotechnology (catalog sc-377324).

Briefly, frozen sections of colonic tissue were trimmed and homogenized in RIPA buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NA-40, 0.25% sodium-deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF. A total of 20 μg of protein from the intestinal homogenate was mixed with gel-loading buffer, boiled for 5 min, and loaded onto a 12% polyacrylamide gel. Electrophoresis was then

conducted, and the protein was transferred to a nitrocellulose membrane. Then, the membrane was pre-incubated in 1 × Tris-buffered saline containing 5% skim milk at room temperature for 2 h. Subsequently, the membrane was incubated at 4 ℃ overnight with rabbit primary antibody against either P2Y1R (1:500) or β-actin (1:1000, control) in 1 × Tris-buffered saline containing 5% skim milk. After washing, the membranes were incubated with anti-rabbit IgG (1:3000) at room temperature for 2 h. Bands were visualized with a SuperSignal Substrate Chemiluminescence Kit (Pierce 34077).

Changes of visceral hypersensitivity upon intervention with P2Y1R agonist or antagonist

Drug preparation: The P2Y1R agonist MRS2365 (2157/1, R&D Systems China Co., Ltd., China) and the antagonist MRS2179 (ab120414, ABCAM Corporation) were dissolved in phosphate-buffered saline at concentrations of 0.1 to 100 μmol/L, respectively, which had been proven effective in other studies[18,19]. The drugs were administered by intra-colonic instillation using a 0.2 cm diameter tube that was inserted into the colon 8 cm from the anus and held still for 10 min per day. All the rats were shortly narcotized by inhalation with ether before instillation of drugs and CRD was carried out when they recovered consciousness.

Drug concentration selection: Five groups of AA rats, three groups of PBS rats and three groups of naïve rats (n = 8 for each) were set for MRS2365 or MRS2179 treatment each. AA rats were randomly divided into an untreated control group and four treated groups (0.1, 1, 10 and 100 μmol/L). The PBS and naïve groups were divided into an untreated control group and two treated groups (10 and 100 μmol/L). The drugs were administered for 7 d at 10 mL/kg body weight every day. CRD and EMG were carried out at 2, 12, 24, 48, 72 and 168 h from the initiation of treatment to select the most effective concentrations. Our preliminary experimental results showed that distension volumes could cause abdominal responses beginning at 0.8 mL and get the highest response at 1.2 mL. Therefore, we chose 1.2 mL to examine the down-regulatory role of MRS2179 in CRD course, and 0.8 mL for the up-regulatory role of MRS2365.

Drug intervention: AA rats were treated with MRS2365 and/or MRS2179 at their selected concentrations. AA rats were divided into four groups (n = 8 each): no treatment (PBS), MRS2179, MRS2365 and combined treatment (first MRS2365, then MRS2179). The regimen lasted 7 d, and drugs were administered at a dose of 10 mL/kg body weight every day. CRD was carried out under the distension volume of 1.2 mL. The AWR and EMG were observed at 2, 12, 24, 48, 72 and 168 h from the initiation of treatment.

Statistical analysis

All examinations were performed in duplicate. Statistical analyses were performed using SPSS 17.0 software. Quantitative data are expressed as the mean ± SE. The one-way ANOVA approach was used to compare two groups. Two-way ANOVA was used for multiple group comparisons. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

IBS model assessment

Because IBS characteristically appears with no inflammation or other organic problems in the colon, and a post-inflammatory model has been proven to demonstrate the major features of IBS after acute inflammation in the colon subsides[17], we chose to use this procedure to create a post-inflammatory animal model. The AWR score was significantly increased from distension volumes of 0.8 (3.0 ± 0.54) mL in the AA model compared with the two control groups and overlapped at 1.2 (4.0 ± 0.02) mL on a highest score of 4 which is the highest score that AWR defined (two-way ANOVA, $P < 0.001$, Figure 1A). The areas under the EMG curves (AUC) were significantly increased from volume of 0.8 (0.31 ± 0.11) to 1.2 ml (1.55 ± 0.15) in the AA rats compared with the control groups ($P < 0.001$, Figure 1B). The responses in PBS rats were also increased compared with naïve rats although there was no significant change ($P = 0.224$).

The water content of stools (Figure 1C) from naïve rats, PBS rats and AA rats were weighed and calculated to determine the stool features. Compared with naïve rats, there was an increase in the stool water content in the AA rats (55.1% ± 4.8% *vs* 25.6% ± 4.6%, one-way ANOVA, $P < 0.01$, n = 8), indicating much looser stools from the AA rats. No such effect was observed in the naïve rats and PBS rats (25.9% ± 5.4%).

The results of MPO activity assay (Figure 1D) of colonic tissue in the AA group showed that the enzyme activity was dramatically increased (1.153 ± 0.09 *vs* 0.190 ± 0.01 compared with naïve rats) on the 2nd d of modeling (one-way ANOVA, $P < 0.001$, n = 3); however, no significant difference was found between the PBS and naïve groups on the 2nd d of modeling and among the three groups on the 7th d of modeling (one-way ANOVA, $P = 0.861$, n = 3). These two examinations indicated that an obvious inflammatory reaction appeared on the 2nd d after the AA injection, and the inflammatory reaction gradually recovered and showed almost no inflammation on the 7th d. The model finished on day 8 and showed a clear feature of IBS that had no organic lesion.

Histological examination (Figure 1E and F) showed that many inflammatory cells such as neutrophils and lymphocytes were found in the lamina propria of distal colonic tissues on the 2nd d of modeling. In contrast, no remarkable inflammatory features were observed in the two control groups on the 2nd d of modeling and

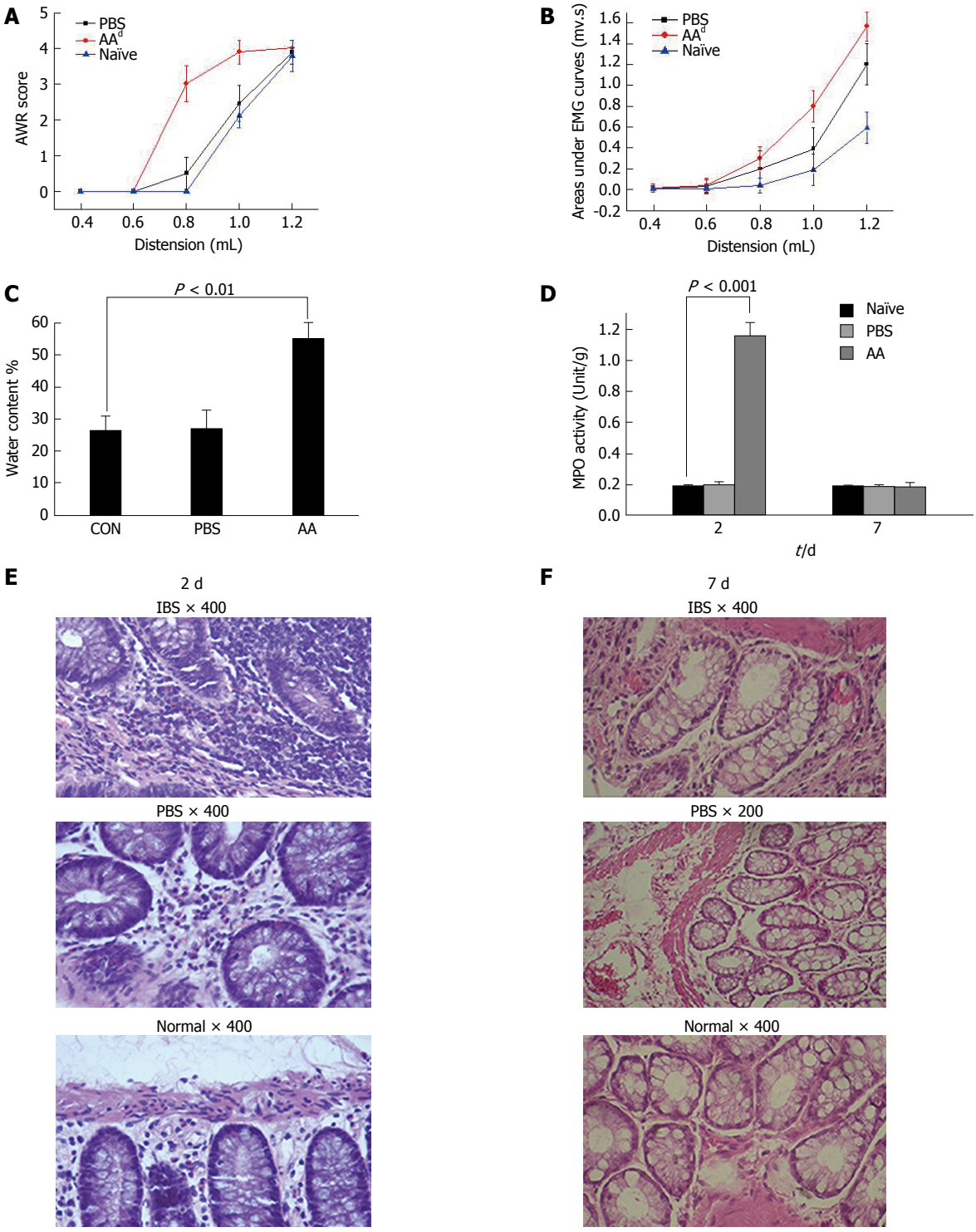


Figure 1 Model assessment using histological sections, myeloperoxidase activity, stool water content and visceral hypersensitivity in the acetic acid rats. A and B: The AWR score was significantly increased at distension volumes of 0.8 and 1.0 mL in the AA model compared with the two control groups and overlapped at 1.2 mL on a highest score of 4. The AUC significantly increased at volumes of 0.8, 1.0 and 1.2 mL in the AA rats compared with the control groups. No significant change was found in the PBS group compared with naïve rats; C: The water contents of stools from the naïve, PBS and AA groups were compared (one-way ANOVA, $P < 0.01$). AA rats showed much looser stool, and the water content was much higher; D: Tissue MPO activity on the 2nd and 7th d of modeling. MPO activity of the AA group was increased on the 2nd d of modeling. No statistically significant difference was detected between the PBS and naïve groups on the 2nd and 7th d of modeling among the AA, PBS and naïve groups (one-way ANOVA, $P < 0.001$ *vs* the control groups); E and F: Histology sections of colonic tissue from AA, PBS and naïve rats on the 2nd and 7th d. On the 2nd d of modeling in the AA group, histological inflammatory features including mucosal hemorrhage, submucosal edema, and inflammatory infiltration in the lamina propria and submucosa were observed. No remarkable inflammatory features were observed in the two control groups on the 2nd d of modeling and in all the three groups (AA, PBS and naïve rats) on the 7th d of modeling. [d]$P < 0.001$ *vs* the control groups. AWR: Abdominal withdrawal reflex; AUC: Area under the electromyography curve; AA: acetic acid; IBS: Irritable bowel syndrome; EMG: Electromyography.

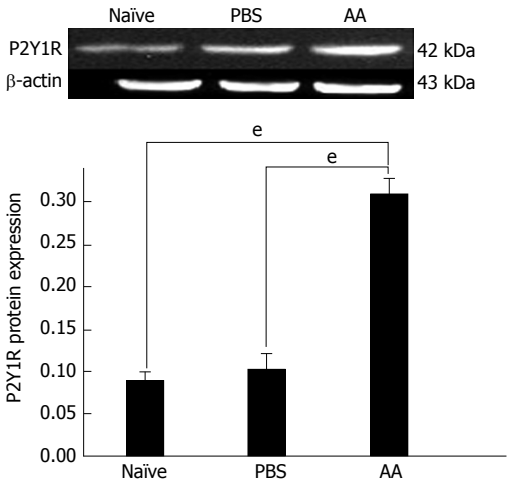


Figure 2 Western blot analysis of P2Y1R expression in the acetic acid, PBS and naïve rats. The average expression of P2Y1R (as determined by densitometry) was significantly higher in the AA group than in the control groups. No significant changes was found between the PBS group and naïve group. β-actin served as an internal control. $^{e}P < 0.001$ *vs* the control groups. AA: acetic acid.

in all the three groups (AA, PBS and naïve rats) on the 7th d of modeling.

High expression of P2Y1R

Compared with naïve rats (0.094 ± 0.016) and PBS rats (0.105 ± 0.013), the protein levels of P2Y1R in the distal colon of AA rats (0.319 ± 0.02) were significantly higher (one-way ANOVA, $P < 0.001$, Figure 2). The P2Y1R expression in PBS rats was higher than that in naïve rats although there was no significant change ($P = 0.398$).

MRS2179 concentration selection

The AUC was detected by two-way ANOVA, in which the dose of the drug ranging from 0 to 100 μmol/L was used as the first factor and the time as the second factor. MRS2179 produced a dose-dependent analgesic effect in the CRD test on AA rats ($P < 0.0001$, Figure 3A). A significant decrease was observed at concentrations of 0.1 to 100 μmol/L at 2 to 168 h time points. Maximal effects of MRS2179 were observed at 100 μmol/L at 72 h after administration (from a mean baseline AUC value of 1.587 ± 0.099 mv·s to 0.140 ± 0.089 mv·s).

While on naïve and PBS rats, MRS2179 at doses of 10 and 100 μmol/L was found to be effective after 48 h and 24 h ($P < 0.05$, Figure 3B and C). One-way ANOVA showed significant effects between AA rats and naïve/PBS rats at doses of 10 and 100 μmol/L ($P < 0.0001$, Figure 3D and E), and effects between naïve and PBS rats were not significant for the two doses ($P = 0.872$ and $P = 0.959$, respectively, Figure 3D and E).

MRS2365 concentration selection

Two-way ANOVA indicated that MRS2365 produced a dose-dependent analgesic effect in the CRD test on AA rats ($P < 0.0001$, Figure 4A). MRS2365 significantly increased AUC at concentrations of 0.1 to 100 μmol/L at 2 to 168 h time points. MRS2365 at 10 and 100 μmol/L showed the most effective increase. Maximal effects of MRS2365 were observed at 10 (AUC value from 0.30 ± 0.089 to 1.973 ± 0.127 mv·s, $P < 0.01$) and 100 μmol/L at 72 h (AUC value from 0.290 ± 0.079 to 1.983 ± 0.195 mv·s, $P < 0.01$).

On naïve and PBS rats, MRS2365 at doses of 10 and 100 μmol/L was found to be effective at 2 to 168 h time points ($P < 0.05$, Figure 4B and C). Significant effects were found between AA and naïve/PBS rats at doses of 10 and 100 μmol/L (one-way ANOVA, $P < 0.0001$, Figure 4D and E), while effects between naïve and PBS rats were not significant for the two doses ($P = 0.792$ and $P = 0.826$, respectively).

MRS2179/MRS2365 intervention

The most effective concentrations above (MRS2179 at 100 μmol/L and MRS2365 at 10 μmol/L) were applied in the drug intervention experiments for the four groups. AWR scoring (Figure 5A) showed that MRS2179 could significantly decrease the abdominal responses (two-way ANOVA, $P < 0.0001$). Differences between the MRS2179 group (1.88 ± 1.45) and the untreated AA rats (3.88 ± 0.33), MRS2365 group (3.96 ± 0.19) or the combined treatment group (3.28 ± 0.11) were significant ($P < 0.01$). No difference was detected among untreated AA rats, MRS2365-treated rats and rats in the combination group ($P = 0.224$).

Significant differences of AUC were also detected (Figure 5B). Compared with the untreated group, a significant decrease was detected in the MRS2179 group (two-way ANOVA, $P < 0.001$), which started at a baseline value of 1.52 ± 0.22 mv·s at time 0 h, peaked to 0.21 ± 0.15 mv·s at 72 h and continued to persist to 168 h. A significant increase was detected in the MRS2365 group ($P < 0.001$) which started at time 0 h (1.60 ± 0.20 mv·s), peaked at 72 h (2.98 ± 0.16 mv·s) and persisted to 168 h. Compared with the MRS2179 group, the difference for the MRS2365 groups and combination group was significant ($P < 0.001$). Compared with the combination group, significant differences were detected for untreated AA rats ($P < 0.05$) and MRS2365-treated rats ($P < 0.01$).

DISCUSSION

Colonic hypersensitivity in patients with IBS has been repeatedly confirmed[15,20,21], and lower colonic pain thresholds exist in most IBS patients[22]. This study investigated the possible role of P2Y1R in visceral hypersensitivity in a rat model of experimental IBS. Our results provided evidence that P2Y1R is involved in generation of visceral hypersensitivity in IBS.

This study was conducted in a post-inflammatory IBS rat model that has been proven to demonstrate

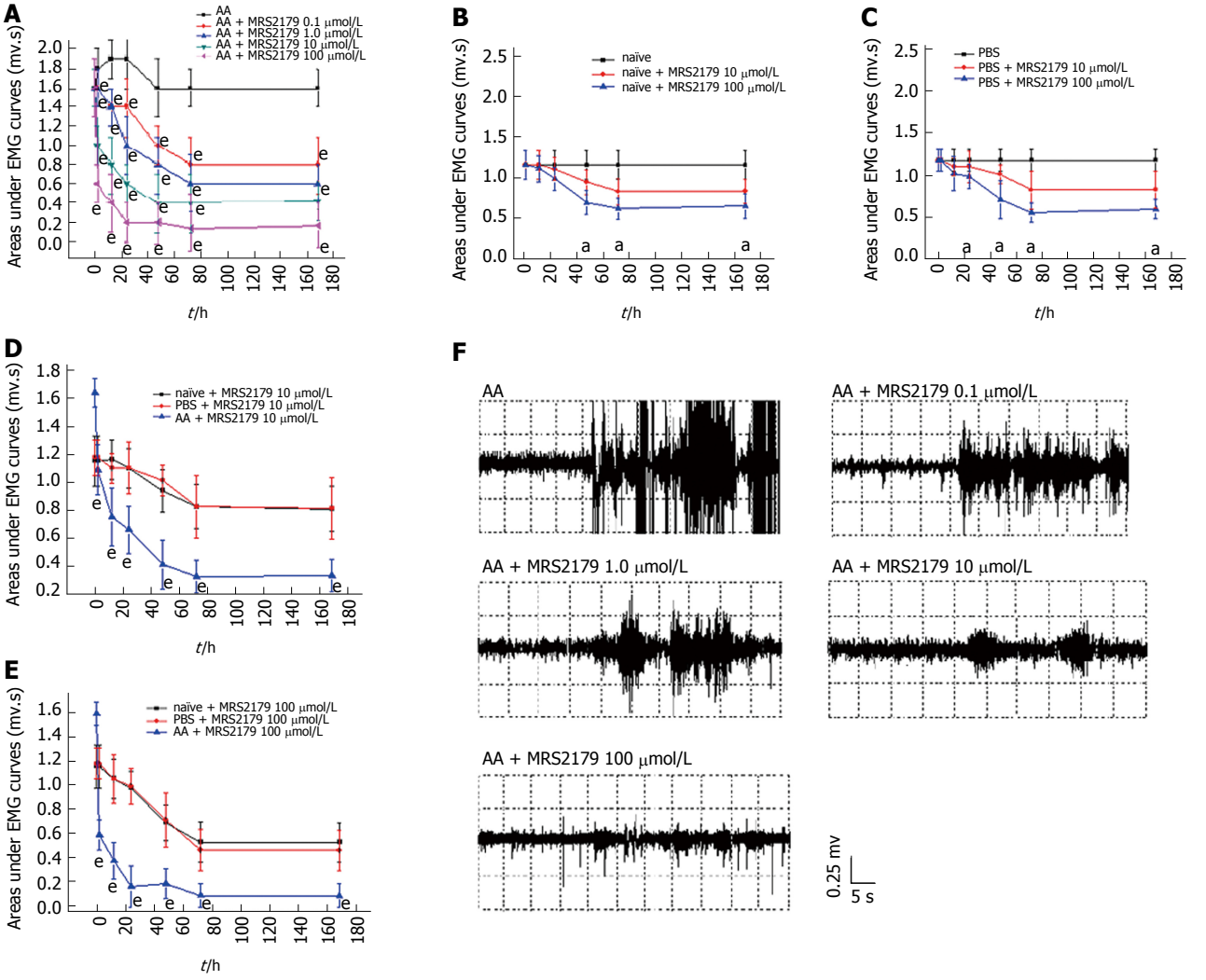


Figure 3 Concentration selection of MRS2179. A: Treatment with the specific P2Y1R antagonist MRS2179 evoked a pain-related visceral motor response in AA rats upon colorectal distension. A significant decrease was observed at concentrations of 0.1 to 100 μmol/L at 2 to 168 h time points. MRS2179 at 100 μmol/L produced the most pronounced result. Maximal effects of MRS2179 were observed at 100 μmol/L at 72 h from a mean baseline AUC value of 1.587 ± 0.099 m.vs to 0.140 ± 0.089 m.vs; B: On naïve rats, MRS2179 at doses of 10 and 100 μmol/L was found to be effective after 48 h; C: On PBS rats, MRS2179 at doses of 10 and 100 μmol/L was found to be effective after 24 h; D and E: AUC of naïve, PBS and AA rats treated with MRS2179 at doses of 10 and 100 μmol/L. There were significant differences between AA rats and naïve/PBS rats at doses of 10 and 100 μmol/L, although differences between naïve and PBS rats were not significant for the two doses; F: AUC of AA rats treated with MRS2179 (from doses of 0.1 to 100 μmol/L) at 72 h time point. $^{a}P < 0.05$, $^{e}P < 0.0001$ *vs* the control group. AUC: Area under the electromyography curve; AA: acetic acid; EMG: Electromyography.

the major features of IBS after subsidence of the acute inflammation in the colon and thus can be used to measure abdominal behaviors in conscious rats[17]. The MPO activity assay and histological examinations in this study proved that inflammation appeared on the 2nd d of model induction but was totally absent on the 7th d. However, the examination of abdominal behaviors and visceral hypersensitivity showed stronger AWR and abdominal EMG responses during the colorectal distention procedure. The AA rats also had a higher stool water content. However, although the differences between naïve rats and PBS rats were not significant, there was also a tiny increase of EMG and AWR scores in PBS rats, which was not expected. These may be caused by epithelial injury resulting from intubation because no intubation was done here for naïve rats, but PBS rats need to be treated by PBS in the anus of the colon. The differences almost disappeared when they were both treated with drugs by intubation in the following part of experiment. Overall, these results showed reserved hypersensitivity and abnormal fecal habits in this post-inflammatory IBS model, and these observations were similar with the reported visceral hypersensitivity in IBS patients.

P2YRs have been claimed to be expressed in intrinsic sensory nerves in the gut[23,24], where sensory neurons can evoke enteric reflex activities and mediate visceral pain *via* the spinal cord and brainstem[25]. Up to now it remains unclear whether P2Y1R is involved in generation and/or modulation of the lower pain thresholds of IBS. In this study, the higher expression of P2Y1R in the colon of AA rats emphasized their relationship with an elevated abdominal response towards mechanical stimuli. This occurrence may be

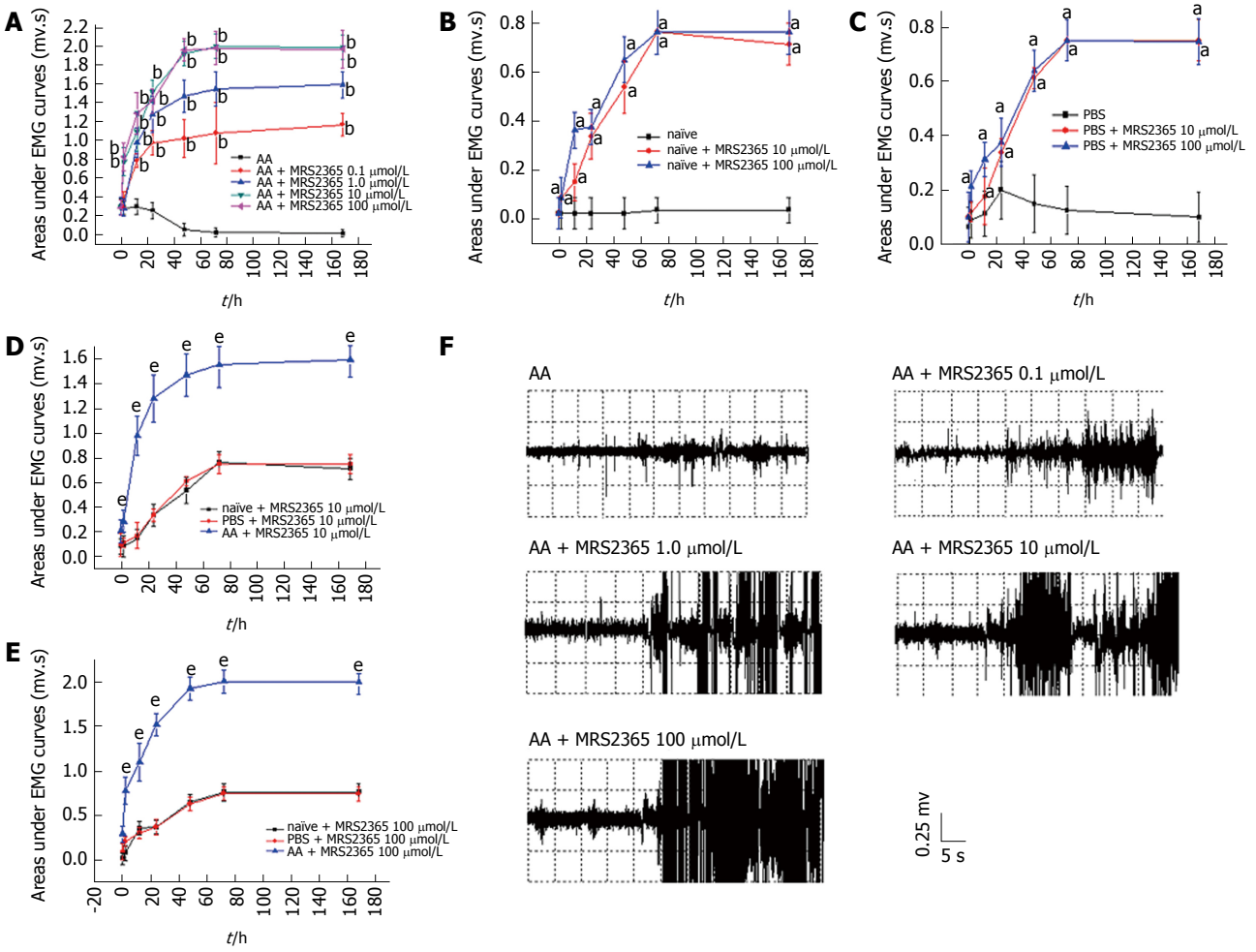


Figure 4 Concentration selection of MRS2365. A: Treatment with the specific P2Y1R agonist MRS2365-evoked pain elicited a visceral motor response in AA rats upon colorectal distension. The AUC was significantly increased in all of the MRS2365 groups (0.1, 1.0, 10 and 100 μmol/L) compared with the AA control group. The MRS2365 doses at 10 and 100 μmol/L elicited the most effective result. There was no significant difference between the concentrations of 10 and 100 μmol/L; B and C: On naïve and PBS rats, MRS2365 at doses of 10 and 100 μmol/L was found to be effective at 2 to 168 h time points ; D and E: AUC of naïve, PBS and AA rats treated with MRS2365 at doses of 10 and 100 μmol/L. Significant differences were found between AA and naïve/PBS rats at doses of 10 and 100 μmol/L, while differences between naïve and PBS rats were not significant for the two doses; F: AUC of AA rats treated with MRS2365 (from doses of 0.1 to 100 μmol/L) at 72 h time point. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.0001$ *vs* the control group. AUC: Area under the electromyography curve; AA: acetic acid; EMG: Electromyography.

related with excitation of sensory neurons located in submucosal and myenteric ganglia. Related studies[26,27] have suggested a possible mechanism that ATP might indirectly act on TRPV1Rs (which has received much attention in IBS) mediated by the P2Y1R in pain models of chronic ischemia. To further determine the role of P2Y1R, we studied changes of abdominal responses by functional regulation.

Both agonists and antagonists of the P2Y1R have been shown to reduce pain and hypersensitivity. Andó *et al*[18] demonstrated that MRS2365 had an analgesic effect on thermal pain threshold of neuropathic, acute pain and inflammatory pain. Chen *et al*[19] showed that MRS2179 could reverse tactile allodynia and spontaneous pain in a rat model of cancer-induced bone pain. They have indicated a bi-directional regulation of P2Y1R in pain. However, in our study, MRS2365 could effectively increase the colonic hypersensitivity status of AA rats, whereas MRS2179 led to a decrease in response to CRD test evoked visceral pain. The results showed an active role of P2Y1R in the generation/modulation of visceral hypersensitivity in the AA rats. This is in accordance with some other reports which indicated that P2Y1R is involved in the generation/modulation of nociceptive signals such as hypersensitivity in the gut[28-30]. It is not clear what exact role of P2Y1R plays in other pain events, but it will leads to high sensitivity in this IBS rat model. Moreover, specific antagonists of P2Y1R may have potential therapeutic value in treating abdominal pain in IBS.

Combined treatment of MRS2365 and MRS2179 showed a down-regulatory effect in AA rats. MRS2179 could not only effectively antagonize the elevated abdominal responses led by MRS2365, but also cause some more decrease. As a result, MRS2365 is a specific agonist of P2Y1R, whereas MRS2179 is not just a competitive antagonist of P2Y1R, but also selectively antagonizes other receptors, like P2X3R and P2Y2R[31,32]. Although we did not detect the expression

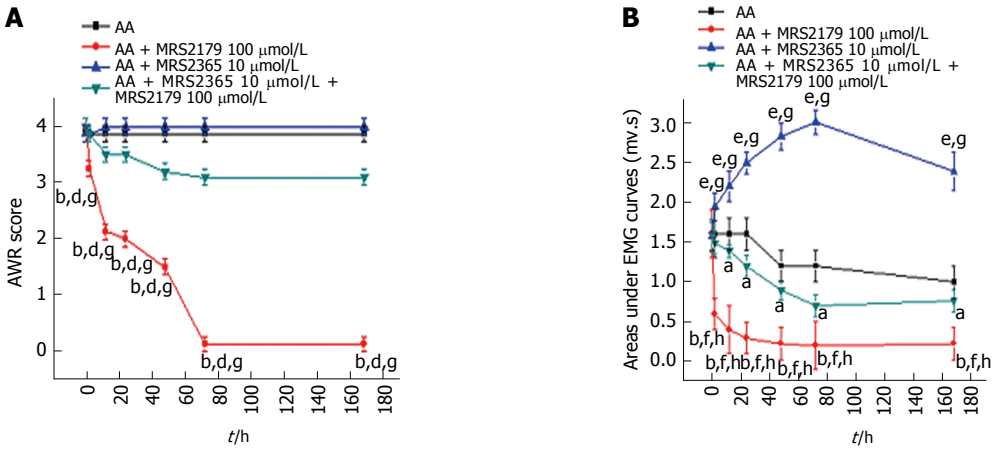


Figure 5 Drug intervention in acetic acid rats. A: AWR scores showed that MRS2179 could significant decrease the abdominal responses. Differences between the MRS2179 group and the untreated AA rats, MRS2365 group or the combined treatment group were significant. No difference was detected between untreated AA rats and MRS2365 treated rats or rats of the combination group; B: Significant differences of EMG curves were also detected. A significant decrease was detected in the MRS2179 group, which reached a peak at 72 h and continued to persist at 168 h. A significant increase was detected in the MRS2365 group (peaked at 72 h). The difference between the MRS2179 and MRS2365 groups or rats of the combination group was significant. Compared with rats of the combination group, significant differences were detected for untreated AA rats and the MRS2365 group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the untreated control group; $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* the MRS2365 group; $^{g}P < 0.01$, $^{h}P < 0.001$ *vs* the combination group. AWR: Abdominal withdrawal reflex; AUC: Area under the electromyography curve; AA: acetic acid; EMG: Electromyography.

of P2X3R and P2Y2R, our data suggested that besides P2Y1R, other receptors (which could be inhibited by MRS2179) might also play active roles in visceral hypersensitivity in the AA rats.

Studies on pain events have suggested interesting relationship between P2Y1R and P2X3R. P2Y1R was co-expressed with P2X3R and TRPV1Rs[31,33] in small diameter neurons, and was indicated to be an "ATP counterbalance" following mutual activation of P2Y1R and P2X3R. In the colon, enhanced expression of P2X3R in colon-specific sensory neurons has been found and chronic functional visceral hyperalgesia was associated with potentiation of ATP-evoked responses[34]. However, P2Y1R in this study showed an active role in causing visceral hypersensitivity, but not acted as a "counterbalance". It might be in line with an explanation[10-12] that P2Y1R may provide a high-sensitive system to respond to minor touch-induced fluctuations in extracellular ATP mediated by P2X3R subjected to mechanical stimuli. It may fit with a recent study[35], suggesting that activation of P2Y1R could exert inhibitory control on P2X3R expression and increased P2X3R-mediated nociceptive flinch behaviors by promoting activity of P2Y1R downstream of p38. More research will be needed to clarify the mechanism of P2Y1R and P2X3R in pain of IBS.

P2Y2R is another subtype which has been demonstrated to dedicate to nociceptive events. Studies[30,36] have shown that P2Y1R and P2Y2R transcripts were detected in 80% and 49%-56% of retrogradely labeled colonic neurons, respectively, and agonists of P2Y1R and P2Y2R can evoke comparable sensitization of sensory neurons. However, one study on IBS-D patients[37] suggested that P2Y2R was more likely related to IBS pain. And another study on rat lumbar dorsal root ganglion neurons[38] showed that TRPV1R was co-expressed with P2Y2R. It is controversial whether P2Y1R or P2Y2R is more important for IBS pain. Our data suggested that both receptors may be involved in pain of the AA rats, but more research needs to be done to confirm this speculation.

Comorbidities or extra-intestinal symptoms are frequently in a sub-type of IBS patients. Some pain and hypersensitivity events in IBS patients, like chronic pelvic pain[39], primary headache[40] and higher reactivity to food antigens[41], might be also related to purinergic signaling. More research needs to be done to figure out whether there are connections between P2 receptors and these events, and it might help to understand the mechanism of P2Y1R involved in visceral pain in IBS.

Previous studies[29,42] indicated that P2Y1R may be responsible not only for post-infectious IBS, but also inflammation, like IBD. This study was conducted in a post-infectious IBS rat model and it was a question whether the higher expression of P2Y1R in AA rats was caused by inflammation? The model has been proven to demonstrate the major features of IBS after subsidence of the acute inflammation in the colon and thus can be used to measure abdominal behaviors in conscious rats[17]. The remained high visceral sensitivity without inflammation was a precondition of the study. However, P2Y1R could not be discounted as a potential mechanism contributing to anti-inflammation. It has been indicated in previous studies[36,43] that P2YRs could sensitize visceral afferents to evoke increased firing of colonic sensory of neurons to gut distension which is typically during, or following, an inflammatory insult. To clarify the possible anti-inflammatory role of P2Y1 in this post-inflammatory IBS model, further investigation needs to be done in other IBS models which are not post infectious.

In conclusion, we provided the evidence that P2Y1R is involved in generation and modulation of visceral hypersensitivity in IBS, and specific antagonists of P2Y1R may have potential therapeutic value in treating abdominal pain in IBS. A limitation of the study is that the localization of P2Y1R in the colon of IBS-like rats was not detected, which could help to better understand its role in visceral hypersensitivity. Moreover, studies on other P2 receptors, as well as their connections with the pain and hypersensitivity events in IBS, would help to strengthen our results.

COMMENTS

Background

Visceral hypersensitivity is considered to be a major feature of irritable bowel syndrome (IBS), the mechanism of which is not yet clear. P2Y1R is part of a high-sensitivity response to mechanical stimulation and is believed to dedicate to visceral hypersensitivity in IBS. In this study, the expression of P2Y1R was detected in a post-infectious IBS rat model, and the role of P2Y1R in the development of visceral hypersensitivity was detected by regulating its function.

Research frontiers

P2Y1R has been shown to mediate nociceptive signaling and suggested to provide a high-sensitivity system to mechanical stimulation in functional gastrointestinal disorders, including IBS. However, whether P2Y1R participates in the generation and regulation of visceral hypersensitivity in IBS remains unclear. The results of this study provide the evidence of P2Y1R in IBS.

Innovations and breakthroughs

Using a post-inflammatory IBS rat model, we determined that P2Y1R is involved in the generation and modulation of visceral hypersensitivity in IBS. Moreover, specific antagonists of P2Y1R may have potential therapeutic value in treating abdominal pain in IBS.

Applications

P2Y1R may be positively involve in the generation and modulation of visceral hypersensitivity in IBS. Its specific antagonists may be used for treating abdominal pain and other relative discomfort in IBS.

Terminology

IBS is a kind of functional gastrointestinal disorder, which is characterized by hypersensitivity and pain before defecation in the relative absence of colon inflammation or structural changes.

Peer-review

In the manuscript, the authors demonstrated that P2Y1R is involved in the generation and modulation of visceral hypersensitivity in IBS. This study is interesting in its scientific question, and it contributes to the knowledge of the IBS.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Randomized controlled trial of uncut Roux-en-Y *vs* Billroth Ⅱ reconstruction after distal gastrectomy for gastric cancer: Which technique is better for avoiding biliary reflux and gastritis?

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Author contributions: Yang D and Wang Q designed the research; Yang D, He L and Wang Q performed the research; Yang D, Tong WH and Su TR collected the clinical data; Yang D and Jia ZF analyzed the data; Yang D, He L and Wang Q wrote the paper.

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

Conflict-of-interest statement: All authors declare no conflict of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at 18844097668@163.com. Participants gave informed consent for data sharing.

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Abstract

AIM

To identify which technique is better for avoiding biliary reflux and gastritis between uncut Roux-en-Y and Billroth Ⅱ reconstruction.

METHODS

A total of 158 patients who underwent laparoscopy-assisted distal gastrectomy for gastric cancer at the First Hospital of Jilin University (Changchun, China) between February 2015 and February 2016 were randomized into two groups: uncut Roux-en-Y (group U) and Billroth

II group (group B). Postoperative complications and relevant clinical data were compared between the two groups.

RESULTS

According to the randomization table, each group included 79 patients. There was no significant difference in postoperative complications between groups U and B (7.6% *vs* 10.1%, P = 0.576). During the postoperative period, group U stomach pH values were lower than 7 and group B pH values were higher than 7. After 1 year of follow-up, group B presented a higher incidence of biliary reflux and alkaline gastritis. However, histopathology did not show a significant difference in gastritis diagnosis (P = 0.278), and the amount of residual food and gain of weight between the groups were also not significantly different. At 3 mo there was no evidence of partial recanalization of uncut staple line, but at 1 year the incidence was 13%.

CONCLUSION

Compared with Billroth II reconstruction, uncut Roux-en-Y reconstruction is secure and feasible, and can effectively reduce the incidence of alkaline reflux, residual gastritis, and heartburn. Despite the incidence of recanalization, uncut Roux-en-Y should be widely applied.

Key words: Gastric cancer; Uncut Roux-en-Y; Billroth II; Bile reflux; Alkaline gastritis

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Core tip: Because of the challenge of recanalization, the uncut Roux-en-Y reconstruction is still controversial and needs further study. This study is the first randomized controlled trial concentrating on uncut Roux-en-Y *vs* Billroth Ⅱ reconstruction after distal gastrectomy for gastric cancer. This study aimed to compare uncut Roux-en-Y and Billroth Ⅱ reconstruction in terms of postoperative complications, including biliary reflux and gastritis. Despite the incidence of recanalization, uncut Roux-en-Y reconstruction is secure and feasible, and can effectively reduce the incidence of alkaline reflux, residual gastritis, and heartburn.

Yang D, He L, Tong WH, Jia ZF, Su TR, Wang Q. Randomized controlled trial of uncut Roux-en-Y *vs* Billroth Ⅱ reconstruction after distal gastrectomy for gastric cancer: Which technique is better for avoiding biliary reflux and gastritis? *World J Gastroenterol* 2017; 23(34): 6350-6356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6350.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6350

INTRODUCTION

There remains no clear consensus regarding the preferred reconstructive surgical procedure after laparoscopy-assisted distal gastrectomy (LADG) for gastric cancer[1,2]. Compared with Japan[3] and Korea[4], early gastric cancer only accounts for a small percentage in China, and most gastric cancer cases are found in advanced stages at the initial diagnosis. It is inappropriate for surgeons to perform Billroth I anastomosis after subtotal gastrectomy. In 2005, Uyama first combined LADG with uncut Roux-en-Y reconstruction; however, its use remains controversial[5,6]. In our department, we usually prefer Billroth II and uncut Roux-en-Y reconstruction.

The current study aimed to compare these two reconstruction techniques in terms of postoperative complications, including biliary reflux and gastritis.

MATERIALS AND METHODS

This is a randomized controlled trial which was evaluated and approved by the ethics committee at our institution, and registered in clinicaltrials.gov with the number NCT02694081. Between February 2015 and February 2016, a total of 158 patients with gastric cancer treated at the First Hospital of Jilin University (Changchun, China), who met the inclusion criteria and provided informed consent, were randomized into one of two groups: uncut Roux-en-Y group (group U) or Billroth Ⅱ group (group B). Randomization was done after laparoscopic exploration with the randomization table, which was produced using SPSS v18.0 for Windows software by the Division of Clinical Research at our hospital. Patients as well as investigators (assessing outcomes and analyzing data) were masked. The inclusion criteria were: (1) distal gastric cancer diagnosed by endoscopy, CT scan, and pathology study; (2) patients who underwent LADG; and (3) age between 18 and 75 years. The exclusion criteria were: (1) late-stage gastric carcinoma or pyloric obstruction; (2) preoperative esophageal reflux symptoms, esophagitis, or hiatal hernia; and (3) systemic disease including diabetes, severe chronic lung disease, cirrhosis, or esophageal varices.

All included patients underwent LADG with D2 lymphadenectomy, which was performed by the same surgical team. For reconstruction, a 5-cm mini-laparotomy was made to complete a delta-shaped Billroth II anastomosis using a 80 mm linear stapler[7]. In the uncut Roux-en-Y group, gastrojejunostomy was performed at 25 cm distal to the Treitz ligament, and jejunum-jejunum anastomosis at 40 cm from the afferent limb. The blade of the linear stapler (Covidien GIA8038S, Medtronic, Minneapolis, MN, the United States of America) was removed to perform the uncut procedure of the afferent jejunal limb, 5 cm proximal to the gastrojejunostomy in the jejunum (Figure 1A and B).

During the postoperative period, omeprazole 40 mg was given to all patients twice a day. Ambulation was encouraged from the first day after operation, and the nasogastric tube was kept in place for 5 d. All patients

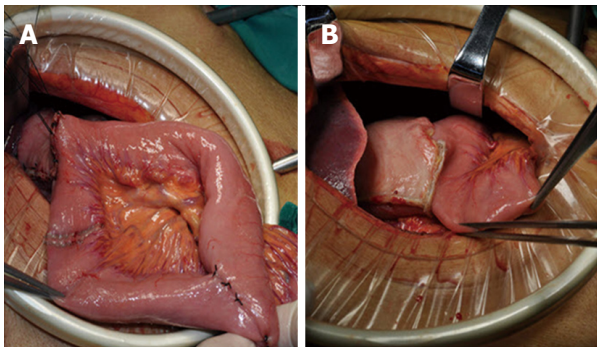


Figure 1 Two kinds of reconstruction after laparoscopy-assisted distal gastrectomy. A: Uncut Roux-en-Y reconstruction; B: Billroth Ⅱ reconstruction. All included patients underwent LADG with D2 lymphadenectomy, which was performed by the same surgical team. Then, two groups underwent different reconstructions as shown.

Table 1 Clinical and pathological data of the patients

Variable	Group U	Group B	*P* value
Age (yr)	58.0 ± 11.4	61.8 ± 11.4	0.030
Gender			0.287
Male	60 (75.9)	54 (68.4)	
Female	19 (24.1)	25 (31.6)	
Pathological tumor stage			0.822
ⅠB	3 (3.8)	2 (2.5)	
ⅡA	31 (39.2)	27 (34.2)	
ⅢB	28 (35.4)	29 (36.7)	
ⅢA	17 (21.5)	21 (26.6)	
Operative time (min)	154.8 ± 17.8	145.5 ± 15.1	0.001
Blood loss (mL)	74.1 ± 26.7	74.0 ± 36.6	0.980

Values are presented as number (%) or mean ± SD.

received uniform diet guidance after discharge.

Demographic data, clinical outcomes, and follow-up data were collected. Change of potential of hydrogen (pH) in the remnant stomach was recorded at 8:00 am on the day before surgery as well as 1-5 d after surgery.

Three months later, an upper esophagogastroduodenal series was performed in each patient with 100 mL of meglumine diatrizoate. The full emptying rate at 30 min and the ratio of partial recanalization were collected.

Twelve months later, items were monitored as follows: (1) number of patients with heartburn symptoms; (2) changes in body weight within 1 year; and (3) gastric residue, residual gastritis, and biliary reflux (RGB; monitored by endoscopy). Combined with standard RGB[8], a modified biliary reflux classification in three grades was applied-grade 0: absence of bile (Figure 2A); grade 1: small amount of bile located in the bottom of residual stomach without overflow (Figure 2B); and grade 2: bile spilled into the jejunum with tidal rhythm (Figure 2C).

Gastric tissue biopsies were taken to compare the degree of gastritis at 2 cm from anastomosis and then evaluated by two pathologists. Classification included three grades: grade 0, normal mucosa with a small amount of lymphocytes and transparent microscopic field (Figure 3A); grade 1, intermediate between grades 0 and 2 (Figure 3B); and grade 2, acute inflammation with fully infiltrated tissue by lymphocytes or inflammatory cells (Figure 3C).

Statistical analysis

All statistical analyses were performed using SPSS v18.0 for Windows software. Continuous variables are expressed as mean and SD and compared by Student's *t*-test. Categorical variables were analyzed by Pearson χ^2 test. The pH variables were compared by repeated measures analysis of variance. A two-tailed *P* value < 0.05 was considered statistically significant.

RESULTS

According to the randomization table, each group included 79 patients. Baseline data are shown in Table 1. There were no significant difference in gender or pathological data ($P > 0.05$), but the average age of group U patients was older than group B patients (58.0 ± 11.4 *vs* 61.8 ± 11.4 years, $P = 0.030$). The surgical time was slightly longer in group U (154.8 ± 17.8 *vs* 145.5 ± 15.1 mins, $P = 0.001$), but there was no difference in blood loss (74.1 ± 26.7 *vs* 74.0 ± 36.6 mL, $P > 0.05$).

There was no significant difference in postoperative complications between the two groups (7.6% *vs* 10.1%, $P = 0.576$). One patient in each group underwent reoperation because of intra-abdominal bleeding. In group U, a patient with ileus required reoperation after 1 mo of conservative treatment. In group B, a patient received emergency endoscopy to insert a stomach tube into the afferent loop to release pressure due to A-loop syndrome. For both groups, no gastroparesis syndrome was found during the postoperative period (Table 2). According to the Clavien-Dindo classification for surgical complications[9], in group U grade Ⅰ complications were recorded in 3.8%, grade Ⅱ in 1.3%, and grade ⅢB in 2.5% of the cases. In group B grade Ⅰ complications were recorded in 3.8%, grade II in 2.5%, grade ⅢA in 1.3%, and grade ⅢB in 2.5% of the cases. There was still no significant difference between the two groups ($P = 0.954$).

The stomach pH was lower in group U patients, with a significant statistical difference ($P < 0.05$) (Table 3).

During the postoperative period, all stomach pH values in group U patients were below 7.00. Conversely, all stomach pH values in group B patients were higher than 7.00 (Figure 4).

Three months later, regarding the postoperative esophagogastroduodenal series after 30 min, a higher ratio of full emptying was seen in group B patients (88.2% *vs* 76.6%, $P = 0.061$), but with no statistical significance.

At the end of the 1-year follow-up period, three patients in group U and one patient in group B were

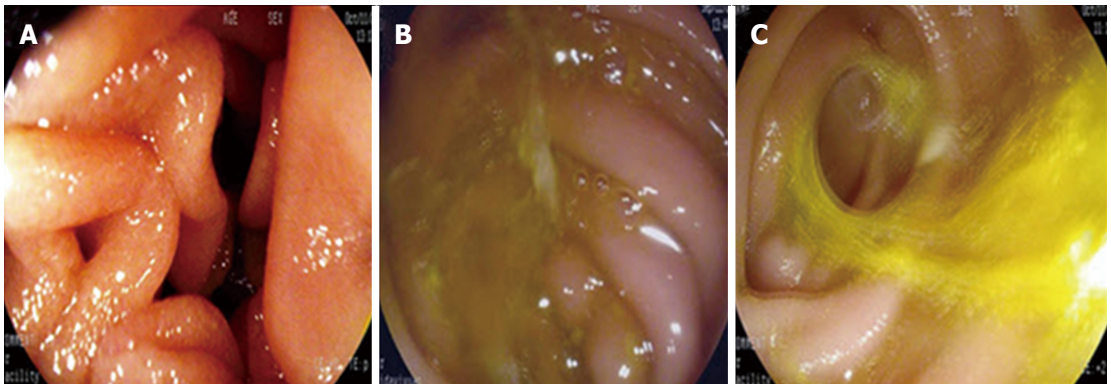


Figure 2 Bile reflux grades. During the endoscopic examination, a modified biliary reflux classification in three grades was applied. A: grade 0, absence of bile (Figure 2A); B: grade 1, small amount of bile located in the bottom of residual stomach without overflow (Figure 2B); C: grade 2, bile spilled into the jejunum with tidal rhythm (Figure 2C).

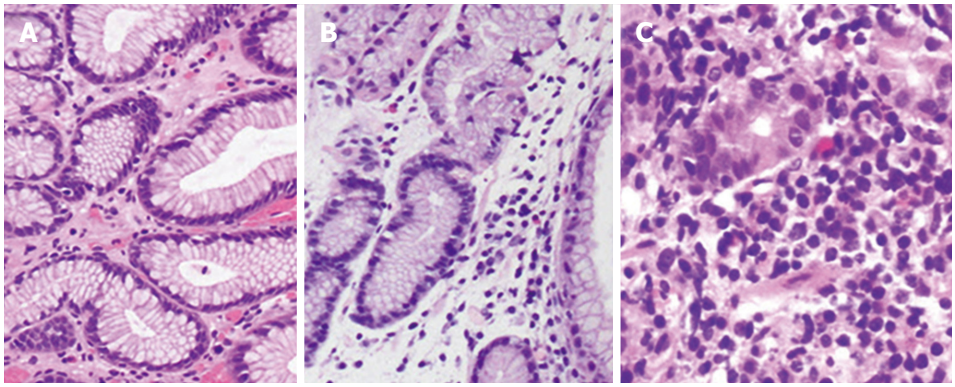


Figure 3 Biopsy for gastritis. Gastric tissue biopsies were taken to compare the degree of gastritis: A: grade 0 [hematoxylin and eosin (HE) staining, × 200], normal mucosa with small amount of lymphocytes and transparent microscopic field; B: grade 1 (HE, × 200), intermediate between grades 0 and 2 with a moderate amount of lymphocytes or other kinds of inflammatory cells; C: grade 2 (HE, × 400), acute inflammation with fully infiltrated tissue by lymphocytes or other kinds of inflammatory cells.

Table 2 Postoperative complications in the two groups *n* (%)

Item	Group U	Group B	*P* value
Duodenal stump leakage	1 (1.3)	3 (3.8)	0.620
Chylous fistula	1 (1.3)	0	1.000
Ileus	1 (1.3)	1 (1.3)	1.000
Anastomotic bleeding	0	1 (1.3)	1.000
Intra-abdominal bleeding	2 (2.5)	2 (2.5)	1.000
Incision infection	1 (1.3)	0	1.000
Gastroparesis syndrome	0	0	-
A-loop syndrome	0	1 (1.3)	1.000
Total	6 (7.6)	8 (10.1)	0.576

Values are presented as number only, or number (%). A-loop syndrome: Afferent loop syndrome.

Table 3 Perioperative stomach pH values

Time	Group U	Group B	*P* value
Preoperative	1.97 ± 0.19	1.99 ± 0.21	
Day 1	6.38 ± 0.18	7.21 ± 0.36	
Day 2	6.28 ± 0.29	7.35 ± 0.32	
Day 3	6.48 ± 0.38	7.27 ± 0.38	
Day 4	6.64 ± 0.22	7.27 ± 0.35	
Day 5	6.56 ± 0.29	7.24 ± 0.42	
			0.000

Values are presented as mean ± SD. Change of potential of hydrogen (pH) in the remnant stomach was recorded at 8:00 am on the day before surgery as well as 1–5 d after surgery.

lost to follow-up. Besides, seven patients in group U and six patients in group B had died. Therefore, the survival rates at 1 year for group U and B patients were 90.79% and 92.31%, respectively, and showed no significant difference (P = 0.735). The biliary reflux incidence in group B was higher than that in group U patients with a significant difference (60.9% *vs* 90.3%, P = 0.000). The ratio of gastritis in group B (72.2%) was significantly higher than that in group U (55.1%). However, the result of biopsy showed no significant difference (63.8% *vs* 70.8%, P = 0.278), but the linear-by-linear association was significant (P_{trend} = 0.015). Besides, there was no significant difference for the incidence of diarrhea, residual food, or gain of weight between the two groups (Table 4).

DISCUSSION

In 1988, uncut Roux-en-Y reconstruction was first

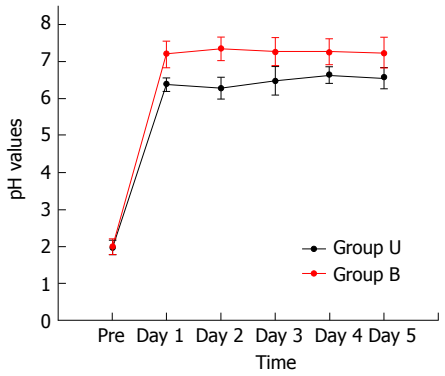


Figure 4 Perioperative potential of hydrogen (pH) in the stomach of the patients. Change of potential of hydrogen (pH) in the remnant stomach was recorded at 8:00 am on the day before surgery as well as 1-5 d after surgery. During the postoperative period, all stomach pH values of group U patients were below 7.00. Conversely, all stomach pH values of group B patients were higher than 7.00.

Table 4 The follow-up data

Item	Group U	Group B	*P* value
3 mo later	*n* = 77	*n* = 76	
Esophagogastroduodenal series			
Full emptying at 30 min	59 (76.6)	67 (88.2)	0.061
Partial recanalization	0	-	-
1 yr later	*n* = 69	*n* = 72	
Heartburn	7 (10.1)	17 (23.6)	0.033
Weight gain (kg)	-0.04 ± 3.6	-0.18 ± 3.8	0.723
Endoscopic finding			
Residual food	8 (11.6)	3 (4.2)	0.178
Grade 0	61	69	
Grade 1	7	3	
Grade 2	1	0	
Grade 3-4	0	0	
Gastritis	38 (55.1)	52 (72.2)	0.044
Grade 0	31	20	
Grade 1	30	35	
Grade 2	8	17	
Grade 3-4	0	0	
Bile reflux	42 (60.9)	65 (90.3)	0.000
Grade 0	27	7	
Grade 1	34	23	
Grade 2	8	42	
Partial recanalization	9 (13.0)	-	-
Biopsy of gastritis	44 (63.8)	51 (70.8)	0.278
Grade 0	25	21	
Grade 1	39	45	
Grade 2	5	6	

Values are presented as number only, or number (%).

reported by Stiegman and Goff[10]. Some studies over the years have confirmed that this reconstruction can preserve myoneural continuity to eliminate Roux stasis syndrome[11-13]. Because uncut Roux-en-Y is a modification of Billroth Ⅱ reconstruction, it makes sense to compare Billroth Ⅱ and uncut Roux-en-Y to determine the better procedure after LADG.

Our study showed there was no significant difference for the incidence of postoperative complications between the two groups (7.6% *vs* 10.1%, *P* > 0.05). Moreover, for the severity of postoperative complications, according to the Clavien-Dindo classification of surgical complications, there was no significant difference (*P* = 0.954). A-loop syndrome does not occurred in the uncut Roux-en-Y group, and the incidence of duodenal stump leakage was lower than that of the Billroth II group. The reason for this may be that the Braun anastomosis effectively relieves the pressure of the afferent loop[14], but more cases should be included to confirm this difference.

For biliary reflux, during the postoperative period, all group U pH values were lower than 7.00, thus representing an acidic stomach environment. In group B patients, all pH values were higher than 7.00, representing an alkaline stomach environment with alkaline reflux, which can be considered an important risk factor for gastric stump cancer[15] (Figure 2). In addition, according to patient outcomes at the 1-year follow-up, the bile reflux incidence in group B patients was significantly higher than that in group U (*P* = 0.000). However, the incidence of bile reflux in group U patients was 60.9%, which is higher than the equivalent statistic in Park and Kim's report (less than 30%)[4]. There are two main reasons that can explain these data. On one hand, the incidence of partial recanalization reached 13.0%. In this study, for partial recanalization, all those cases were first observed by endoscopy, and the result would be confirmed by esophagogastroduodenal series if there was doubt. Recanalization eventually allowed bile access to the gastric remnant. On the other hand, for some other cases, the bile reflux happened through the efferent loop, and a small amount of bile was usually found in the bottom of residual stomach with no overflow (grade 1).

Regarding gastritis, the incidence in group B patients (72.2%) was significantly higher than that in group U patients (55.1%), with a higher incidence of heartburn in group B patients as well (23.6%). These results allow us to conclude that uncut Roux-en-Y can reduce the occurrence of residual gastritis and heartburn proportion in Billroth Ⅱ reconstruction. However, the biopsy results showed no significant difference between the two groups (*P* = 0.278).

We draw two conclusions from these results. First, the pattern of bile reflux is different. The majority of group U patients were classified into grade 1 (49.3%), characterized by a small bile amount usually located in the bottom of the stomach as gastric residue, whereas most group B patients belonged to grade 2 (58.3%), characterized by considerable tidal rhythm. Hence, the stomach bile was fresh and temporary, which may be less corrosive to the gastric mucous membranes. Park showed a correlation between bile reflux and the degree of gastritis[4], but perhaps it depends not only on the amount but also on the pattern of biliary reflux. Second, the follow-up time was too short to show differences in the percentage of residual gastritis on biopsy, so the linear-by-linear association *P* value was also calculated. The result showed a significant

difference (P_{trend} = 0.015), meaning that the severity of residual gastritis for group B was worse than that for group U on biopsy.

For residual food, there was no significant difference between the two groups (11.6% *vs* 4.2%, $P > 0.05$). For gastrointestinal anastomosis of both procedures, the stoma was extensive along the greater curvature. No gastroparesis syndrome was found during follow-up, which is perhaps related to myoneural continuity. At 3-mo follow-up, the incidence of full emptying at 30 min reached 76.6% and 88.2% in group U and B patients, respectively (Table 4). As a result, no retention of afferent loop stump was found during the follow-up, and the incidence of residual food was lower than that in other studies[4,16].

This study adopted body weight change to evaluate postoperative nutritional status of patients, and 1 year later, the weight change values of group U and group B patients were -0.04 ± 3.6 kg and -0.18 ± 3.8 kg, respectively, with no significant difference. Moreover, there was no significant difference in survival rates of group U and group B patients after 1 year (90.79% *vs* 92.31%, respectively; $P > 0.05$).

In conclusion, the uncut Roux-en-Y digestive reconstruction procedure is secure and feasible. Moreover, it can effectively reduce the incidence of alkaline reflux, residual gastritis, and heartburn seen in classical Billroth II procedure. Besides, the uncut technique still needs improvement so that the risk of staple line dehiscence is minimized, with a longer follow-up period to reevaluate the exact risk. Despite the incidence of recanalization[17-21], uncut Roux-en-Y should be widely applied.

COMMENTS

Background

In 1988, uncut Roux-en-Y reconstruction was first reported by Stiegman and Goff. Some studies over the years have confirmed that this reconstruction can preserve myoneural continuity to eliminate Roux stasis syndrome. However, because of the challenge of recanalization, the uncut Roux-en-Y is still controversial and really needs further study. Since uncut Roux-en-Y is a modification of Billroth II reconstruction, it makes sense to compare Billroth II and uncut Roux-en-Y to determine the better procedure after laparoscopy-assisted distal gastrectomy (LADG).

Research frontiers

Compared with Japan and Korea, early gastric cancer only accounts for a small percentage in China, and most gastric cancer cases are found in advanced stages at the initial diagnosis. It is inappropriate for surgeons to perform Billroth I anastomosis after subtotal gastrectomy. In 2005, Uyama first combined LADG with uncut Roux-en-Y reconstruction, and since then it has been the hotspot for many years. However, its use remains controversial. Some surgeons believe that it is better than Billroth II and Roux-en-Y reconstructions, while others do not.

Innovations and breakthroughs

This study is the first randomized controlled trial concentrating on uncut Roux-en-Y *vs* Billroth II reconstruction after distal gastrectomy for gastric cancer. It aimed to compare uncut Roux-en-Y and Billroth II reconstruction in terms of postoperative complications, including biliary reflux and gastritis. Despite the incidence of recanalization, uncut Roux-en-Y reconstruction is secure and feasible, and can effectively reduce the incidence of alkaline reflux, residual gastritis, and heartburn.

Applications

There remains no clear consensus regarding the preferred reconstructive surgical procedure after LADG for gastric cancer. In this study, uncut Roux-en-Y reconstruction is secure and feasible, and can effectively reduce the incidence of alkaline reflux, residual gastritis, and heartburn. Therefore, uncut Roux-en-Y should be widely applied in the future. Besides, the uncut technique still needs improvement so that the risk of staple line dehiscence is minimized, with a longer follow-up period to reevaluate the exact risk.

Peer-review

Interesting comparison of two techniques of postgastrectomy reconstruction.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Drainage fluid and serum amylase levels accurately predict development of postoperative pancreatic fistula

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Abstract

AIM

To investigate potential biomarkers for predicting postoperative pancreatic fistula (POPF) after pancreaticoduodenectomy (PD).

METHODS

We prospectively recruited 83 patients to this study. All patients underwent PD (Child's procedure) at the Division of Hepatobiliary and Pancreas Surgery at the First Bethune Hospital of Jilin University between June 2011 and April 2015. Data pertaining to demographic variables, clinical characteristics, texture of pancreas, surgical approach, histopathological results, white blood cell count, amylase and choline levels in the serum, pancreatic/gastric drainage fluid, and choline and amylase levels in abdominal drainage fluid were included in the analysis. Potential correlations between these parameters and postoperative complications such as, POPF, acute pancreatitis, hemorrhage, delayed gastric emptying, and biliary fistula, were assessed.

RESULTS

Twenty-eight out of the 83 (33.7%) patients developed POPF. The severity of POPF was classified as Grade A in 8 (28%) patients, grade B in 16 (58%), and grade C in

4 (14%), according to the pancreatic fistula criteria. On univariate and multivariate logistic regression analyses, higher amylase level in the abdominal drainage fluid on postoperative day (POD)1 and higher serum amylase levels on POD4 showed a significant correlation with POPF ($P < 0.05$). On receiver operating characteristic curve analysis, amylase cut-off level of 2365.5 U/L in the abdominal drainage fluid was associated with a 78.6% sensitivity and 80% specificity [area under the curve (AUC): 0.844; $P = 0.009$]. A cut-off serum amylase level of 44.2 U/L was associated with a 78.6% sensitivity and 70.9% specificity (AUC: 0.784; $P = 0.05$).

CONCLUSION

Amylase level in the abdominal drainage fluid on POD1 and serum amylase level on POD4 represent novel biomarkers associated with POPF development.

Key words: Pancreaticoduodenectomy; Complication; Amylase; Pancreatic fistula

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Core tip: In this study, we sought to identify biomarkers that could help predict the risk of postoperative pancreatic fistula (POPF) after pancreaticoduodenectomy. Diagnosis of POPF was based on the International Study Group of Pancreatic Fistula criteria. Association between POPF and various clinical and biochemical parameters was assessed. Amylase level in the abdominal drainage fluid on postoperative day 1 and serum amylase level on postoperative day 4 showed a significant association with POPF and represent novel biomarkers associated with POPF development.

Jin S, Shi XJ, Wang SY, Zhang P, Lv GY, Du XH, Wang GY. Drainage fluid and serum amylase levels accurately predict development of postoperative pancreatic fistula. *World J Gastroenterol* 2017; 23(34): 6357-6364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6357.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6357

INTRODUCTION

Pancreaticoduodenectomy (PD) is a common operative approach for treatment of various malignant diseases of the distal biliary duct, the head of pancreas, and the peri-ampullar region. It is also widely acknowledged as one of the most challenging surgical procedures. PD is associated with substantial perioperative mortality (2%-5% even in high-volume centers) and morbidity (30%-50%)[1,2]. Postoperative pancreatic fistula (POPF) is one of the most important complications of PD; reported incidence rates range from 2% to 25%[3]. POPF often leads to further complications, such as hemorrhage and abdominal abscess, and is associated with increased length of hospital stay[3,4]. POPF and other PD-associated complications have necessitated the development of new surgical modalities, like a combination of pancreaticojejunostomy with duct-mucosa pancreaticojejunostomy; however, the incidence of POPF following PD continues to be a concern[5].

Various pre-operative and intra-operative factors, such as preoperative jaundice and diameter of the pancreatic duct, are associated with POPF; however, these factors are not reliable predictors of POPF[6,7]. Biochemical markers in serum and drainage fluid may reflect the disease progression, and it is of translational significance to investigate their correlation with clinical characteristics and their potential value as predictors of POPF. In this study, we sought to identify potential predictors of POPF development, which may help optimize the treatment of these patients in clinical practice.

MATERIALS AND METHODS

Study design

We prospectively recruited 83 patients to this study. All patients underwent PD at the Department of Hepatobiliary and Pancreatic Surgery at the First Bethune Hospital of Jilin University between June 2011 and April 2015. Preoperative, intraoperative and postoperative data were collected for each patient. Preoperative variables of interest included age, sex, history of diabetes, jaundice, plasma protein levels and pre-operative intervention for jaundice (if any). Intraoperative variables included pancreatic consistency, diameter of pancreatic duct, and the technique used for pancreatic anastomosis. Postoperative data included the results of pathological examination, pancreatic and gastric drainage volume, and complications after operation, such as POPF, hemorrhage, acute pancreatitis, delayed gastric emptying (DGE), and biliary fistula (Table 1).

Severity of POPF was classified as grade A, B or C, as defined by the International Study Group of Pancreatic Fistula[8]. Association between surgical outcomes and results of routine serological and biochemical investigations was assessed. The study protocol complied with the principles of the Declaration of Helsinki, and was approved by the Institutional Ethics Committee at the First Hospital of Jilin University. All patients had provided informed consent prior to their enrolment.

Details of clinical management

Forty-three patients received PD with a pancreatic drainage tube (size: 6, 7 or 8) anastomosed to the pancreatic parenchyma during the *pancreaticojejunostomy*; the tube was then drawn out through the distal bowel. In the remaining 40 patients, the standard Child's procedure was performed with placement of external pancreatic drainage tube (size: 6, 7 or 8), which was fixed to the pancreatic parenchyma

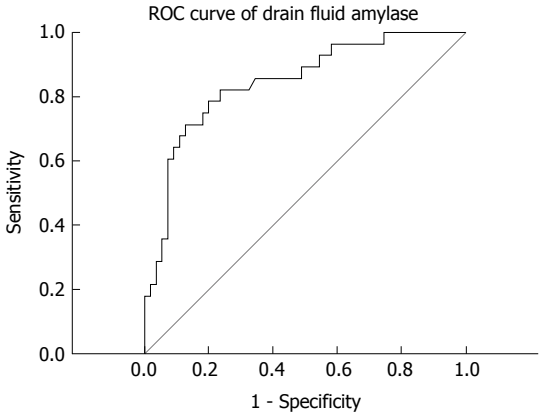


Figure 1 Receiver operating characteristic curve analysis of amylase activity in drainage fluid. A cut-off value of 2365.5 U/L was associated with 78.6% sensitivity, 80% specificity, 66.7% PPV and 88% NPV for POPF (AUC: 0.844, P = 0.009). ROC: Receiver operating characteristic; POPF: Postoperative pancreatic fistula; NPV: Negative predictive value; PPV: Positive predictive value; AUC: Area under the curve.

Table 1 Definitions of postoperative complications after pancreaticoduodenectomy

Complication	Definition
POPF	Drainage fluid amylase activity on or after postoperative day 3 is at least three times the upper limit in normal serum
Ascites	Ultrasound evidence of ascites depth > 5 cm
Hemorrhage	Requires postoperative transfusion of ≥ 2 U isogenic red blood cells
Biliary fistula	Abdominal drainage produces bilious fluid at 50 mL/d after surgery
DGE	Indwelling stomach tube for > 10 d

POPF: Postoperative pancreatic fistula; DGE: Delayed gastric emptying.

and left in the bowel. All patients were treated with cefoperazone and sulbactam (3.0 g/Q12 h) for the first 4 postoperative days (PODs) to prevent infection. Postoperatively, prophylactic intravenous octreotide was administered at a dose of 0.6 mg/24 h for 3 d to reduce the amount of pancreatic secretion.

Statistical analysis

Statistical analyses were conducted using SPSS software (version 19.0; SPSS Inc., IBM, Armonk, NY, United States). Univariate and multivariate analyses were performed to identify factors significantly related to POPF. Receiver operating characteristic (ROC) curve analysis was performed to assess the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the identified variables. A P value of less than 0.05 was considered indicative of statistical significance.

RESULTS

Incidence of POPF

Out of 83 patients, 28 (33.7%) eventually developed POPF. The severity of POPF was classified as grade A in 8 (28%) patients, grade B in 16 (58%) and grade C in 4 (14%) (Table 2).

Table 2 Summary of postoperative complications

Complication	Cases, n = 30	Incidence, %
POPF	28	33.7
Type A	8	28
Type B	16	58
Type C	4	14
Ascites	4	10
Hemorrhage	7	17.5
Biliary leakage	1	2.5
DGE	1	2.5
Postoperative acute pancreatitis	1	2.5

POPF: Postoperative pancreatic fistula; DGE: Delayed gastric emptying.

High amylase level predicts POPF development

We first categorized the patients as POPF and non-POPF groups, and then compared the levels of different parameters between the two groups (Table 3). On univariate analysis, amylase level in the abdominal drainage fluid on POD1 and serum amylase level on PODs 1 and 4 were correlated with POPF development (P < 0.05) (Table 4).

Multivariate logistic regression analysis confirmed that amylase level in abdominal drainage fluid on POD1 and serum amylase level on POD4 were independent predictors of POPF (Table 5). On ROC curve analysis, a cut-off amylase level of 2365.5 U/L in the abdominal drainage fluid on POD1 as predictor of POPF was associated with 78.6% sensitivity, 80% specificity, 66.7% PPV and 88% NPV [area under the curve (AUC): 0.844; P = 0.009] (Figure 1). Similarly, a cut-off serum amylase level of 44.2 U/L on POD4 was associated with a 78.6% sensitivity and 70.9% specificity (AUC: 0.784; P = 0.05; Figure 2). The specificity of these associations was further highlighted by the observation that neither white blood cell (WBC) counts nor choline and albumin levels correlated with POPF development.

DISCUSSION

The Whipple procedure for PD for the treatment of benign and malignant tumors in the head of pancreas and the peri-ampullar region is one of the most technically challenging surgical procedures, with long duration of surgery and a high rate of associated complications[9,10]. The reported rates of postoperative complications following PD range from 30%-70%, and postoperative mortality rates have remained approximately 5% even in high-volume centers[11]. POPF accounts for 2%-25% of all complications of PD[12]. The advent of novel surgical modalities for pancreaticojejunostomy, such as the duct-mucosa pancreaticojejunostomy or pancreatic duct stent

Table 3 General risk factors for pancreatic leakage

Variable	POPF (n = 14)	Non-POPF (n = 26)	$t/z/\chi^2$	P value
Age in yr	54.18 ± 6.945	54.04 ± 10.447	0.065	0.948
Sex				
Male	20 (71.4)	32 (58.2)	1.391	0.337[1]
Female	8 (28.6)	23 (41.8)		
Blood loss in mL	300 (200, 575)	200 (200, 600)	-1.266	0.205
Pancreatic duct diameter in mm				
> 3	11 (39.3)	33 (60.0)	3.196	0.104[1]
< 3	17 (60.7)	22 (40.0)		
Preoperative low plasma protein				
Yes	7 (25.0)	12 (21.8)	0.106	0.786[1]
No	21 (75.0)	43 (78.2)		
Preoperative relief of jaundice				
Yes	4 (14.3)	4 (7.3)	1.048	0.433[1]
No	24(85.7)	51 (92.7)		
Preoperative jaundice				
Yes	18 (64.3)	34 (61.8)	0.048	1.000
No	10 (35.7)	21 (38.2)		
Surgical type				
External drainage of PD	12 (42.9)	27 (49.1)	0.289	0.647[1]
Internal drainage of PD	16 (57.1)	28 (50.9)		
Diabetes				
Yes	2 (7.1)	0 (0.0)	4.026	0.111[1]
No	26 (92.9)	55 (100.0)		
Duration of surgery in min	267.5 (240.0, 327.5)	280.0 (235.0, 335.0)	-0.082	0.935
Hospitalization period in days	15.00 (13.00, 18.00)	21.50 (18.00, 27.75)	-4.385	< 0.001
Drainage fluid amylase level on POD1 in U/L	6017.5 (2494.5, 11752.5)	890.0 (350.0, 1500.0)	-5.110	< 0.001
Serum amylase level in U/L				
POD1	379.5 (157.5, 627.5)	124.0 (80.0, 165.0)	-4.091	< 0.001
POD4	30.0 (30.0, 50.0)	61.5 (45.5, 150.0)	-4.435	< 0.001
Serum albumin				
POD1	29.5 (25.3, 34.3)	30.7 (26.6, 33.0)	-0.125	0.900
POD3	30.1 (25.7, 34.6)	30.4 (27.1, 33.2)	-0.111	0.912
POD5	30.8 (27.8, 34.4)	31.4 (28.4, 36.9)	-0.718	0.473
Choline				
POD1	4957 (4022, 4957)	5242 (4048, 6715)	-0.876	0.381
POD3	3481 (2626, 4518)	4056 (3345, 5020)	-1.425	0.154
POD5	3268 (2696, 4537)	4024 (3248, 5069)	-1.580	0.114
WBC count				
POD1	14.73 (10.84, 18.75)	15.49 (12.42, 18.41)	-0.751	0.452
POD3	14.90 (11.74, 18.64)	13.54 (10.34, 16.58)	-1.112	0.266
POD5	11.99 (9.65, 15.56)	11.24 (9.17, 14.79)	-0.510	0.610
Pathology examination result			2.445	0.485
Cholangiocarcinoma	12 (42.9)	29 (52.7)		
Pancreatic carcinoma	3 (10.7)	8 (14.5)		
Ampullary carcinoma	3 (10.7)	7 (12.7)		
Other	10 (35.7)	11 (20.0)		

Data are presented as n (%). [1]Fisher's exact test. POPF: Postoperative pancreatic fistula; PD: Pancreaticoduodenectomy; WBC: White blood cell; POD: Postoperative day.

implantation, has not resulted in major improvement. Therefore, early detection of POPF development and adequate timely intervention is important to improve surgical outcomes in these patients[13-17]. There is a paucity of tools to identify patients who are at risk of development of POPF. Here, we report our prospective study to identify potential predictors of POPF. The results suggest that monitoring of amylase levels in drainage fluid and serum as biomarkers is quite promising.

Various factors have been linked to POPF in previous studies. These include pancreatic consistency, pancreatic duct diameter, and the results of assessment by the pathologist[18-22]; however, their predictive value has been shown to be relatively poor and not intuitive. In the present study, we observed an obvious correlation between amylase levels in the ascitic fluid on POD1 and the serum amylase level on POD4 with the occurrence of POPF. Cloyd *et al*[20] reported that serum amylase level on POD1 (> 140 U/L) predicts POPF with 81.5% sensitivity, 55.5% specificity, 29.3% PPV, and 93% NPV[23]. Likewise, some researchers demonstrated that drainage fluid amylase level on POD1 (> 350 U/L) can predict POPF with a 79% specificity, 100% sensitivity, 41% PPV, and 100% NPV. Furthermore, Popiela *et al*[24] showed that the drainage

Table 4 Results of univariate analysis showing risk factors for postoperative pancreatic fistula

Factor	β	SE	*Wald* χ^2	OR (95%CI)	*P* value
Age in yr	0.023	0.037	0.397	1.024 (0.952-1.101)	0.529
Sex, female/male	-0.586	0.500	1.375	0.557 (0.209-1.482)	0.241
Blood loss in mL	0.000	0.001	0.257	1.000 (0.999-1.001)	0.612
Pancreatic duct diameter, > 3 mm/< 3 mm	-0.841	0.475	3.135	0.431 (0.170-1.094)	0.077
Preoperative low plasma protein, yes/no	0.178	0.545	0.106	1.194 (0.410-3.476)	0.744
Preoperative relief of jaundice, yes/no	0.754	0.749	1.012	2.125 (0.489-9.227)	0.314
Preoperative jaundice, yes/no	0.106	0.482	0.048	1.112 (0.432-2.861)	0.826
Internal or External drainage of PD	0.251	0.468	0.289	1.286 (0.514-3.214)	0.591
Diabetes, yes/no	21.952	28420.722	0.000	3.417E9 (0.000)	0.999
Pathology examination result, ampullary carcinoma/ pancreatic carcinoma/cholangiocarcinoma	0.241	0.182	1.757	1.273 (0.891-1.819)	0.185
Duration of surgery in min	0.000	0.003	0.008	1.000 (0.995-1.005)	0.928
Drainage fluid amylase level, POD1 > 5000 U/L	0.000	0.000	10.293	1.000 (1.000--1.000)	0.001
Serum amylase level					
POD1 > 140 U/L	0.004	0.001	9.982	1.004 (1.001-1.006)	0.002
POD4 > 140 U/L	0.013	0.005	7.752	1.013 (1.004-1.022)	0.005
Serum albumin					
POD1	0.000	0.045	0.000	1.000 (0.916, 1.092)	0.994
POD3	-0.006	0.044	0.020	0.994 (0.911, 1.084)	0.887
POD5	-0.023	0.044	0.277	0.977 (0.897, 1.064)	0.599
Choline					
POD1	-0.016	0.041	0.149	0.984 (0.908, 1.067)	0.700
POD3	0.033	0.046	0.521	1.034 (0.945, 1.131)	0.470
POD5	0.007	0.053	0.016	1.007 (0.908, 1.117)	0.898
WBC count					
POD1	0.000	0.000	1.153	1.000 (1.000, 1.000)	0.283
POD3	0.000	0.000	1.298	1.000 (0.999, 1.000)	0.255
POD5	0.000	0.000	2.026	1.000 (0.999, 1.000)	0.155

POPF: Postoperative pancreatic fistula; PD: Pancreaticoduodenectomy; WBC: White blood cell; POD: Postoperative day.

Table 5 Results of multivariate logistic regression analysis showing risk factors for postoperative pancreatic fistula

Predictor	β	SE	*Wald* χ^2	OR (95%CI)	*P* value
Amylase level, POD1 > 5000 U/L	0.000	0.000	6.728	1.000 (1.000--1.000)	0.009
Serum amylase, level POD1 > 140 U/L	0.001	0.002	0.243	1.001 (0.998-1.004)	0.622
Serum amylase level, POD4 > 140 U/L	0.009	0.004	3.826	1.009 (1.000-1.017)	0.050

POPF: Postoperative pancreatic fistula; POD: Postoperative day.

fluid amylase level (> 5000 U/L) on POD1 is a reliable predictor of POPF. Kawai *et al*[25] also found that the ratio of total amylase in drainage fluid could predict POPF. Our results are consistent with these previous publications, which indicate that amylase level in ascitic fluid on POD1 and blood amylase on POD4 could be used to identify patients who are most likely to suffer from this complication after PD.

Of note, we did not observe a significant association of albumin and WBC counts with POPF. Kawai *et al*[25] reported that serum albumin levels (< 3.0 g/d) and serum WBC count (> 9800 mm^3) on POD4 predicted grades B and C POPF with a 69% sensitivity, a 96% specificity, an 88% PPV, and an 85% NPV. Relles *et al*[26] found that serum albumin (< 2.5 mg/L) and blood urea nitrogen (> 10 mg/dL) on POD1 were important predictors of perioperative morbidity following PD[27]. However, in our study, the level of serum albumin through POD1, POD3 and POD5 did not correlate with the development of POPF. Considering that acute stress can affect serum albumin level and WBC counts[28], these may not be appropriate measures to gauge the risk of POPF. A prospective trial showed that postoperative albumin levels were not associated with risk during abdominal operation[29]. Welsch *et al*[29] also reported poor specificity of serum WBC as a predictor of POPF. Our results are consistent with these reports. In addition, no significant association of POPF with cholinesterase level was observed on POD1, POD3 and POD5, which suggests that POPF may not have an obvious relationship with the postoperative change in hepatic reserve.

In our study, a cut-off amylase level of 2365.5 U/L in ascitic fluid on POD1 predicted POPF with 78.6% sensitivity, 80% specificity, 66.7% PPV, and 88% NPV. We hypothesize that in patients with POPF, the higher level of amylase in drainage fluid on POD1 derives from the failed pancreaticojejunal anastomosis, which

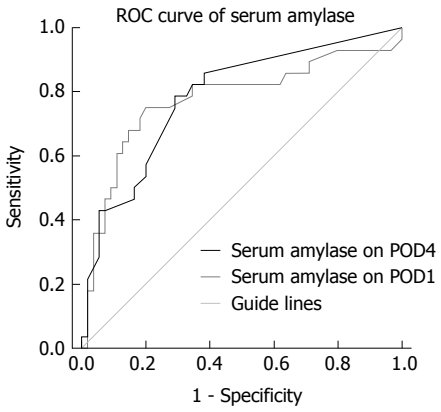


Figure 2 ROC curve analysis of serum amylase activity. A cut-off value of 44.2 U/L on POD4 was associated with a 78.6% sensitivity and 70.9% specificity for POPF (AUC: 0.784, P = 0.05). ROC: Receiver operating characteristic; POPF: Postoperative pancreatic fistula; AUC: Area under the curve.

leads to POPF.

Serum amylase has been used for diagnosis of acute pancreatitis for > 70 years; however, increased serum amylase levels are also observed in other diseases of salivary glands, oviduct epithelium and proximal duodenum, which limits its specificity for the diagnosis of pancreatitis. Intuitively, this should be less of a concern with respect to drainage fluid or generally following PD[30]. Indeed, in our study, serum amylase on POD4 showed a significant correlation with POPF, although serum amylase on POD1 did not effectively predict POPF. We found that serum amylase on POD4 of 44.2 U/L was an effective maker; however, this value is within the normal range of serum amylase, and thus serum amylase level on POD4 higher than 100 U/L is probably more appropriate. Serum amylase on POD4 with a discriminatory threshold of 100 U/L can predict POPF with 42.9% sensitivity, 55% specificity, 75% PPV, and 76.1% NPV. In the early postoperative stage, ischemic injury to pancreatic tissues caused by surgical damage to blood vessels, intraoperative and postoperative hypovolemia, and surgical stress leads to elevation of serum amylase[31]. However, no statistically significant correlation was observed between serum amylase level on POD1 and pancreatic leakage, and no correlation was found between serum amylase and POPF in theory, whereas absorption of exudates from pancreatic anastomosis cannot explain such a phenomenon. On the other hand, we believe that elevation in serum amylase level on POD4 reflects the preliminary establishment of a collateral pancreatic anastomosis, and that correction of hypovolumia may reflect the condition of pancreatic juice derived from accessory pancreatic duct or residual pancreatic section instead of the obstructed main pancreatic duct. Moreover, the erosion effect of amylopsin and trypsin on blood vessel can also increase the absorption speed and the amount of amylopsin into the blood, which reflects the increased serum amylase. Thus, blood amylase level on POD4 is of certain predictive value for the occurrence of POPF.

In our study, we found that amylase activity in drainage fluid on POD1 and serum amylase activity on POD4 could accurately predict POPF, whereas serum albumin and prealbumin did not show a significant predictive value. Measurement of amylase level in drainage fluid on POD1 and the serum amylase level on POD4 is a relatively easy and economical method. Our results support the use of amylase to predict POPF.

This may allow for timely interventions, such as increased duration of antibiotic and octreotide therapies, that may help prevent POPF and allow for timely risk-communication to the patient. It is also another procedure in the processing carried out in our medical center.

COMMENTS

Background

Identification of patients who are at high risk of postoperative pancreatic fistula (POPF) in the immediate postoperative period after is a key imperative to improve surgical outcomes of pancreaticoduodenectomy (PD). Biochemical markers in serum and drainage fluid may reflect disease progression, and it is of translational significance to investigate their correlation with clinical characteristics and their potential use to predict the risk of POPF. In this study, we sought to identify potential predictors of POPF development, which may help optimize the treatment of such patients in clinical practice.

Research frontiers

Although anastomosis techniques used for PD significantly improves, the incidence of PD-associated POPF remains relatively high. POPF is a major threat to patients who undergo PD. Thus, early and accurate prediction of POPF is essential to achieve optimal surgical outcomes.

Innovations and breakthroughs

Previous studies have found several biochemical factors, such as serum amylase level on postoperative day (POD)1, drainage fluid amylase level on POD1, and a combination of serum albumin and leukocyte count as predictors of POPF. However, some of these markers have low positive predictive value. We demonstrate that amylase level in drainage fluid on POD1 and serum amylase level on POD4 are better predictors of POPF than those proposed earlier.

Applications

Measurement of amylase level in serum and drainage fluid is relatively straightforward and inexpensive. These two investigations can help identify patients who are at an increased risk of POPF. This may allow for timely interventions, such as increased duration of antibiotic and octreotide therapies, that may help prevent POPF. It is also another procedure in the processing carried out in our medical center.

Terminology

Although several biochemical factors have been shown to predict POPF in previous studies, we found that just amylase levels in drainage fluid on POD1 and serum amylase level on POD4 could accurately predict POPF, whereas serum albumin and prealbumin were found to have negligible predictive value.

Peer-review

In this manuscript, the authors correlate the postoperative clinical factors with POPF rate, including prospectively 83 patients with PD, and analyzed the potential correlation between biomarkers and postoperative complications such a pancreatic fistula.

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CASE REPORT

Interventional endoscopic ultrasound for a symptomatic pseudocyst secondary to gastric heterotopic pancreas

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Author contributions: All authors contributed to the acquisition of data, writing, and revision of this manuscript.

Institutional review board statement: This case report was reviewed and approved by the Hangzhou First People's Hospital Institutional Review Board.

Informed consent statement: The patient and his family provided informed written consent.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

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Abstract

Heterotopic pancreas (HP) is a relatively uncommon entity that is defined as pancreatic tissue without a true anatomical or vascular connection to the pancreas. HP does not cause symptoms in most cases but can occasionally produce various manifestations, including nausea, vomiting, abdominal pain, and even heterotopic pancreatitis. Here, we report an unusual case in which heterotopic pancreatitis complicated by the formation of a pseudocyst that caused gastric outlet obstruction was diagnosed based on serum hyperamylasemia and findings from endoscopic ultrasonography (EUS)-guided fine needle aspiration (EUS-FNA) cytology. EUS-guided single pigtail stent insertion was successfully performed for recurrent gastric outlet obstruction. The patient has remained healthy and symptom-free during 4 years of surveillance. In the context of the relevant literature, the described case is a rare case of HP complicated by a pseudocyst treated *via* EUS-FNA and stent insertion.

Key words: Heterotopic pancreas; Pseudocyst; Gastric outlet obstruction; Endoscopic ultrasound-guided fine needle aspiration

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Core tip: Heterotopic pancreas (HP), which is defined as the atypical presence of pancreatic tissue without an anatomical or vascular connection to the pancreas,

is relatively rare. As an intra- or submucosal lesion, HP is typically asymptomatic and found incidentally. We report a case involving a 40-year-old man with gastric outlet obstruction secondary to pseudocyst formation in HP.

Jin HB, Lu L, Yang JF, Lou QF, Yang J, Shen HZ, Tang XW, Zhang XF. Interventional endoscopic ultrasound for a symptomatic pseudocyst secondary to gastric heterotopic pancreas. *World J Gastroenterol* 2017; 23(34): 6365-6370 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i34/6365.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i34.6365

INTRODUCTION

Heterotopic pancreas (HP) is defined as the presence of pancreatic tissue in an abnormal location without anatomical or vascular connection to the orthotopic pancreas. HP was first described by Jean-Schultz in 1729 and was confirmed histologically by Klobb[1]. Other terms for HP, such as ectopic pancreas, aberrant pancreas, and accessory pancreas, have been used in the literature[2]. In most cases, HP is asymptomatic and found incidentally[1], but it can occasionally produce non-specific symptoms that depend on its location, size, and types of complications. Here, we describe a case involving a 40-year-old man with gastric outlet obstruction secondary to heterotopic pancreatitis with pseudocyst formation.

CASE REPORT

A 40-year-old man was admitted to a community hospital for upper abdominal pain accompanied by nausea and vomiting. He also complained of abdominal distention and moderate fever (38.3 ℃). Physical examination revealed minimal tenderness in the epigastric region without a palpable mass or peritoneal signs, and the patient's vital signs were unremarkable. Routine blood tests showed a significant elevation of the leukocyte count and neutrophil ratio, and biochemical examinations revealed serum amylase of 460 U/L and urine amylase of 3500 U/L. Transabdominal ultrasound was initially performed when the patient was diagnosed with pancreatitis, but no gallbladder stones or common bile duct stones were detected. The patient was treated for acute pancreatitis; his treatments included fasting, injection of a proton pump inhibitor, and somatostatin. All symptoms disappeared 3 d later but recurred after the patient ingested fluids. Subsequently, the patient was transferred to our hospital for further diagnosis. A computed tomography (CT) scan of the abdomen revealed manifestations of a normal pancreas and an ill-defined enhancing mass at the gastric antrum that was causing circumferential narrowing of the pyloric channel (Figure 1A). In an esophagogastroduodenoscopy (EGD) examination, a sub-epithelial lesion without overlying umbilication or dimpling was found in the prepyloric region (Figure 1B). The pyloric channel was only wide enough to allow for the passage of an Olympus Q260 endoscope (OD 9.2 mm), but the duodenum was normal. Radial endoscopic ultrasonography (EUS), which was used as an additional diagnostic tool, confirmed the presence of a cystic lesion measuring 21 mm × 25 mm (Figure 1C) originating from the third layer with anechoic contents and debris. Subsequently, EUS-guided fine needle aspiration (EUS-FNA) was successfully performed (Figure 1D), and a total of 25 mL of clear fluid was aspirated to the greatest possible extent with negative pressure. Cystic fluid analysis revealed an amylase level of 83770 U/L and a carcinoembryonic antigen level of 3.7 μg/L; a cytologic examination of smears revealed clusters of benign-appearing ducts and small acini mixed with inflammatory cells (Figure 2).

The patient was allowed to ingest low-fat fluids 5 d after the procedure, but the aforementioned symptoms immediately re-emerged, and serum amylase increased to 512 U/L. A narrowed pyloric channel was observed in a second EGD examination. The patient refused surgical treatment. Balloon dilation, bougie dilation, and needle-knife puncture of the cyst were not initially selected. In accordance with an endoscopic drainage procedure used for pancreatic pseudocysts, we successfully performed cyst cavity (20 mm × 23 mm) puncture, wire exchange, and the gradual insertion of a single pigtail stent (5 Fr × 4 cm) using a 19-gauge needle (EchoTip Ultra, ECHO-19; Cook Endoscopy, Winston-Salem, NC, United States) (Figure 1E and F). Informed consent was obtained from the patient and his family prior to this endoscopic procedure. No abnormal signs or symptoms were noted during or immediately after the procedure. Shortly thereafter, the patient's abdominal pain and vomiting subsided, and his leukocyte count and serum amylase returned to normal. The patient was discharged on the sixth day after the second drainage procedure.

Five months later, the patient returned for a follow-up examination without any complaints. EGD surveillance revealed no stent at the original position, and radial EUS confirmed the presence of a smaller anechoic cystic lesion measuring 3 mm × 5 mm (Figure 3A and B). The stent had completely passed, as confirmed by abdominal radiography (Figure 4). The patient has remained healthy and symptom-free during 4 years of follow-up.

DISCUSSION

In autopsy studies, HP has generally had an incidence ranging from 0.5%-13.0% and been more common in 30- to 50-year-old subjects, with male predominance[3]. HP is most frequently located in the stomach

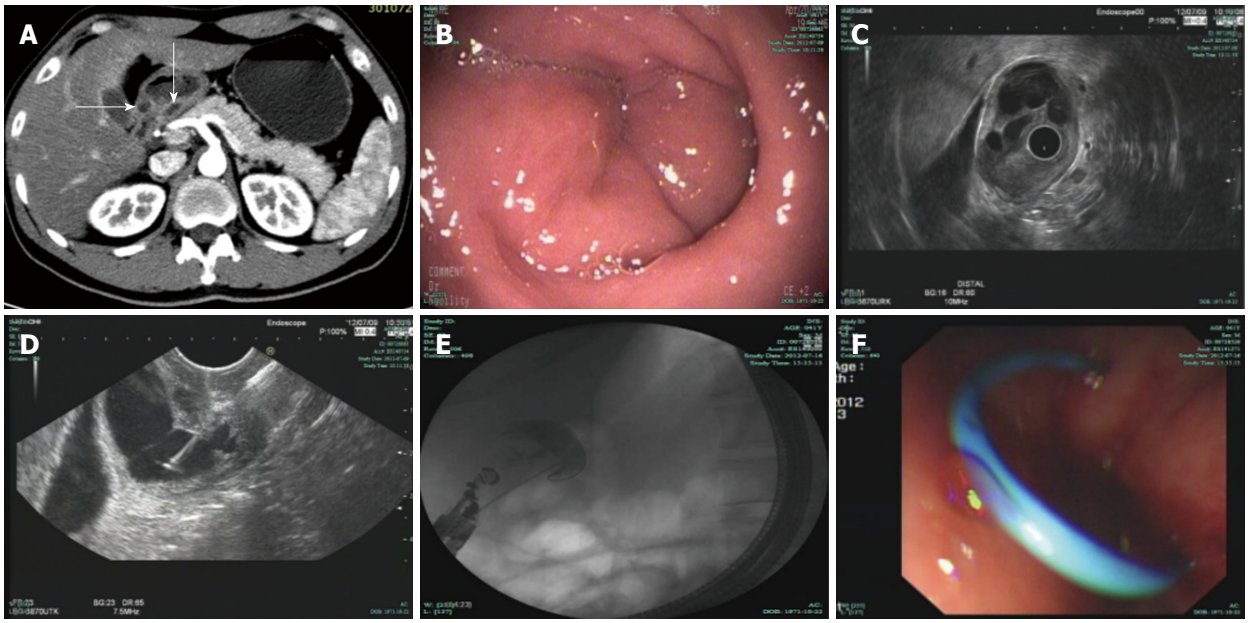


Figure 1 Computed tomography scan and endoscopic treatment of the lesion. A: Contrast-enhanced CT revealed manifestations of a normal pancreas and an ill-defined enhancing mass (arrows) at the gastric antrum that caused circumferential narrowing of the pyloric channel; B: EGD showed a narrowed pyloric channel with normal mucosa; C: Radial EUS imaging confirmed the presence of a cystic lesion measuring 21 mm × 25 mm that originated from the third layer with anechoic contents and debris; D: EUS-guided fine needle aspiration; E: Cyst cavity radiography and guide-wire exchange; F: Successful insertion of a single pigtail stent (5 Fr × 4 cm). CT: Computed tomography; EUS: Endoscopic ultrasonography; EGD: Esophagogastroduodenoscopy.

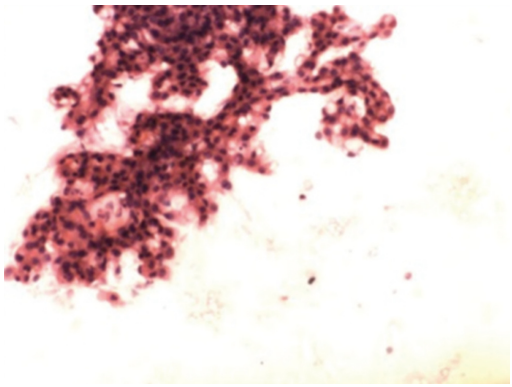


Figure 2 Cytologic examination with hematoxylin-eosin staining (low-power field). A cytologic examination of smears revealed clusters of benign-appearing ducts and small acini mixed with inflammatory cells.

(25%-38%), followed by the duodenum (17%-36%) and the jejunum (15%-21.7%); it is rarely found in the esophagus, common bile duct, spleen, mesentery, mediastinum or fallopian tube[4]. In the gastrointestinal tract, HP is most frequently located only in the submucosa (54%) and is less frequently found in both the submucosa and muscularis propria (23%), only the muscularis propria (8%), the subserosa (11%), and the whole wall (4%)[1].

HP has been classified into four types by Heinrich and Gaspar-Fuentes[5]. Type Ⅰ HP shows the presence of ducts, acini, and islets of Langerhans cells; type Ⅱ HP shows only ducts and acini; type Ⅲ HP has only ducts; and type Ⅳ HP has only islets. Most cases of HP are type Ⅱ. The pathogenesis of HP remains unclear, although the two most widely accepted hypotheses are the misplacement and metaplasia theories. The misplacement theory proposes that ectopic pancreatic tissue develops from misplacement during foregut rotation, whereas the metaplasia theory suggests that heterotopia arises from a pancreatic metaplasia of the endoderm that migrates to the submucosa during embryogenesis[6].

HP is typically asymptomatic; however, depending on its location and size, HP can occasionally cause clinical symptoms, the most common of which are vomiting and abdominal pain. Peptic ulceration and gastrointestinal bleeding are rare presentations, as are malignant transformation, pancreatitis, pseudocyst formation, gastric outlet obstruction, jejunal intussusception, and ileus[3,7-10]. A thorough search of the English literature revealed five cases of gastric heterotopic pancreatitis in the last 20 years, including our case[11-14] (Table 1). These cases involved three male patients and two female patients. The median age of the patients was 32 years. Epigastric pain was reported in four cases, and vomiting occurred in three cases. In our case, the patient presented with recurrent epigastric pain and vomiting, which were mainly induced by gastric outlet obstruction induced by heterotopic pancreatitis. The symptoms of pain and fever in our case might have been attributable to inflammation or chemical irritation of the involved tissue and enzyme secretion.

It is typically complex and challenging to preoperatively diagnose HP due to its varied presentations. Gastric HP is difficult to radiologically diagnose; however, the double contrast barium meal technique may characteristically show a focally raised mucosal

Table 1 Reported cases of gastric heterotopic pancreatitis

Author	Year	Age/gender	Symptom(s)	Size (mm)	Location	Serum amylase (IU/L)	Preoperative diagnosis	Diagnostic method	Therapy
Matsushita	1997	33/M	Epigastric pain	20	N/A	59	SMT	Surgery	Enucleation
Hirasaki	2005	32/M	Epigastric pain	35	Angulus	262	SMT	Surgery	Partial resection
Matissek	2012	15/F	Vomiting	30	Pylorus	144	SMT	Surgery	Gastroduodenostomy
Matsumoto	2012	21/F	Epigastric pain and vomiting	26	Antrum	122	HP	EUS-FNA	Laparoscopic partial resection
Our case	2017	40/M	Epigastric pain and vomiting	25	Pylorus	460	HP	EUS-FNA	EUS-guided stenting

EUS: Endoscopic ultrasonography; FNA: Fine needle aspiration; HP: Heterotopic pancreas; N/A: Not available; SMT: Submucosal tumor.

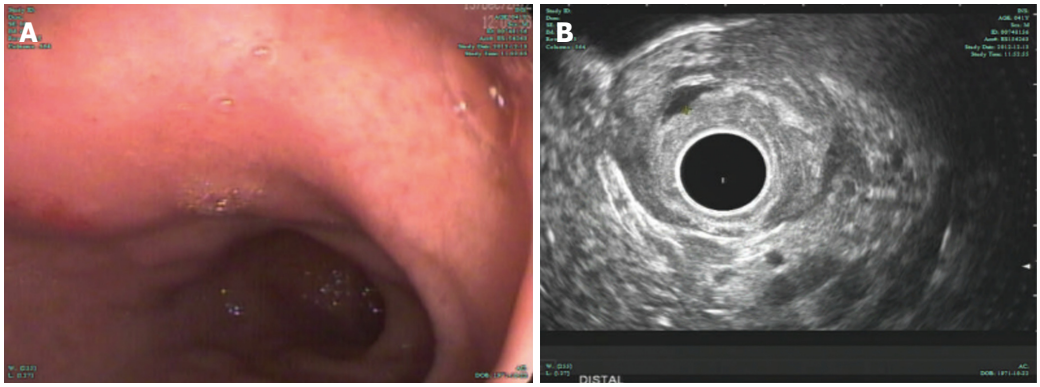


Figure 3 Esophagogastroduodenoscopy and endoscopic ultrasonography surveillance 5 mo later. A: EGD surveillance 5 mo after stent insertion; B: Radial EUS confirmed the presence of a small anechoic cystic lesion measuring 3 mm × 5 mm. EUS: Endoscopic ultrasonography; EGD: Esophagogastroduodenoscopy.



Figure 4 Abdominal radiograph. A normal abdominal radiograph confirmed excretion of the stent without stent impaction.

area with associated superficial ulceration within the gastric antrum[15]. CT can localize lesions with normal pancreatic tissue but cannot distinguish HP from other submucosal tumors[16]. Upper endoscopy generally reveals a broad-based, umbilicated, submucosal lesion, with superficial biopsies being non-diagnostic in the vast majority of cases[17]. EUS has been reported to be helpful in diagnosing HP; EUS-FNA in particular is especially effective for confirming a diagnosis of HP, with a reported sensitivity of 80%-100%[18,19]. A novel sampling method using submucosal endoscopy with a mucosal flap was recently reported by Kobara *et al*[20]. Submucosal dissection allows for the collection of tissue samples even from small lesions (< 2 cm in diameter) under direct endoscopic view. This method was used to eventually prove that a gastrointestinal submucosal tumor (SMT) was a gastric duplication cyst with HP and an ectopic submucosal gland[21]. This approach can be used for preoperative diagnosis of an SMT, albeit with certain limitations.

Symptomatic HP should be respected. However, the optimal management of asymptomatic HP remains debatable. Surgical resection has been reported in most HP cases[11-14]. In the aforementioned cases (Table 1), four patients underwent partial resection of the stomach because this approach was preferable for the preservation of gastric function. The prognosis after surgery was generally favorable, and complete symptom remission was achieved in these cases. Endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) have also been reported for symptomatic HP; these approaches offer the possibility of localized treatment for HP with relatively low mortality and morbidity rates[22]. Zhong *et al*[23] reported 60 cases of gastric HP that were treated using ESD or EMR; in particular, 14

patients underwent EMR, and 46 patients underwent ESD. Complications occurred in 12 (20%) patients, including arterial bleeding in nine patients and pneumoperitoneum in three patients; all of these patients were treated conservatively. However, it has been reported that in total, 23% of gastric HP cases are located in the muscularis propria, the subserosa, or the whole wall; the technical difficulty and complication rates of endoscopic procedures may be significantly increased for HP at these locations[1]. Recently, EUS has been increasingly used for pancreatic fluid collection, biliary and pancreatic duct drainage, gallbladder drainage, and other applications[24]. The roles of interventional EUS continue to expand, and growing global experience supports the feasibility and efficacy of this approach[25,26]. In our case, an endoscopic view of the lesion did not reveal the typical umbilicated indentation that has been recognized as characteristic of a central ductal orifice. The absence of communication between the glandular epithelium and the gastric lumen can be attributed to the retention of exocrine secretions that subsequently led to recurrent episodes of acute obstructive pancreatitis, the formation of a retention pseudocyst, and eventually recurrent gastric outlet obstruction. EUS-guided stenting was used to relieve the gastric outlet obstruction by creating an artificial fistula in the pseudocyst. Consequently, this stenting not only rapidly relieved clinical symptoms but also resulted in long-term conversion of symptomatic HP to asymptomatic HP that no longer required surgical treatment. To our knowledge, no previous reports have described heterotopic pancreatitis treated using interventional EUS.

In summary, this case demonstrates the use of a novel endoscopic approach to successfully manage a gastric outlet obstruction secondary to heterotopic pancreatitis with pseudocyst formation. Based on this case, we suggest that HP should be considered in the differential diagnosis of a submucosal gastric mass. EUS-guided intervention can be beneficial for both diagnosing and providing symptomatic relief of heterotopic pancreatitis with pseudocyst formation, and endoscopic treatment should be recommended in selected cases.

COMMENTS

Case characteristics

A 40-year-old male complained of upper abdominal pain, nausea, and vomiting.

Clinical diagnosis

Heterotopic pancreatitis with pseudocyst formation located in the prepyloric region causing gastric outlet obstruction.

Differential diagnosis

Hyperplastic polyp, gastric adenoma, gastrointestinal stromal tumor, and lymphoma.

Laboratory diagnosis

Laboratory workup revealed a significant elevation of the leukocyte count and neutrophil ratio, serum amylase of 460 U/L, and urine amylase of 3500 U/L.

Imaging diagnosis

The abdominal computed tomography scan showed an ill-defined enhancing mass at the gastric antrum. Endoscopic ultrasound (EUS) revealed a cystic lesion measuring 21 mm × 25 mm originating from the third layer with anechoic contents and debris.

Pathological diagnosis

Histology revealed heterotopic pancreatic tissue in the prepyloric mucosa with clusters of benign-appearing ducts and small acini mixed with inflammatory cells.

Treatment

EUS-guided stenting.

Related reports

A total of four cases of gastric heterotopic pancreatitis have been reported in the literature. However, this is the first report of heterotopic pancreatitis with pseudocyst formation treated using interventional EUS.

Experiences and lessons

Heterotopic pancreas (HP) is a rare entity, and should be considered in the differential diagnosis of a submucosal gastric mass. Although surgical resection has been reported in most symptomatic HP cases, interventional EUS can sometimes be an effective minimally invasive treatment procedure for HP.

Peer-review

The authors describe an interesting and rare case of HP complicated with pseudocyst. Heterotopic pancreatitis with pseudocyst formation has been reported previously, but this case is unique in that endoscopic drainage was performed, which has not been previously described to our knowledge.

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EDITORIAL

Organization of future training in bariatric gastroenterology

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Abstract

A world-wide rise in the prevalence of obesity continues. This rise increases the occurrence of, risks of, and costs of treating obesity-related medical conditions. Diet and activity programs are largely inadequate for the long-term treatment of medically-complicated obesity. Physicians who deliver gastrointestinal care after completing traditional training programs, including gastroenterologists and general surgeons, are not uniformly trained in or familiar with available bariatric care. It is certain that gastrointestinal physicians will incorporate new endoscopic methods into their practice for the treatment of individuals with medically-complicated obesity, although the long-term impact of these endoscopic techniques remains under investigation. It is presently unclear whether gastrointestinal physicians will be able to provide or coordinate important allied services in bariatric surgery, endocrinology, nutrition, psychological evaluation and support, and social work. Obtaining longitudinal results examining the effectiveness of this ad hoc approach will likely be difficult, based on prior experience with other endoscopic measures, such as the adenoma detection rates from screening colonoscopy. As a long-term approach, development of a specific curriculum incorporating one year of subspecialty training in bariatrics to the present training of gastrointestinal fellows needs to be reconsidered. This approach should be facilitated by gastrointestinal trainees' prior residency training in subspecialties that provide care for individuals with medical complications of obesity, including endocrinology, cardiology, nephrology, and neurology. Such training could incorporate additional rotations with collaborating providers in bariatric surgery, nutrition, and psychiatry. Since such training

would be provided in accredited programs, longitudinal studies could be developed to examine the potential impact on accepted measures of care, such as complication rates, outcomes, and costs, in individuals with medically-complicated obesity.

Key words: Obesity; Bariatrics; Bariatric surgery; Intragastric balloon; Endoscopic sleeve gastroplasty; Nutrition; Endoscopy; Training

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Core tip: A world-wide rise in the prevalence of obesity and obesity-related medical conditions needs to be addressed. Newer endoscopic methods will be incorporated into the clinical practice of gastrointestinal physicians, although the long-term impact of these endoscopic methods on medically-complicated obesity remains under investigation. As a potential long-term approach, development of a curriculum designed to incorporate one year of subspecialty training in bariatrics to the present training of gastrointestinal fellows needs to be reconsidered. Longitudinal studies could be performed to examine the impact of subspecialty training on medical complications, outcomes, and costs in treating individuals with medically-complicated obesity.

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INTRODUCTION

The rising prevalence of obesity is a worldwide disorder, and therefore this problem will require a collaborative global approach. One commonly utilized definition of overweight and obesity is based upon the body mass index or BMI, which is calculated from an individual's height and weight (see Table 1). An extensive study has shown that the worldwide percentage of adults who are overweight (BMI 25-29.9 kg/m^2) or obese (BMI 30.0 and higher) increased from 28.8% of men in 1980 to 36.9% of men in 2013, and increased from 29.8% of women in 1980 to 38.0% of women in 2013[1]. Based on their data, the authors by extrapolation estimated that 2.1 billion individuals were overweight or obese by 2013. Similar results were recently reported in a study of 195 countries[2]. In more than 70 countries, the prevalence of obesity has doubled since 1980[2].

Obesity-related medical conditions (see Table 2) include insulin-resistant diabetes mellitus, obstructive sleep apnea, mechanical or degenerative osteoarthritis, non-alcohol steatohepatitis, hypertension, dyslipidemia with secondary coronary artery disease, pseudotumor cerebri, and likely gastroesophageal reflux, deep vein thrombosis, and asthma[3]. Obesity has also been consistently associated with increased risk of kidney, breast, endometrial, colorectal, pancreatic, esophageal, and gall bladder carcinoma[4]. Excess body weight contributes to a significant percentage of cases of cancer[4]. With the increase in the incidence of obesity in Asia, The Gut and Obesity in Asia Workgroup has developed an experts' consensus on obesity and gastrointestinal diseases in Asia[5]. In a worldwide systematic review, it was reported that obesity accounts for 0.7% to 2.8% of a country's total healthcare expenditures and that individuals with obesity have at least 30% higher healthcare costs[6]. In the United States, the medical care costs of obesity in 2008 were estimated to be $147 billion[7].

As one can ascertain from this rise in the worldwide prevalence of obesity, the previously suggested solutions with regards to this serious medical problem have not been effective. One suggested approach is education, with the inference that individuals will then make responsible choices when eating several times every day[8]. In the United States, the National Institutes of Health and the United States Preventive Services Task Force in 1998 and in 2012 suggested behavioral interventions, increased physical activity, and improved dietary intake[9,10]. Expectations of results from these recommendations however must be balanced against the findings of multiple meta-analyses[11-13] that have examined the potential benefits of behavioral interventions, diet and activity programs in individuals with excess weight. Reported "long term" results are for studies of only 12 to 18 mo. Unfortunately, the published weight loss results are modest with 1.5 to 5 kg of weight loss at 12 mo[11] after adopting behavioral interventions (which can include self-monitoring, setting weight loss goals, addressing barriers to change, and strategizing long-term change in lifestyle). These meta-analyses mention that there is higher weight loss reported for those individuals participating in dietary only programs compared to individuals involved in exercise or activity only programs. Combined dietary and physical activity programs tailored to individual metabolic and dietary response needs appear to be more beneficial than solely dietary programs. However, adding physical activity programs appear to increase overall weight loss by only an additional 0 to 3 kg.

Our review of previous contributions in this field included specific searches on PubMed (www.ncbi.nlm.nih.gov/pubmed) on June 11, 2017. The terms "gastroenterology fellow training" yielded 111 abstracts; the terms "gastroenterology fellow nutrition" yielded 35 abstracts; the terms "gastroenterology fellow obesity" yielded 8 abstracts; the terms "nutrition training gastrointestinal medicine" yielded 251 abstracts; and

Table 1 Obesity defined by body mass index

BMI (kg/m^2)	Definition	Obesity class
18.5-24.9	Normal	
25-29.9	Overweight	
30-34.9	Obesity	I
35-39.9	Obesity	II
40.0-	Obesity	III

BMI: Body mass index.

Table 2 Obesity related medical disorders

Diabetes mellitus
Obstructive sleep apnea
Degenerative osteoarthritis
Non-alcohol steatohepatitis
Hypertension
Dyslipidemia with coronary artery disease
Pseudo-tumor cerebri
Gastroesophageal reflux
Deep vein thrombosis
Asthma
Carcinomas (kidney, breast, endometrial, colorectal, pancreatic, esophageal, gallbladder)

the terms "obesity training gastrointestinal medicine" yielded 97 abstracts. All abstracts and all manuscripts consistent with the topic of this present manuscript were reviewed by one of us (TRK).

ONGOING ROLE OF GASTROINTESTINAL PHYSICIANS IN OBESITY CARE AND TRAINING

Gastrointestinal physicians and general surgeons both deliver gastrointestinal care to individuals with medically-complicated obesity, including the performance of endoscopy. These physicians, after completing traditional training programs, are uniformly trained in or familiar with available bariatric care. This notion is not a new concern. A survey of gastroenterologists published in 1976 revealed that obesity was included as one of the five most common diagnostic areas[14]. The author suggested that his findings could be the basis for planning education, training, and research activities in gastroenterology. An editorial published 26 years later described a commitment of the American Gastroenterological Association to nutrition and obesity, including a summary of elements included in advanced or level 2 training in nutrition[15]. A 2006 study in Canada of gastrointestinal clinicians and gastrointestinal fellows reconfirmed the opinion that training in management of obesity was inadequate[16]. A follow-up survey of Canadian gastrointestinal fellows published in 2009 revealed that gastrointestinal fellows considered their knowledge of nutrition and obesity to be suboptimal[17]. A written examination completed by these gastrointestinal fellows confirmed that their knowledge base indeed was suboptimal, especially in the areas of nutritional support, knowledge of micronutrients and macronutrients, obesity, and nutrition in gastrointestinal diseases. Similar findings have been reported in studies of gastrointestinal fellows in Iran[18] and in the United States[19]. One response to these long-standing concerns and weaknesses in nutrition training has been the publication of a potentially extensive curriculum in nutrition as part of the core curriculum for fellows or trainees in gastroenterology[20].

Training of gastrointestinal fellows internationally has involved programs with a traditional pathway which include disease-related training and endoscopy[21], and programs that combine basic elements of gastrointestinal training with time for advanced training in a subspecialty of gastroenterology or hepatology.

The traditional involvement of gastrointestinal clinicians in obesity has involved two levels of medical care: (1) direct care of gastrointestinal medical disorders related to obesity including non-alcohol steatohepatitis, gastroesophageal reflux, colorectal carcinoma, pancreatic carcinoma, esophageal carcinoma, and gall bladder carcinoma; and (2) consultants in collaboration with providers who treat patients for obesity, for example in the care of individuals who have undergone bariatric surgery. More recently, due to advances in therapeutic endoscopy, gastrointestinal physicians have begun to assume a role as the provider directly treating individuals for obesity. The question to now be considered is what pathways should be utilized to expand the role of gastrointestinal physicians in the care of individuals with medically-complicated obesity.

INCORPORATION OF BARIATRIC MEDICINE INTO ONGOING GASTROINTESTINAL PRACTICE

There are two major approaches for a process by which gastrointestinal physicians can evolve into physicians with primary responsibility for the treatment of individuals with medically-complicated obesity. A recent White Paper from the American Gastroenterology Association proposes incorporation of methods for obesity care and "weight management" into existing gastroenterological practices[22]. This approach immediately raises the question of how much weight loss is required for the appropriate treatment of medically-complicated obesity. An overview of treatment of individuals with non-alcohol steatohepatitis summarizes a proposed goal of loss of 10% of total body weight[23]. This weight loss goal can be difficult to reach in individuals with obesity through the use of dietary and activity programs. In agreement with recently published meta-analyses[11-13], a recent study from Cuba again demonstrated that only 10% of participants involved in dietary and activity

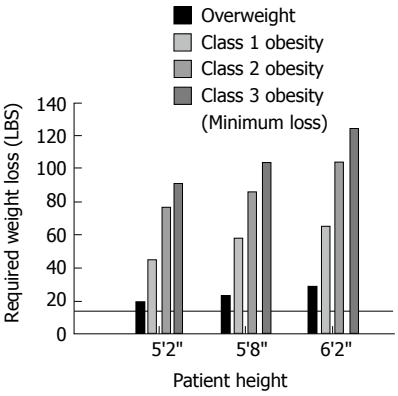


Figure 1 The vertical bars depict weight loss required to reach the normal weight range in overweight and obese individuals of difference heights. The solid line approximates the best published weight loss obtained through diet and activity programs.

Table 3 Weight loss medications approved by the United States Food and Drug Administration

Medication	Published weight loss
Short term use	Range from 3.6 to 8.1 kg
Benzphetamine	
Diethylpropion	
Phendimetrazine	
Phentermine	
Long term use	
Orlistat	2.6 kg
Liraglutide	4 to 6 kg
Lorcaserin	3.2 kg
Naltrexone and bupropion	5 kg
Phentermine and topiramate	8.8 kg

programs were able to lose 10% or more of their total body weight[24]. Again in agreement with ongoing experience[23], liver biopsies revealed that 90% of the participants who lost 10% or more of their total body weight had resolution of non-alcohol steatohepatitis. This study did not address the follow up concern which of course is long-term maintenance of weight loss, or an individual's weight regain.

When considering treatment of individuals with medically-complicated obesity, Figure 1 summarizes weight loss (in pounds) required to reach the normal weight range in overweight individuals and in obese individuals of difference heights. The solid line approximates the best published weight loss obtained through dietary and activity programs. Figure 1 provides a graphic depiction of the extent of weight loss required to improve serious medical disorders in individuals with Type Ⅱ obesity and Type Ⅲ obesity. For this reason, we have previously suggested that bariatric surgery utilizing vertical sleeve gastrectomy will likely become a major consideration for the treatment of nonalcoholic steatohepatitis in those individuals with Type Ⅱ obesity and Type Ⅲ obesity[25].

As step-up therapy for the treatment of medically-complicated obesity, the United States Food and Drug Administration has approved nine medical therapies for weight loss (see Table 3)[26-29]. There are four sympathomimetic drugs, which increase adrenergic stimulus, that have been approved for short term (12 wk) of medical therapy. This group includes Benzphetamine, Diethylpropion, Phendimetrazine, and Phentermine. There are five medical therapies approved for long-term medical therapy, which includes Orlistat, Liraglutide, Lorcaserin, Naltrexone with Bupropion, and Phentermine with Topiramate. As shown in Table 3, the published weight loss results reported for these medications are modest, ranging from 2.6 to 8.8 kg. Prescribing providers should be familiar with the significant risks involved in using these medications. For example, Orlistat can lead to deficiencies of fat soluble vitamin A, vitamin D, vitamin E, and vitamin K. Lorcaserin should not be used with monoamine oxidase inhibitors or selective serotonin reuptake inhibitors. Topiramate is associated with oral clefts when taken by pregnant women.

Table 4 Potential endoscopic methods to treat medically-complicated obesity

Method	Potential physiological mechanisms
Delivery of a Weight Loss Device	
Intragastric Balloon	Stomach distension; reduce volume required for satiety; delay gastric emptying
Orbera[1]	
ReShape[1]	
Obalon[1]	
Duodeno-jejunal bypass sleeve	Delay Gastric Emptying; Induce Malabsorption
Aspiration therapy	Reduce Intragastric Nutrients
Use of intraluminal suturing	
Endoscopic sleeve gastroplasty	Induce early satiation; delay gastric emptying
Transoral gastroplasty	Reduce volume required for satiety

[1]Listed in order of dates the devices were approved by United States Food and Drug Administration.

The next available step-up therapies for treatment of medically-complicated obesity involve the utilization of newer endoscopic techniques that are now available. These techniques can be divided into either the delivery of a weight loss device or the use of intralumenal suturing to promote weight loss[30] (see Table 4). Individualized care with scheduled post-procedural follow up is important for long-term optimization of an endoscopic weight loss program, as has been supported by studies of adjustable gastric banding[31]. Individuals might also benefit from transition to the use of a well selected long-term weight loss medication.

Upper endoscopy has been utilized for placement of an intragastric balloon, a duodeno-jejunal bypass sleeve, or an aspiration tube. Other than The AspireAssist described below, all of these devices are meant to be removed at 6 to 12 mo after their placement. The United States Food and Drug Administration approved the

first intragastric balloon, the Garren-Edwards bubble in 1985. Interest in intragastric balloon has grown due to the United States Food and Drug Administration' s approval of 3 separate intragastric balloon systems in the past 2 years: the Orbera balloon, the ReShape balloon, and the Obalon balloon. All three balloon systems must be removed after 6 mo. Clinical research has examined factors important for weight loss with the use of an intragastric balloon[32]. The mechanism for weight loss from the fluid filled intra-gastric balloon, *e.g.,* delayed gastric emptying, has also been identified[33]. A duodenojejunal bypass sleeve, EndoBarrier, (GI Dynamics, Inc., Lexington, MA, United States) is available in some countries. This bypass sleeve is a fluoroscopically assisted and endoscopically implanted, removable, and impermeable fluoropolymer sleeve with a nitinol anchor. At upper endoscopy, the 60 cm long device is deployed from the duodenal bulb and into the jejunum under fluoroscopic guidance. In 2016, the United States Food and Drug Administration approved The AspireAssist produced by Aspire Bariatrics, King of Prussia, PA, United States which provides a long term option for patients in the higher body mass index range. At upper endoscopy, a specialized aspiration tube is placed percutaneously into the stomach. This aspiration tube has both an intragastric portion with holes to permit aspiration as well as a skin port. Individuals with an aspiration tube are instructed to aspirate their gastric contents 20 min after a meal containing more than 200 kcal. The device is not meant to be placed in an individual with an eating disorder.

The endoscopic sleeve gastroplasty (ESG), first reported from the Mayo Clinic, Rochester, MN, United States in 2013, has eclipsed all prior failed efforts at altering the gastric lumen endoscopically[34,35]. There have been over 2500 ESG procedures performed worldwide with patient follow-up now exceeding 2 years, and a recent multicenter study using this technique to produce an endoscopic sleeve gastroplasty reported 24-mo weight loss data[36]. Publications with individual and multicenter experiences are appearing and demonstrate not only sustained weight loss but improvement in co-morbidities. There is also published experience discussing mechanisms by which ESG is important a patients' weight loss[37]. Others[38,39] are attempting to duplicate successful weight loss using technologies differing from the existing endoscopic suturing device (OverStitch, Apollo Endosurgery, Austin, TX, United States) presently used for the ESG.

The advantages of development of an endoscopic procedure to mimic the popular and effective vertical sleeve gastrectomy are clear, *e.g.* reduced risk of anesthesia and reduced risk of a gastric leak or perforation. The importance of obtaining long term weight loss data to support durability is supported by results from the surgical literature. In the original report from the Mayo Clinic, Rochester, MN, United States of the surgical non-banded vertical gastroplasty, at 4 years of follow up, only 31% of patients were judged to have persistent excess weight lost[40]. In additional studies, risk factors important for treatment failures after vertical gastroplasty were examined[41]. Even with the availability of this information, a study (published 16 years later) involving seven years of follow up after vertical banded gastroplasty reported staple line disruption in 54% of patients[42]. Sixty percent of patients after vertical banded gastroplasty underwent a major re-operation during this seven year follow up period.

After practicing gastrointestinal physicians incorporate these newer endoscopic techniques into their care of individuals with obesity, a major question will arise as to whether reliable follow up will be obtained. In examination of other endoscopic quality measures, the recommendation that endoscopists should track their adenoma detection rates from screening colonoscopy has been supported by results of interval cancers published in 2010[43]. However, in a 2015 survey of training programs in the United States, 63.8% of programs did not assess adenoma detection rates in their trainees[44]. It is similarly likely that it will be difficult to obtain longitudinal results designed to examine the effectiveness of an ad hoc approach for incorporation into an established gastrointestinal practice of new endoscopic methods for treating individuals with medically-complicated obesity.

COMPLICATIONS DURING BARIATRIC CARE

It is expected[45] that physicians providing bariatric care will properly evaluate, diagnose, and treat individuals who develop complications from their weight loss therapy.

Prevention of complications from weight loss therapy begins by screening patients for their suitability to undergo treatment of medically-complicated obesity. Gastrointestinal physicians will need to decide how they will integrate physiologic (*e.g,* low satiety or rapid gastric emptying), psychological, or psychiatric assessment of eating disorders and underlying psychiatric illness into their practice. For example, it is reported that the prevalence of obesity is higher individuals with schizophrenia[46,47].

Following bariatric surgery, a Danish study has described increased risks of self-harm, psychiatric service use, and the occurrence of mental disorders[48]. With regards to the potential for addiction, persistent opioid abuse can result from even minor surgical procedures[49]. A systematic review of postoperative bariatric patients suggested the presence of increased

alcohol use and increased admissions to substance abuse treatment facilities[50]. Physicians involved in primary weight loss therapy will need a program to screen for these potential problems and regimens for appropriate and sufficient treatment.

SUBSPECIALTY TRAINING OF GASTROINTESTINAL FELLOWS IN BARIATRICS

A second, long-term approach for incorporation of bariatric care into gastrointestinal practice would involve adaptation of the training of gastrointestinal fellows for treatment of and long-term control of medically-complicated obesity. Such an approach is supported by studies demonstrating improved bariatric outcomes in hospitals with accredited bariatric fellowships[51]. The evolution of a field of medicine requires individuals who focus on the evaluation of alteration of proposed methods and treatments. This potential approach should be facilitated by the gastrointestinal trainees' prior internal medicine residency training in subspecialties that provide care for individuals with medical complications of obesity, including endocrinology, cardiology, nephrology, and neurology. It is expected that gastrointestinal fellows trained in advanced endoscopy should be capable of learning the proper application of most endoscopic techniques for weight loss therapy in individuals with medically-complicated obesity. Gastrointestinal fellows will also need training to manage post-operative complications (*e.g.,* leaks, fistulas, and endolumenal revision of the Roux en Y gastric bypass and the sleeve gastrectomy) in patients who have undergone bariatric surgery.

The training time commitment would depend upon the specific bariatric care that the future gastrointestinal physician would plan to deliver. The issue of development of a curriculum incorporating one year of subspecialty training in bariatrics to the present training of gastrointestinal fellows needs to be revisited. A one year period of subspecialty training in bariatrics could incorporate additional rotations with collaborating providers in bariatric medicine, bariatric surgery, nutrition, and psychiatry. The trainees could then be instructed in postoperative care of patients who have undergone bariatric surgery. Trainees could be trained in the management of adjustable gastric bands, postoperative nutritional disorders[52], postoperative symptoms and, as mentioned above, complications after Roux-en-Y gastric bypass surgery[3], and the management of major complications of vertical sleeve gastrectomy including gastric perforation or leak[53] and vomiting/dysphagia[54].

As an advantage of this potential organization, since such training would be provided in accredited programs, longitudinal studies could be developed to examine the potential impact on accepted measures of care, such as complication rates, outcomes, and costs, in individuals with medically-complicated obesity.

CONCLUSION

The prevalence of obesity continues to rise worldwide. This rise increases the incidence of obesity-related diseases, and these obesity-related diseases are an important component of health care costs in countries across the world. Diet and activity programs are largely inadequate for the long-term treatment of medically-complicated obesity. Gastrointestinal physicians to date have not been extensively involved in weight loss programs in individuals with medically-complicated obesity. It is certain that gastrointestinal physicians will incorporate new methods into their endoscopic practice for treatment of individuals with medically-complicated obesity. This change will involve incorporation of new endoscopic techniques into present gastrointestinal practices. Improved bariatric care will likely require an organized curriculum in gastrointestinal training programs. Involvement in weight loss programs induces significant psycho-social disorders. Obtaining optimal results will require practice patterns that screen for recognized pre-disposing gastric physiological disorders and psycho-social disorders, and develop ongoing protocols for the management of complications of bariatric care. Optimizing strategies for the long-term maintenance of weight loss after the use of newer endoscopic techniques remain important ongoing issues.

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EDITORIAL

Resistance to clarithromycin and gastroenterologist's persistence roles in nomination for *Helicobacter pylori* as high priority pathogen by World Health Organization

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Author contributions: Amin TBA collected the primary data and designed the first draft and finally wrote the manuscript.

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Abstract

Due to the increasing prevalence of clarithromycin resistance, future of management of *Helicobacter pylori* (*H. pylori*) infections need to be recognized. To now, clarithromycin was the best effective, well-tolerated and safe antibiotic used in treatment of the bacterium, but, increasing trend of resistance reduced efficacy of recommended regimens. Indeed, gastroenterologists are mostly unable to start appropriate therapy-according to the sensitivity profile-due to the certain difficulties in routine *H. pylori* culture procedure and being time consuming method. This announcement by World Health Organization (WHO) was an onset to reconsider current challenging dilemma about *H. pylori* clarithromycin resistant isolates. Therefore, investigating of various factors affecting this nomination by WHO is highly welcomed. In fact, WHO enumerated more than 16 pathogens which seriously threats human life and public health, thus better management or effective guidelines are necessary. Here for the first time, we nominated this phenomenon as "gastroenterologist's persistence" which should be equally investigated as antibiotic resistance. The ability of gastroenterologists to win the game against *H. pylori* infections is highly influenced by their collaboration with diagnostic laboratories to apply susceptibility patterns before any prescription. In conclusion, closer collaboration between two important partners (gastroenterologists and microbiologists) in management of *H. pylori* infection may hopefully trigger an era to remedy current crisis in clarithromycin resistance, a later gastric cancer can be practically preventable.

Key words: Resistance; Clarithromycin; *Helicobacter pylori*; World Health Organization

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Core tip: Due to the increasing prevalence of clarithromycin resistance, future of management of *Helicobacter pylori* (*H. pylori*) infections need to be rewritten. To now, clarithromycin was the best tolerated and safe antibiotic used in treatment of the bacterium, but, increasing trend of resistance reduced efficacy of therapeutic regimens recommended. In this paper, we discussed that why persistence of gastroenterologists is a critical item need to be considered if we really aim to increase efficacy of prescribed antibiotics against *H. pylori*.

Abadi ATB. Resistance to clarithromycin and gastroenterologist's persistence roles in nomination for *Helicobacter pylori* as high priority pathogen by World Health Organization. *World J Gastroenterol* 2017; 23(35): 6379-6384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6379.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6379

INTRODUCTION

Helicobacter pylori (*H. pylori*) is an infectious agent colonizes half of the world population and must be eradicated with suitable antibiotics[1,2]. Of twenty years ago, *H. pylori* was recognized as a major cause of peptic ulcers, thus many of these clinicians attempted to treat those colonized individuals with antibiotic[3]. Later, the World Health Organization (WHO) recommended that bacterial elimination can be a useful strategy to decrease mortality of gastric cancer in the world. Subsequently, in the first half of 2017, WHO listed the clarithromycin resistant-*H. pylori* in the category of high priority which requires intense attention on the treatment. This announcement by WHO is an onset to reconsider current challenging dilemma about *H. pylori* clarithromycin resistant isolates. Therefore, investigating of various factors affecting this nomination by WHO is highly welcomed. In fact, WHO enumerated more than 16 pathogens which seriously threats human life and public health, thus better management or effective guidelines are necessary. New visions of *H. pylori* treatment as well as the better insight of uncertain prescription of antibiotics in the fight against this bacterium is guaranteeing the solution to address the dilemma. Until 2004, *H. pylori* standard triple therapy include proton pump inhibitor (PPI), amoxicillin and clarithromycin providing the acceptable eradication rate, however, increased prevalence of antibiotic resistance hampered continuing the triple therapy for next years. As such, newer formulations were in the focus of researches among the gastroenterologists and microbiologists. As first line treatment for *H. pylori*-positive subjects, bismuth quadruple therapy or concomitant therapy consisting of a PPI, clarithromycin, amoxicillin, and metronidazole are now highly recommended by many international guidelines[4-11]. Therefore, it seems that clarithromycin has an inevitable role in *H. pylori* treatment and even bacterial resistance seems not be able to remove its existence from therapeutic lines. In next section, we review the importance of clarithromycin and how the bacterium became resistant to this commonly used drug in treating the *H. pylori*.

H. PYLORI THERAPY-CLARITHROMYCIN AS KEY ANTIBIOTIC

Due to the traditional microbiologic rule, an infectious agent should be treated with appropriate antibiotics following the susceptibility tests. Despite many problematic infectious bacteria, the story is different for *H. pylori*. Notably, the success chance of regimen include clarithromycin, if a resistant bacterium exists, is less than 40%. This disappointing rate can apparently reflect the irrefutable role of clarithromycin as a core antibiotic in *H. pylori* therapy. The problem with this critical role is that antimicrobial resistance to this drug is sharply increasing and our hopes to have successful eradication regimens (*i.e.,* consistent treatment success > 90%) including the clarithromycin is unfortunately falling.

CLARITHROMYCIN MECHANISM OF ACTION AND RESISTANCE

Clarithromycin is a bacteriostatic antibiotic mostly used in childhood to treat upper respiratory infections, but, its application to treat *H. pylori* is the most used indication[12,13]. The main action mode of clarithromycin as one of the wide-spectrum antibiotics used in *H. pylori* therapy is to prevent protein translation. Current knowledge about mechanisms of resistance to clarithromycin has shown the importance of several point mutations in the peptidyltransferase region encoded in domain V of 23s rRNA[14-16]. In 1996, versalovic *et al*[14] has shown that responsible mutations in the peptidyltransferase region cause an inhibition in binding space between the ribosomal subunit dedicated to specific antibiotic-related protein synthesis and clarithromycin.

Following the first exposure to the clarithromycin, spontaneously mutations (in both 23S rRNA operons) confer *H. pylori* resistance phenotype and genotype. The direct impact of these mutations is emergence of *H. pylori* strains resistant to clarithromycin. To now, two major mutations A2142G and A2143G were listed as main cause of antibiotic resistance in clinical isolates[17]. Indeed, the problem is started since the first exposure is not occurred against *H. pylori*. Clarithromycin is usually used to treat upper respiratory infections, therefore, colonized *H. pylori* is exposed in childhood

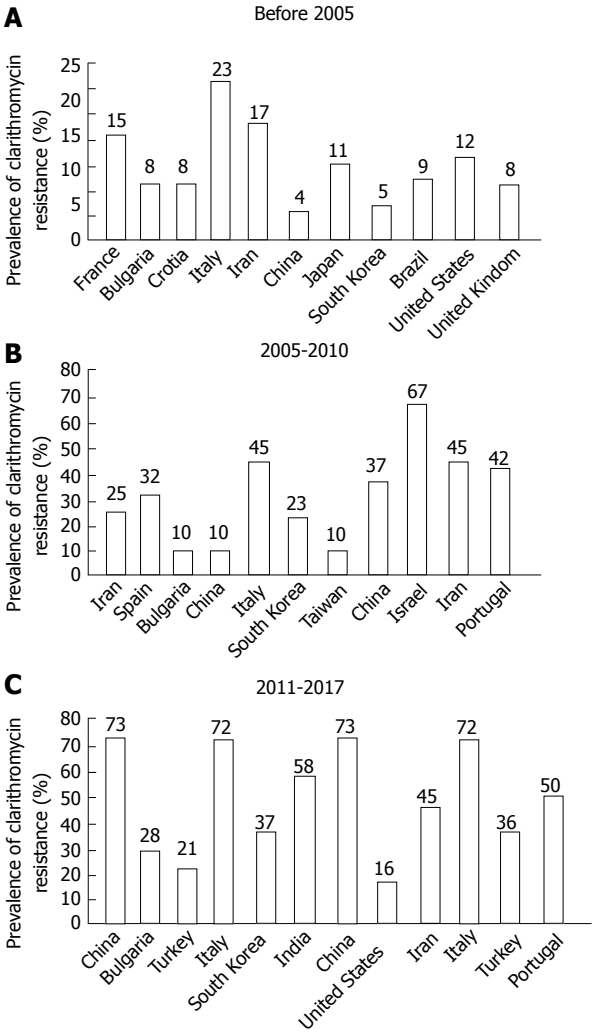


Figure 1 Resistance to clarithromycin among the *Helicobacter pylori* isolates in various countries. A: Before 2005; B: 2005-2010; C: 2011-2017.

already, while no gastroduodenal disorders happened! Another problematic side of this scenario is that antibiotic stress can easily increase the frequency of inducing mutations in cells. This fact in light of relevant usage of clarithromycin for upper respiratory infections in childhood can excuse why we need to be worried about wide-spread prescription of clarithromycin in clinical practice. In brief, our bacteria are almost resistant before we start to use clarithromycin against them in anti-*H. pylori* therapy. In other hands, efflux pump system is also a potential mechanism that confers clarithromycin resistance[18]. Current findings are insisting on at least four families of gene clusters of efflux pumps in *H. pylori* isolates[18,19]. Of them, the family that is found only in Gram-negative bacteria, is the resistance-nodulation-cell division (RND). Bina *et al*[20] was first reported this family of efflux pumps, but additional data are missing to prove exact role of these structures to induce antibiotic resistance in *H. pylori*. The present data show that existence of efflux pumps in *H. pylori* strains can have synergic effect to induce antibiotic resistance in parallel with 23S rRNA mutations. The different angle of the problem is that while we still publishing the papers indicating on increased the emergence of clarithromycin resistance, our holistic view on the rationale for skyrocketing increase in resistance to clarithromycin is missing. Because of antibiotic resistance is a dynamic phenomenon, it may be found with outstandingly different rates even in a state of a country. In reality, the spread of clarithromycin resistant-*H. pylori* has another dimension which remains often undiscussed. Given a sharp increase in clarithromycin resistance during the last decade worldwide (P value < 0.011), *H. pylori* become difficult to eradicate even using various treatment guidelines (Figure 1). Within these three 5 years duration, we can understand the clarithromycin is getting more and more useless antibiotic against *H. pylori* in many countries.

CLARITHROMYCIN RESISTANCE: MOLECULAR DETECTION METHODS

Indeed, in absence of reliable culture-based method to produce data on susceptibility tests, molecular methods were developed rapidly in last decade[17]. Based on available molecular methods tracking 23S rRNA gene, a long list of methods have been developed to quickly identify resistance in colonized *H. pylori* strains to the clarithromycin (Table 1).

Apart from progress have been made, still we are lacking in a quick method with accurate findings. Taking together, real-time PCR can be proposed as best option to be used in hospitals and even smaller centers. During the last years, many companies started to produce this machine and now it is way cheaper than 10 years ago (almost 10 folds). Therefore, we are suggesting to apply this machine to see what kind of mutations are existed 23S rRNA gene and forthcoming susceptibility profile will be easier to present.

NEW DIMENSION OF PROBLEM: GASTROENTEROLOGIST'S PERSISTENCE

Face to face with the problem, we definitely need gastroenterologists more actively involved. So far, due to the clinical and official settings, many of microbiologists do not have any access to the patients. The data for antibiotic susceptibility tests mainly come from laboratories which are dominated by microbiologists. Thus, as a historic conflict between clinicians and basic medical scientists, lack of connection can result in blind antibiotic prescription by the gastroenterologists. Another evidence for this conflict is that almost none of famous microbiologists are co-authored in recently published guidelines on treatment or diagnosis of the *H. pylori.* Moreover, the story is exacerbated when the microbiology journals have no scope for publishing such guidelines. This existed, but, unwanted gap deserves an intense attention to improve management of patients carrying *H.*

Table 1 Molecular methods to identify mutations induce clarithromycin resistance in clinical *Helicobacter pylori* isolates

Methods	Advantages	Disadvantages	PCR-based method	Ref.
Real-time PCR	Quick and reliable High specificity, high sensitivity	Relatively expensive	Yes	[21]
PCR-LiPA	Fast and cheap	Moderate specificity and sensitivity	Yes	[22]
DNA sequencing	Produce many information,	Expensive, Time-consuming protocol	Yes	[23]
3′-mismatch PCR	Fast and high specificity	Produce limited data on the gene, not practically useful	Yes	[24]
RFLP	High specificity, high sensitivity	Risk of contamination Low reproducibility	Yes	[15,25]
FISH	High specificity, high sensitivity	Need invasive approach so not good for children	No	[26,27]

FISH: Fluorescence in situ hybridisation; RFLP: Restriction fragment length polymorphism; PCR-LiPA: PCR line probe assay.

pylori with resistance to clarithromycin. As we described above, in infectious diseases, treatment is highly recommended based on susceptibility pattern, a fact does not follow in the case of *H. pylori*. In other words, treatment of *H. pylori* in many of clinics are based on personal experiences by the gastroenterologists than antibiotic susceptibility tests determined in a microbiology laboratory. With this regard, an incoherent connection between those two populations is a major cause of inappropriate drug prescription ending in increased antibiotic resistance worldwide. Therefore, WHO announced that we are facing with the new generation of resistant *H. pylori* isolates to clarithromycin which endangers effectiveness of current chemotherapy against the bacterium. By continuing the current situation, the replacement of *H. pylori* by WHO in a critical level of priority in a ranking of threatening pathogen is not far from the mind. The next uninvestigated part of the problem is a natural persistence of clinicians on the application of susceptibility patterns reported by microbiologists! For example, metronidazole resistance is not under 60% in many of regions worldwide[28-31], but any trials can show intrinsic tendency among the clinicians who aim to use this ineffective drug for eradicating the *H. pylori*. Taking together, our knowledge about antibiotic resistance has evolved nicely during the relatively short time we treating the *H. pylori*, however, we need new insight on how to increase the reliability among the clinicians to use susceptibility pattern to efficiently eliminate the bacterium especially in patients at least with severe gastroduodenal disorders. Here for the first time, we called this phenomenon as "gastroenterologist's persistence" which should be equally investigated as antibiotic resistance. The ability of gastroenterologists to win the game against *H. pylori* infections is highly being influenced by their collaboration with diagnostic laboratories to apply susceptibility patterns before any prescription. Therefore, two major targets should be considered, (1) antibiotic resistance; and (2) clinician's persistence. Invasive nature of gastroscopy is a considerable factor that influence on rare access to culture-based information on susceptibility tests. Defeating most of the recommended guidelines to eliminate the *H. pylori* can be a major output of neglecting the clinician's persistence.

CONCLUSION

Nowadays, therapeutic failures in anti-*H. pylori* regimens has emerged as major concern for both gastroenterologists and microbiologists. The recent announcement by WHO should be considered as an alarming sound that indicates on emergence of clarithromycin-resistant strains causing global *H. pylori* treatment failure. Since clarithromycin has a critical role in the success of therapeutic regimens against the *H. pylori*, the spread of its resistant strains can easily hamper our attempts to eradicate this microbe. Conclusively, it is not the exaggeration to consider this antibiotic as the main drug in the treatment of *H. pylori*. We need to repeat that all records insisting on using the clarithromycin before recommending as anti-*H. pylori* agent should be considered as serious alarming item. Although the ideal and effective therapy to eliminate the *H. pylori* is not available, but optimizing the current formulations especially, cautious application of clarithromycin in regions with resistance rate > 20% is a likely successful approach. New research direction to produce and suggest novel antibiotics are welcomed to compensate the lacking in antimicrobial agents listed as therapeutic options. In future, application of MALDI-TOF as a novel method can be used directly to detect mutations from biopsy sample; a suggestion which is not far away. To my knowledge, this is one the first paper that pinpoints the gap between gastroenterologists and microbiologists following nomination for *H. pylori* as high priority pathogen by WHO. To be honest, although the persistence of gastroenterologists is a critical factor to increase antibiotic resistance among *H. pylori* strains, but, difficulties in bacterial culture, time consuming procedure of culture and high cost for rapid detection

of resistance are the list which belongs to another side of involving individuals. Finally, closer collaboration between two important partners (gastroenterologists and microbiologists) in management of *H. pylori* infection may hopefully trigger an era to remedy current crisis in clarithromycin resistance, a later gastric cancer can be practically preventable.

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REVIEW

Time to clinical response and remission for therapeutics in inflammatory bowel diseases: What should the clinician expect, what should patients be told?

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Abstract

An awareness of the expected time for therapies to induce symptomatic improvement and remission is necessary for determining the timing of follow-up, disease (re)assessment, and the duration to persist with therapies, yet this is seldom reported as an outcome in clinical trials. In this review, we explore the time to clinical response and remission of current therapies for inflammatory bowel disease (IBD) as well as medication, patient and disease related factors that may influence the time to clinical response. It appears that the time to therapeutic response varies depending on the indication for therapy (Crohn's disease or ulcerative colitis). Agents with the most rapid time to clinical response included corticosteroids, calcineurin inhibitors, exclusive enteral nutrition, aminosalicylates and anti-tumor necrosis factor therapy which will work in most patients within the first 2 mo. Vedolizumab,

methotrexate and thiopurines had a longer time to clinical response and can take several months to achieve maximal efficacy. Factors affecting the time to clinical response of therapies included use of concomitant therapy, disease duration, smoking status, disease phenotype and advanced age. There appears to be marked variation in time to clinical response for therapies used in IBD which is further influenced by disease and patient related factors. Understanding the expected time to therapeutic response is integral to inform further decision making, maintain a patient-centered approach and ensure treatment is given an appropriate timeframe to achieve maximal benefit prior to cessation.

Key words: Crohn's disease; Clinical pharmacology; Ulcerative colitis; Thiopurines; Inflammatory bowel disease; Biologics; Nutrition

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Core tip: There appears to be marked variation in time to clinical response for therapies used in inflammatory bowel disease which is further influenced by disease and patient related factors. The most rapid response can be expected with corticosteroids, calcineurin inhibitors, exclusive enteral nutrition, aminosalicylates and anti-tumor necrosis factor therapy (within 2 mo), while methotrexate, thiopurines and vedolizumab can take several months to achieve maximal response. There is a lack of reporting of the time to response of therapies in clinical trials for inflammatory bowel disease and this remains an area that should be addressed in future studies.

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INTRODUCTION

The therapeutic armamentarium for inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), continues to expand, providing valuable additional opportunities to achieve optimal long term outcomes for patients. Equally, however, there is added complexity, commensurate with the number of options, an enhanced understanding of the risks and benefits, plus the differential effects of treatments on objective disease outcomes (*e.g.*, mucosal healing), clinical remission and/or patient-reported outcomes.

Yet when better outcomes are potentially achievable, there are higher expectations. It is increasingly important with current therapeutics that the physician plans ahead, given delays in escalating treatment are likely just as common and detrimental as delays in diagnosis are in IBD[1]. Hence an awareness of the approximate time expected to achieve a treatment goal is fundamental to making decisions such as whether to persist with a therapy or switch to an alternative. Equally, one does their patient a disservice by prematurely switching therapies before an agent is given an appropriate length of time to achieve efficacy.

Achieving an optimal time to therapeutic response has further benefits in the doctor-patient relationship, as it allows the clinician to provide the patient a cogent framework of the expected period to see response to a new drug and hopefully empower the patient to persevere with, and maintain adherence to therapy. This is particularly relevant for therapies that have a longer time to therapeutic response, such as the immunomodulators, where it might take several months to reach maximal therapeutic efficacy without the patient necessarily experiencing any symptom benefit for a significant part of this.

Hence, time-to-therapeutic response is an important yet underestimated factor in the day-to-day management of IBD and has not been a major focus of attention in the literature to date. This review attempts to address this unmet need by analyzing the available literature relating to the expected time-to-clinical-response for currently available therapies in IBD and measures that can assist the clinician in determining whether a medication has reached its therapeutic potential. We will also analyze disease and patient related factors that may impact on the time-to-clinical-response of therapies. Therapies discussed in the review will include corticosteroids, aminosalicylates (5-ASA), thiopurines, methotrexate, anti-tumor necrosis factor (anti-TNF) therapies, vedolizumab, calcineurin inhibitors and exclusive enteral nutrition.

DEFINING TIME TO CLINICAL RESPONSE AND REMISSION

The concept of time-to-clinical-response is schematically represented in Figure 1. Given the lack of focus on time to response in previous literature, there is no broadly accepted definition. Table 1 provides a summary of some of the important components of time to response. These include the earliest time at which a patient can expect a response, the time at which most patients (*i.e.*, greater than 50%) expecting to benefit from therapy will achieve a response and the time point where therapeutic benefit remains improbable, the so-called time to futility. For this review, time-to-clinical-response refers to the time from the initiation of therapy until the patient achieves a clinical response. It only pertains to patients who attain a clinical response and can thereby aid the

Table 1 Expected time to clinical response for therapeutic agents used in the management of inflammatory bowel disease

Agent	Earliest published clinical response	Earliest published objective response	Time to response in most patients[1]	Time to futility	Use of therapeutic drug monitoring	Comments
Mesalazine (oral)	1 wk[21]	3 wk[148]	4 wk	> 12 wk	N	A higher dose may lead to a more rapid response
Prednisolone (oral)	2 wk[28]	2 wk[28]	3 to 7 wk	8 wk	N	May take longer for CD
Corticosteroids (IV)	3 d[31]	1 wk[127]	3-5 d	7-10 d	N	
Infliximab (IV)	1 wk[48]	8 wk[149]	2-8 wk	> 6 mo	Y	
Adalimumab (SC)	4 wk[59]	8 wk[59]	4-8 wk	> 6 mo	Y	Response time better with 160/80 mg *vs* 40/40 mg induction dosing
Certolizumab (SC)	2wk[61]	10 wk[64]	10 wk	> 16 wk	N	
Golimumab (SC)	6 wk[65]	6 wk[65]	6 wk	> 14 wk	Y	
Certolizumab (SC)	2 wk[61]	10 wk[64]	10 wk	> 16 wk	N	
Vedolizumab (IV)	6 wk[78]	6 wk[78]	19 wk	12 mo	N[2]	Response time may be better for UC *vs* CD
Thiopurines (oral)	2 wk[80]	3 mo[88]	10 to 12 wk	> 6-9 mo	Y	Endoscopic response may take much longer than clinical response
Methotrexate (oral or SC)	9 wk[111]	12 wk[110]	9 wk	> 6 mo	N	Response time and efficacy may be better in 1) CD *vs* UC, 2) SC *vs* oral
Cyclosporin (IV then oral)	1 wk[127]	1 wk[127]	4 to 5 d	> 14 d	Y	
Tacrolimus (oral)	2 wk[122]	2 wk[122]	2 wk	4 wk	Y	
EEN (oral)	10 d[131]	4 wk[139]	3 to 4 wk	8 wk	N	

[1]Clinical response reported in at least 50% of patients who achieve a response to therapy; [2]Therapeutic drug monitoring is not yet widely available. EEN: Exclusive enteral nutrition; IV: Intravenous; SC: Subcutaneous; Y: Yes; N: No; CD: Crohn's disease; UC: Ulcerative colitis.

clinician in judging the likelihood of a response being achieved based upon the elapsed time on therapy. Where available, estimates of timeframes in which the majority of patients who ultimately respond to therapy will be expected to respond to therapy will be reported. The time to futility of therapy is reported in Table 1, however, this will not be a primary focus of this review.

The methods of determining when a "response" has occurred are heterogeneous and include both clinical symptoms and endoscopic (or objectively-assessed) findings. The correlation between symptomatic improvement and achievement of endoscopic remission differs between UC and CD, with improvement in symptoms correlating better with mucosal healing in UC than CD[2-5]. There are data to support early clinical remission, albeit not response, to be predictive of endoscopic improvement and healing at 12 mo[6]. The value of symptomatic improvement, however, cannot be discounted from a patient's perspective given the correlation with long-term steroid-free remission and the inherent part that alleviation of symptoms plays in improving quality of life[5,7]. Moreover, the complex interplay of patient symptoms and structural damage in IBD is being increasingly recognized with both symptoms and endoscopic findings important factors in determining overall disease severity and burden[8].

Thus, response is a multi-faceted concept and this review will primarily address the time-to-clinical-response and time-to-clinical-remission, given that the focus here is to engender a patient-centered approach when clinicians discuss therapeutic options with their patients. Time to endoscopic response and remission will also be reported where data are available, although this is a secondary focus.

Given the heterogeneity across studies in defining clinical response, clinical remission and endoscopic remission, we have used broad outcome measures of "clinical or endoscopic improvement" or "clinical or endoscopic remission or mucosal healing" as defined by the authors of each study. For the purpose of this review, clinical response will consider symptom improvement only rather than an improvement in symptoms and laboratory indices.

A summary of the relative time-to-therapeutic-response of different therapies for IBD is presented in Table 1 and Figure 1. Medication related factors that influence the time to response of different therapies are discussed within each of the therapeutic classes.

AMINOSALICYLATES

Time to clinical response

Aminosalicylates are more effective at inducing response and remission in patients with mild-moderate ulcerative colitis than placebo[9], but their evidence for efficacy in patients with Crohn's disease is poor[10,11]. Therefore, with regards to ulcerative colitis, available data indicate that it generally takes two to four weeks to achieve clinical response with oral and/or topical aminosalicylates. Mesalazine induces endoscopic remission in 67% of patients at 4 wk for active colitis for both 2 and 4 g preparations, while another study found higher endoscopic remission rates of 78% and 69% after 8 wk with multimatrix mesalazine 4.8 g and 2.4 g, respectively, suggesting some patients who will achieve endoscopic remission may take up to 8 wk[12,13].

Therapy-related factors affecting time-to-response

Key issues for aminosalicylates include whether the formulation, the dose and/or the route(s) of delivery

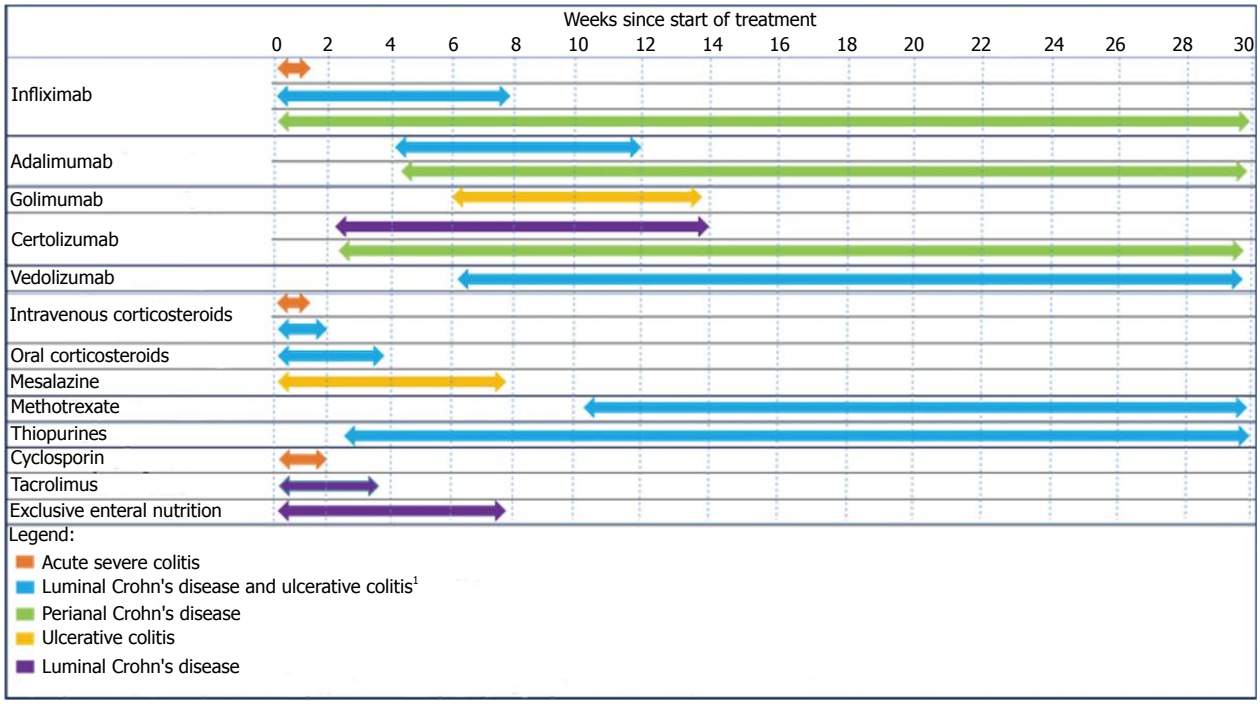


Figure 1 Schematic representation of the range of expected time of clinical response for therapies based on indication. [1]This symbol is used to represent the expected time to response for therapies that can be used in either condition.

influence the speed of onset of action.

Formulation: Trials of sulfasalazine, olsalazine and balsalazide in mild to moderate UC demonstrated an improvement in clinical symptoms and endoscopic response with 2 to 3 wk of therapy in most patients[14-17]. In contrast according to published data, coated mesalazine preparations demonstrated a somewhat slower clinical and endoscopic improvement within 4 to 8 wk[18,19]. Two head-to-head studies suggested that an equimolar dose of balsalazide resulted in a more rapid clinical and endoscopic response than delayed-release mesalazine (using Eudragit S-coated tablets) therapy for patients with left-sided disease[17,20].

Dose: Time to therapeutic response for aminosalicylates may also be dose-dependent as demonstrated by Orchard *et al*[21] who found that 4.8 g daily of mesalazine (delayed release, Asacol MR®) improved and resolved symptoms more rapidly than 2.4 g daily (median 7 d *vs* 9 d, 19 d *vs* 29 d respectively). Another study found a numerically faster time-to-clinical-response with mesalazine 4.5 g than 3 g or 1.5 g daily[22]. Kamm *et al*[13] described numerically higher endoscopic remission rates of 78% after 8 wk with mesalazine MMX 4.8 g *vs* 2.4 g daily (69%), but whether this equates to faster response in those who achieve remission was not reported[12,13].

Route of delivery: Combined oral and topical mesalazine was associated with more rapid resolution of rectal bleeding (mean 11.9 d) than with oral mesalazine only (25.5 d) for left-sided colitis in the only randomized study reporting this end-point[23]. Another randomized trial of sixty patients with distal UC comparing oral mesalazine with mesalazine enemas or combination topical and oral treatment found a median time to resolution of rectal bleeding of 8 d on combination therapy and bleeding were significantly lower after ten days with either topical or combination therapy compared to oral mesalazine only. The findings indicate that topical therapy alone or in combination with oral therapy achieves symptom resolution more rapidly than oral therapy[23,24]. The efficacy of topical 5-ASA does not appear dose-dependent in the single study where this was specifically examined, but rapidity of response was not addressed[25].

CORTICOSTEROIDS

A clinical response to steroids should be expected within 1 to 4 wk of commencing therapy for both CD and UC (not applicable to acute severe colitis) with response occurring more rapidly with intravenous than oral therapy[26,27]. There are several types of corticosteroids available for the treatment of IBD and the influence of route of administration and type of corticosteroid are relevant to determining the time to response, as discussed below.

Time to clinical response

CD: Although most patients with CD can expect a response to high dose oral corticosteroids within 4 wk, some data suggest a more prolonged course

may be necessary to capture response. For example, clinical response after three to seven weeks of 1 mg/kg per day oral prednisolone in a prospective cohort study of 146 patients with active ileocolonic or colonic CD increased from 63% to 92% between weeks 4 and 7 respectively, although only 29% achieved endoscopic remission[2]. These data suggest that a clinician should wait up to 7 wk before deciding that a response to high doses of prednisolone is unlikely if that approach is clinically acceptable.

UC: Response to oral prednisolone is rapid in UC, with 17%-76% achieving clinical remission and 65%-78% endoscopic improvement after 2 wk of oral prednisone in two randomized studies, with the higher response rates noted by Truelove *et al*[28] who used both oral and rectal prednisolone in combination[28,29]. Other studies have also suggested a response within the first two weeks of a tapering dose of oral prednisolone beginning at 40 mg/d in the majority of patients with moderate UC[30].

Therapy related factors affecting time to response

Route of administration: (1) CD: While direct comparisons between intravenous and oral corticosteroids are not available in CD, response appears rapid with intravenous corticosteroids, with 78% of patients having symptom resolution after five days of intravenous hydrocortisone (300 mg daily), which increased to 93% after 10 d in one randomized study comparing intravenous hydrocortisone to corticotrophin, to which response rates were also high (71% and 82% at days 5 and 10, respectively)[27,31]. (2) UC: Moderate UC has been shown to typically improve within five days of intravenous corticosteroids, including patients who failed to respond to high-dose oral prednisolone. Time frames for expected response are well described for acute severe colitis, where most patients appear to respond to therapy within 3 or 5 d of intravenous steroids (methylprednisolone 60 mg/d or hydrocortisone 300-400 mg/d), although these are observational data only[32-35]. A lack of response within 5 d is associated with a higher rate of subsequent colectomies again in observational studies, and therapy beyond 7 d is unlikely to be beneficial[36].

Type of glucocorticoid: (1) CD: Several randomized studies have suggested the mean times to clinical response and remission with budesonide in CD were comparable to systemic corticosteroids, ranging from 22 to 27 d[37-40].

(2) UC: Induction of remission when using budesonide MMX 9 mg daily in mild to moderate UC should occur within 4 to 8 wk of commencing therapy, with 42%-47% of patients achieving an endoscopic or clinical improvement in randomized controlled trials (RCT)[41,42].

Dose: The effect of corticosteroid dose on time to response has not been evaluated. One randomized study assessed response rates with 20 mg, 40 mg and 60 mg daily of oral prednisolone for outpatient management of ulcerative colitis and suggested a higher response rate at both 2 wk and 3-5 wk of follow-up with 40 and 60 mg/d of therapy (both 50% at 2 wk, then 65% at 3-5 wk respectively) compared to 20 mg daily (20% then 30%), but did not specifically assess time to response[30]. Determining the appropriate dose of steroid has traditionally been either empiric or weight-based. Accordingly, corticosteroid dosing evaluated in clinical trials has varied; for instance, studies have used 1 mg/kg/d or 40-60 mg/d of prednisolone, 9 mg of budesonide orally, while for intravenous therapy includes 300-400 mg/d of hydrocortisone (divided doses) or 60 mg/d of methylprednisolone[26,43,44].

TUMOR NECROSIS FACTOR ALPHA INHIBITORS

Pertinent issues relating to time to therapeutic response of anti-TNF therapy include the associations with serum drug levels and antibodies, plus concomitant therapy.

Time to response

Infliximab: (1) CD: Clinical response and remission after administration of infliximab appear to be rapid in luminal CD, taking 8 and 9 d respectively in one observational study of 129 patients[45]. Clinical response rates in RCTs of infliximab in CD were 61% and 81% for weeks 2 and 4 respectively after a single infusion of infliximab[46,47]. Clinical remission rates were reportedly 88% one week after a single infliximab dose for colonic CD although the data were observational only[48]. Rates of mucosal healing in Crohn's disease have ranged from 30%-67% after 6 mo of infliximab, with higher rates typically observed in 'real-world' clinical cohorts than trials[49,50].

(2) UC: Clinical and endoscopic response to infliximab in patients with moderate to severe chronic active ulcerative colitis appears to take several weeks, although this may be due to a lack of reporting of early outcomes after initiation of therapy in the outpatient setting, given that response rates reported for acute severe colitis are generally more rapid than this. Nevertheless, about half of patients previously not responding to either intravenous or oral corticosteroids experienced a clinical response two weeks after the first infusion of infliximab in one prospective uncontrolled study[51]. Such early response rates have not been reported in RCTs, but data from such studies have shown a significantly higher rate of clinical response (69% *vs* 37%), remission (39% *vs* 15%) and mucosal healing (62% *vs* 33%) by week 8 with 5 mg/kg induction dosing versus placebo[52]. For infliximab

use in ulcerative colitis, two large randomized studies of moderately severe ulcerative colitis showed a significantly higher rate of mucosal healing by week 8 with after both 5 mg/kg and 10 mg/kg induction therapy (62% *vs* 33% with placebo)[52].

For acute severe colitis, a clinical response to infliximab therapy should be expected within the first 7 d after therapy[53]. Achieving a higher serum infliximab level during induction has been associated with a higher rate of short term mucosal healing and an accelerated induction regimen of infliximab in acute severe colitis has been associated with a more prolonged time to colectomy than standard induction, although the rapidity of response has not been directly assessed[54,55]. Indeed, recent data suggest that a rebound of higher C-reactive protein, lower albumin and/or symptoms within a few days after the first dose of infliximab should prompt concerns of infliximab non-response and a potentially higher risk of colectomy[55]. A trial is currently underway to assess the utility of an accelerated induction regimen of infliximab in acute severe colitis and this may provide further information on the effect of dose and drug levels on time to response[56].

Adalimumab: (1) CD: An initial response to adalimumab typically occurs within the first few weeks of therapy, as inferred from a phase 2 RCT showing clinical remission rates of 36% at week 4 following induction treatment with 160/80 mg at week 0 and 2 for CD, compared to 12% with placebo[57]. While clinical remission rates were higher from week 1 than placebo in this study (16% *vs* 7% respectively), this only reached statistical significance at week 4. Moreover, the rate of mucosal healing for moderately severe ileocolonic CD with induction 160/80 mg adalimumab followed by 40 mg fortnightly was significantly higher than placebo at 12 wk (27% *vs* 13%) and sustained until 52 wk (24% *vs* 0%) in another RCT[58]. Endoscopic remission rates were 52% and 28% at weeks 12 and 52 respectively in this study, the latter likely reflecting secondary loss of response during maintenance therapy.

(2) UC: Clinical remission and mucosal healing rates with adalimumab induction with 160/80 mg regimen in patients with moderate to severe UC after 8 wk was achieved in 19 and 47% respectively, with separation in clinical remission rates as early as week 4 compared to placebo in a RCT[59]. The lower remission rates in this study may relate to the high proportion (75%) of patients who had failed other therapies prior study enrolment[59]. In another RCT assessing long term remission rates with adalimumab in moderate to severe UC, mucosal healing rates were 41% at week 8 and 25% at week 52 with fortnightly adalimumab 40 mg, compared to 32% and 15% for placebo, respectively[60]. Mucosal healing rates following adalimumab induction for UC have varied between 32% and 47% in RCTs[59,60].

Certolizumab pegol: CD: Certolizumab pegol at a dose of 400 mg given subcutaneously at weeks 0, 2 and 4 wk, about a third of patients with CD will have a clinical response to therapy within 2 wk, increasing to 41% by week 6 based on RCT data[61]. One study found response rates peak at 10 wk of 400mg 4-weekly therapy[62], while another study found response rates, as per a reduction in CDAI of 100, peaked by week 16 and declined thereafter[63]. Endoscopic activity was assessed at week 10 in one prospective, open label clinical trial of patients on 400 mg certolizumab 4-weekly and showed endoscopic remission occurred in 37%, reducing to 27% by week 54 in CD[64].

Golimumab: UC: Golimumab is administered subcutaneously and has been approved for use in ulcerative colitis in many countries. Approximately half of patients will achieve a clinical response by 6 wk with regimens of 100/200 mg and 400/200 mg as induction at weeks 0 and 2 for moderate to severe ulcerative colitis from one large RCT[65]. Observational data suggest that response rates may continue to increase up until week 14, when reported to be between 69% and 86%[66,67]. Mucosal healing appears to be rapid, with 42 and 45% of patients achieving mucosal healing 6 wk after induction therapy with 100/200 mg and 400/200 mg, respectively[65]. Intravenous induction therapy for golimumab does not appear to confer any additional benefit in terms of response rate compared to subcutaneous induction, although time to response of this strategy has not been evaluated[68]. Since there are no data evaluating clinical response rates beyond week 14, the benefits of continuing therapy beyond this time point in patients who have not achieved a response remains uncertain.

Factors affecting time to response

Demographic factors: One study assessed baseline factors that were predictive of a more rapid attainment of clinical remission with induction certolizumab therapy for CD and found that younger age, non-smokers, the absence of previous IBD surgery and a lower disease activity score were associated with a more rapid attainment of clinical remission[69].

Anti-TNF drug levels: Currently, data supporting a correlation between anti-TNF drug levels and time to therapeutic response are limited. For golimumab, drug levels at weeks 2 and 4 were shown to correlate with week 6 clinical response rates, but the effect on time to therapeutic response was not further assessed as response was only evaluated at a single time point in this study[70]. Higher certolizumab plasma concentrations at week 8 are associated with higher rates of clinical remission and endoscopic remission

at week 10, but not a higher clinical response rate[71]. There is a lack of data concerning the relationship between time-to-response for infliximab and adalimumab in relation to drug levels.

Concurrent immunomodulator therapy: While time to response has not been directly compared between anti-TNF monotherapy and in combination with immunomodulator therapy, there was a trend toward a higher rate of clinical remission at week 10 with combination infliximab and azathioprine compared to infliximab alone in the Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease (SONIC) Study, and a significantly higher rate of endoscopic remission at 26 wk with adalimumab and azathioprine, suggesting combination therapy may work faster than either therapy alone[50,72]. Similar findings of more rapid clinical remission have also been found for certolizumab therapy when used with concomitant immunomodulator therapy in CD[69].

VEDOLIZUMAB

Vedolizumab appears to have a generally slower time to response compared to other biologic agents. This may relate to the mechanism of anti-integrin therapy, with inhibition of lymphocyte gut migration taking more time to achieve therapeutic efficacy[73].

Time to response

CD: Clinical remission with vedolizumab appears to take at least 10 to 14 wk in CD. This slower onset of action of vedolizumab, compared to anti-TNF therapies for instance, was evident in the RCTs GEMINI 2 and 3 registration trials of vedolizumab comparing therapy to placebo induction and maintenance therapy in CD[74]. Clinical remission rates increased from 15% to 27% between weeks 6 and 10 while remained stable in the placebo group (12%) at these time points in GEMINI 3 (*i.e.,* those with prior anti-TNF failure), and in GEMINI 2 there was a significant increase in clinical remission after 6 wk compared to placebo[73]. Subsequent real-world observational data have also demonstrated that clinical remission rates tend to increase from week 6 to week 14 and in one study the median time to clinical response was 19 wk[75]. Mucosal healing rates of 30% were attained after a median of 22 wk in CD in one observational study[76]. Furthermore, GEMINI 2 found that almost 40% of initial vedolizumab responders remained in clinical remission to 52 wk although clinical remission rates only became superior to placebo after 30 wk[77].

UC: Response and remission rates appear more rapid in UC than CD. At week 6 after a 2-dose induction, clinical response, remission rates and mucosal healing were significantly higher with vedolizumab than placebo (47%, 17% and 41% *vs* 26%, 5% and 25% respectively) in one RCT[78]. Moreover, maintenance vedolizumab resulted in higher rates of clinical and endoscopic remission at week 52 than week 6 and mean partial Mayo scores continued to decline until week 52, suggesting that maximal response often takes several months.

Therapy-related factors affecting time to response

While immunomodulators appear to reduce the formation of antidrug antibodies to vedolizumab[78], the low proportion of patients who actually formed antidrug antibodies in trials might imply that combination therapy may be unnecessary and hence may not provide additional benefit to time to therapeutic response, in contrast to anti-TNF therapies[78].

THIOPURINES

Thiopurines, including azathioprine and 6-mercaptopurine, appear to exert their effect *via* the metabolites, 6-thioguanine nucleotides (6-TGNs). Thiopurines inhibit the synthesis of DNA, RNA and proteins leading to inhibition of lymphocyte proliferation and apoptosis, thereby immunosuppression[79].

Time to response

CD: Azathioprine or mercaptopurine take at least 4 to 8 wk to achieve clinical remission[80]. A small RCT suggested that a median of 10.7 wk was required to achieve clinical remission with oral azathioprine 2 mg/kg/d[81]. Time to clinical response with 1.5 mg/kg/d of mercaptopurine was assessed in an RCT by Present *et al*[82] in patients with CD failing corticosteroids or sulfasalazine. They demonstrated a wide-ranging time to therapeutic response from two weeks to nine months with a median of 3.1 mo; 19% of cases took more than 4 mo to achieve a sustained clinical response[82]. Similarly, in another RCT, Ardizzone *et al*[83] found a clinical remission rate gain from 33% to 63% from 3 to 6 mo after initiating azathioprine 2 mg/kg/d in patients with corticosteroid-dependent CD. Endoscopic remission rates also appear to be slow to achieve with only 17% of patients on azathioprine in the SONIC study achieving mucosal healing after 26 wk of therapy[50]. Yet another RCT comparing budesonide to azathioprine with 1 year follow up found mucosal healing occurred with thiopurines in 83% of steroid-dependent CD by 12 mo, implying that endoscopic improvement continues to slowly accumulate over extended periods[84].

UC: Both clinical and endoscopic response to thiopurines in UC appear to take a minimum of one month but more typically 3-6 mo for steroid-dependent UC[85,86]. Ardizzone *et al*[87] found that more than half of patients on azathioprine were in steroid-free endoscopic and clinical remission after 6 mo for patients with steroid-dependent UC, which was significantly higher

Table 2 Previously documented and potential/ novel methods of improving time to response to therapy in Crohn's disease and ulcerative colitis

	Clinical scenario	Method	Improves time to response	Improves response rate	Improves tolerability	Published data?	Comments	Ref.
Corticosteroids	CD and UC	Intravenous administration		-	-	Yes		[27,32,150]
Anti-tumour necrosis factor-α	Initial or for flare to recapture response (CD and UC)	Addition of azathioprine			-	Yes		[50]
Thiopurine	CD and UC	Addition of allopurinol	-	-		Yes		[105,108]
		Split dosing of thiopurine	-	-		Yes		[151]
Methotrexate	CD	High dose parenteral with corticosteroids if relapse on lower dose	-		-	Yes	Can recapture response	[152]
Tacrolimus	UC	Target levels of 10-15 ng/mL	-		-	Yes		[123]
Aminosalicylates	UC	Maximize dose			-	Yes		[21]
	Distal UC	Choice of formulation (balsalazide)		-	-	Yes		[17,20]

CD: Crohn's disease; UC: Ulcerative colitis.

than that with mesalazine (53% *vs* 21%) in an RCT. Another RCT showed a significant decrease in a composite disease activity score (including endoscopic, clinical and biochemical findings) at 3 and 6 mo after commencing azathioprine 2.5 mg/kg/d for steroid-dependent UC, implying that time to therapeutic response is likely between 3 and 6 mo[88].

Factors affecting time to response

Metabolite levels: Azathioprine undergoes rapid non-enzymatic conversion to 6-mercaptopurine which is then further metabolized to 6-TGNs in erythrocytes and leukocytes[89]. The therapeutic effect of thiopurines appears commensurate with 6-TGN concentrations, with steady state achieved at two to four weeks, so response can only be expected after this period[90]. Intravenous loading with azathioprine has been associated with a more rapid time to therapeutic response initially (1 wk for clinical response and 4 wk for endoscopic improvement). However, when compared to oral azathioprine at 8 wk, no difference in remission rates or 6-TGN levels had persisted[80,91]. Using dose titration guided by therapeutic drug monitoring, the mean time to therapeutic response decreased from 22 to 19 wk[92]. While prospective studies have not demonstrated a difference in outcomes between patients treated with weight-based and individualized, metabolite-guided dosing of thiopurines[93,94], robust retrospective data elucidated higher rates of clinical remission with 6-TGN levels above 230-260 pmol/8 × 10^8 RBCs[95-97]. Indeed, 78%-90% of patients had improved clinical outcomes from dose optimization after having a sub-therapeutic 6-TGN level[98-100].

Addition of allopurinol: A combination of allopurinol 100 mg and 25%-50% of the standard thiopurine dose has been utilized to overcome a number of thiopurine related side effects and correct an unfavorable metabolite profile (so called hypermethylators), with high clinical efficacy[101-105]. This has piqued interest as to whether combination allopurinol-thiopurine therapy might be able to achieve not only higher rates of, but quicker time to therapeutic response, especially compared to slow-titrating introductory dosing protocols (as advocated by treatment guidelines[26,106] and widely used to mitigate early side effects[107]). The result of controlled trials addressing this issue are awaited[108]. Additional strategies that may affect time to response of therapies are summarized in Table 2.

METHOTREXATE

Methotrexate is a folic acid analogue used in the treatment of multiple autoimmune conditions, including IBD. Methotrexate has been shown effective as an induction and maintenance therapy in CD particularly when administered parenterally. The role of methotrexate in UC is less clear, although a placebo-controlled trial of subcutaneous methotrexate did suggest clinical efficacy in the induction of steroid-free remission[109].

Time to response

CD: A clinical response should be expected within 12 wk on parenterally-administered methotrexate according to an open label, non-randomized trial by Kozarek *et al*[110]. An observational study suggested that the median clinical response time was 9 wk for both oral and parenteral therapy (although 86% were on parenteral therapy in this study) and clinical remission took 22 wk[111]. Despite most patients clinically responding within the first several weeks of therapy, a subgroup may take up to 6 mo to respond[83].

UC: While a placebo-controlled trial of oral methotrexate 12.5 mg in patients with steroid-refractory UC showed no significant difference in clinical remission between the groups, those who reached clinical remission with methotrexate took a mean of 4.1 mo

to do so[112]. Subsequent uncontrolled observations by Kozarek *et al*[110] showed a rapid clinical response (71% within 12 wk) with high-dose intramuscular dosing in UC, albeit in only 7 patients. The METEOR RCT, assessed the efficacy of parenteral methotrexate 25 mg weekly in steroid-dependent UC and found almost a third of patients had corticosteroid-free clinical remission by week 16, which increased to 40% by week 24[109]. Endoscopic remission was achieved in a numerically greater proportion then did the placebo group, but the difference did not reach statistical significance, perhaps due to lack of statistical power.

Factors affecting time to response

Route of administration: It is likely that parenteral methotrexate generally achieves a faster time to therapeutic response in a higher proportion of patients due to greater bioavailability, though supportive evidence is found only in the rheumatological literature[113].

Drug levels: Currently there is no reliable method to apply therapeutic drug monitoring of methotrexate in routine IBD care and this is therefore not useful in predicting time to therapeutic response. Serum methotrexate levels are only detectable for about 24 h post-dose and appear not to correlate with clinical response in IBD[114], adenosine levels do not correlate with efficacy[115], and methotrexate polyglutamates [active metabolite(s) of methotrexate] have displayed inconsistent results[116-118]. Finally, although folic acid is an important adjunct to methotrexate use and may reduce gastrointestinal upset and hepatic dysfunction, it has no apparent impact on time to therapeutic response[119].

CALCINEURIN INHIBITORS

Tacrolimus and cyclosporin are calcineurin inhibitors with evidence in IBD primarily for steroid-refractory UC. Small case series have suggested efficacy of tacrolimus for induction of remission in luminal CD[120]. Cyclosporin is an effective rescue therapy in acute severe colitis and has been used in moderate to severe chronic UC. Oral cyclosporine does not appear to be an effective induction therapy in CD[121].

Time to response

Tacrolimus: (1) UC: When targeting a trough level of 10-15 ng/mL, tacrolimus induces clinical response and mucosal healing rates of 50%-68% and 44%-79% respectively, in patients with UC within 2 wk based on results from two RCTs, with lower rates noted in the larger of these studies[122,123]. In contrast, clinical remission was reported in only 9%-20% after 2 wk in the aforementioned studies, but amongst those who continued tacrolimus, remission rates increased to 29% by week 12, with mucosal healing rates increasing from 67% to 86% over the same time period[122,123]. (2) CD: Small case series suggest a similarly rapid clinical response with tacrolimus in CD refractory to other therapies, occurring within 30-40 d[124,125]. However, time to therapeutic response appears longer than UC, with one study finding that 36% achieved clinical remission by 20 d which increased to 64% at 120 d[125].

Cyclosporin: UC: Clinical response to cyclosporine in acute severe colitis failing to respond to intravenous corticosteroids is usually rapid, with a median response time reported to be 4-5 d, with the vast majority responding within 7 d in two randomized studies[53,126]. Clinical remission rates were approximately 65% with cyclosporine monotherapy and 93% in combination with steroids after 7 d, according to the results of a randomized study by D'Haens *et al*[127] In the above studies, endoscopic response was described within 7 d of initiation, with a continued improvement in endoscopic activity noted between weeks 1 and 4[126,127].

Factors affecting time to response

Dose and drug levels: Targeting high levels (10-15 ng/mL) of tacrolimus for induction appears more effective than low levels (5-10 ng/mL) with possibly a more rapid time to therapeutic response[123]. For ongoing maintenance thereafter, targeting trough levels of 5-10 ng/mL appears sufficient[122-125].

For cyclosporin, no additional benefit in clinical response was achieved with 4 mg/kg compared to 2 mg/kg intravenously in an RCT for acute severe colitis with a median time to response of 4 d in both groups, suggesting that doses above 2 mg/kg do not produce a more rapid time to response[126].

Route of administration: One observational study suggested that oral and intravenous tacrolimus achieved similar rates of clinical response by 14 d in steroid-refractory colitis, with comparable serum tacrolimus levels achieved with both strategies[128]. A retrospective study of oral and intravenous cyclosporin in ulcerative colitis actually found a higher early clinical response rate (exact timing not specified) with oral compared to intravenous cyclosporin (100% *vs* 65%) despite comparable serum drug levels, predominantly due to higher rates of side effects necessitating treatment cessation with intravenous cyclosporin[129]. Time to response was not directly assessed in this study and groups significantly differed with higher proportions of inpatients and intravenous corticosteroid failures in the intravenous cyclosporin group.

EXCLUSIVE ENTERAL NUTRITION

Exclusive enteral nutrition (EEN) involves the administration of a liquid nutrition formula to meet all

Table 3 Factors affecting time to response and response rates of therapies in inflammatory bowel disease

Variable	Parameter	Effect on time to response	Effect on response rate	Medications implicated	Level of evidence[1]	Ref.
Age	> 65 yr	↑	?	Anti-tumour necrosis factor-α (anti-TNF)	2b	Lobatón *et al*[143]
Increased body mass index	BMI > 25	-	↓	Azathioprine	2b	Holtmann *et al*[153]
	Weight > 82 kg	-	↓	Anti-TNF	1b	Reinisch *et al*[59]
Concomitant therapies		↓	↑	Immunomodulators with anti-TNF	1b	Colombel *et al*[50] Sandborn *et al*[69]
Smoking status	Current smoker	↑	↓	Anti-TNF	1b, 2b	Arnott *et al*[154] Sandborn *et al*[69]
Disease duration	> 2 yr	-	↓	Anti-TNF	1b	Colombel *et al*[155] Schreiber *et al*[156] D'Haens *et al*[157]

[1]As per the Oxford level of evidence scoring; The available literature suggests a slower initial response but comparable long-term response rate; The use of "-" denotes an absence of published data addressing this issue.

nutritional requirements, replacing normal diet, either orally or via nasoenteric tube. It is mostly used for induction of remission in CD typically over a duration of 6-8 wk. While most data emanates from pediatric studies, efficacy is also likely in adults[130].

Time to response with EEN seems rapid, with 75% of adult patients with active CD achieving clinical remission after 10 d of an elemental feeding in one small RCT[131]. Other small RCTs have demonstrated clinical remission rates of 25% to 80% within 3-4 wk of commencement[132-138]. In two non-randomized cohort studies utilizing objective disease activity endpoints, 44% of patients achieved mucosal healing after 4 wk of EEN and, in a pediatric cohort, 36% had mucosal healing after 8 wk of EEN[139,140].

ADDITIONAL FACTORS AFFECTING TIME TO CLINICAL RESPONSE

Disease related factors

There are several patient and disease-related factors that appear important when predicting the likelihood of response and time to therapeutic response in individual patients (see Table 3). Agents tend to achieve a more rapid time to therapeutic response in UC than CD. This may relate to the transmural nature of CD thus treatment takes longer to achieve resolution of inflammatory changes. This difference in time to therapeutic response is exemplified by the vedolizumab registration studies, where induction therapy appeared to have higher response rates in UC than CD and the benefits in CD were predominantly observed later during the maintenance phase[73,78].

Patients who have not achieved an adequate response to prior therapies may have a slower response to subsequent therapies. This was noted with vedolizumab induction, where previous anti-TNF failures achieved clinical remission rates that only became significantly different to placebo after 10 wk, in contrast to the overall cohort where clinical remission rates were higher at week 6 compared to placebo[74]. Similar findings of a longer time to clinical remission were found in patients who had received infliximab previously and were subsequently treated with induction certolizumab for CD[69].

Patient related factors

While several additional factors intuitively could affect time to therapeutic response such as nutritional status, age and the intestinal microbiome, published data are lacking. Advancing age is typically associated with a reduced glomerular filtration rate, greater oxidative stress, increased volume of distribution, co-morbid conditions, decreased hepatic metabolism and frailty-any of which may affect treatment choice and a therapy's time to clinical response[141,142]. For instance, one retrospective study found that rates of clinical response to anti-TNF therapy for IBD in patients > 65 years was significantly less at week 10 but not significantly different at 6 mo than matched controls with similar co-morbidities aged < 65 years, suggesting a slower onset of response overall[143]. The limited available evidence also suggests that obesity can affect the rate of response, although the effect on time to response has not been studied. For instance, higher baseline weight was associated with a lower rate of clinical remission following induction therapy with adalimumab in the ULTRA-1 study and has been associated with an earlier loss of response to infliximab and adalimumab in IBD[59,144,145].

Although many of these factors are not modifiable, intervening early in the disease course and/or simple complementary measures such as improving nutrition may allow for a more rapid time to therapeutic response.

DISCUSSION

This review has elucidated multiple important principles

of time to therapeutic response with direct applicability to clinical practice. First, patience is critical (for patients and clinicians alike) to reap the maximal benefits of some agents, particularly thiopurines, vedolizumab and methotrexate, with cumulative gains in response to these agents up to 12 mo after commencement. The clinical benefits indeed might lag significantly beyond the drug reaching therapeutic levels. For instance, this was reflected in a study assessing whether initial intravenous loading of azathioprine might hasten time to response. Indeed, therapeutic levels of 6-TGN were achieved rapidly. However, this did not improve the time to therapeutic response compared to standard oral administration[80]. Thus assessment of response should be delayed until sufficient time has lapsed to reach clinical efficacy. Whilst symptomatic improvement may occur relatively early following the initiation of many therapies, there often appears to be a lag in achieving mucosal healing, which should be considered when interpreting an early endoscopic assessment. This concept was demonstrated in a randomized controlled trial by Sutherland *et al*[146] comparing 4 g aminosalicylate enemas to placebo enemas for the management of distal UC. Clinical response rates and endoscopy were measured at 3 and 6 wk, with response rates reaching 60% by week 3 in the aminosalicylate arm, then plateauing to reach 68% by week 6. The mucosal healing rates over the same period increased from 25% to 42%, suggesting a later rise[24]. Additionally, patients kept a symptom diary for the first two weeks of this trial and rectal bleeding had ceased in over half of patients by day 6, so the symptom improvement may have occurred more rapidly than the time of outcome measurement. Hence, it may be appropriate to delay assessment until after the expected time to therapeutic response has passed.

Secondly, it follows that a prolonged period of bridging therapy such as co-administration of EEN, corticosteroids or even perhaps tacrolimus is an important component of treatment planning, particularly in patients who are acutely unwell, so that relapse is avoided if possible, prior to the expected maximal response of a newly introduced therapy. Thirdly, in agents where a delayed time to therapeutic response is more likely, one must consider whether potential methods of hastening onset of action can be employed, such as thiopurine and allopurinol in combination or perhaps using EEN in combination with an anti-TNF agent in CD as induction therapy.

Significantly, the lack of reporting of time-to-response in clinical trials is a deficiency. Mostly, data must be extrapolated from studies rather than being directly reported. Given its utility in clinical practice and the relatively basic calculations required, this should be addressed in future studies. The expected time-to-response can potentially assist in trial design by providing an estimate of the necessary duration of a study to show maximal efficacy. For example, for vedolizumab for the management of CD, the CDAI-100 response rate was not significant at week 6 and only surpassed placebo around week 28[73]. Similarly, modest early response rates were also noted with another anti-integrin therapy natalizumab at week 10 with response rates increasing during maintenance therapy[147]. The use of week 6 rather than week 10 or later as the response times for vedolizumab has been a criticism of the large randomized studies[77]. Hence, such information can potentially be reported in early studies and thus provide further insight into the rapidity of action of drugs or more widely, drug classes, which can be incorporated into future studies of that therapy, plus into clinical practice.

Finally, the concept of time-to-response is a broad term and it is likely that it can be further divided based on the proportion of patients who have respond to therapy, as shown in Table 1. There is a time point at which most patients will respond and then following this there is progressively diminishing returns in the likelihood of further response, culminating in a point where response is unlikely; the time to futility. This concept remains clinically important, as the yield from persisting with therapy beyond this point is low and thus place patients at higher risk, for minimal or no benefit. Again, such information be ideally included in published data to better aid clinicians with therapeutic decisions.

CONCLUSION

In a chronic disease like IBD, where many therapies are effective yet of relatively slow onset of action, time-to-therapeutic response is of central importance both quantitatively to frame the patient's expectations and the clinician's decision making but also conceptually-encouraging the mindset of forward treatment planning, overlapping bridging therapies and accuracy in determining therapeutic failure. Furthermore, in contrast to the homogenized, group-based reporting of results from the seminal RCTs as the reference standard for each new agent, time to therapeutic response is inherently patient-centric and individualized. This is not only because it has the potential to vary by indication, dosage, demographic, clinical factors and is dependent on which endpoint is chosen (*i.e.*, clinical response, remission, or endoscopic remission) but one can argue that the time to therapeutic response is of far greater relevance to patients and perhaps even to clinicians in day-to-day practice, thus future studies should examine and incorporate this paradigm further.

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ORIGINAL ARTICLE

Basic Study

Pharmacological inhibition of cannabinoid receptor 1 stimulates gastric release of nesfatin-1 *via* the mTOR pathway

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Abstract

AIM

To determine whether Nucb2/nesfatin1 production is regulated by the cannabinoid system through the intracellular mTOR pathway in the stomach.

METHODS

Sprague Dawley rats were treated with vehicle, rimonabant, rapamycin or rapamycin+rimonabant. Gastric tissue obtained from the animals was used for biochemical assays: Nucb2 mRNA measurement by real time PCR, gastric Nucb2/nesfatin protein content by western blot, and gastric explants to obtain gastric secretomes. Nucb2/nesfatin levels were measured in gastric secretomes and plasma using enzyme-linked immunosorbent assay.

RESULTS

The inhibition of cannabinoid receptor 1 (CB1) by the peripheral injection of an inverse agonist, namely rimonabant, decreases food intake and increases the gastric secretion and circulating levels of Nucb2/nesfatin-1. In addition, rimonabant treatment activates mTOR pathway in the stomach as showed by the increase in pmTOR/mTOR expression in gastric tissue obtained from rimonabant treated animals. These effects were confirmed by the use of a CB1 antagonist, AM281. When the intracellular pathway mTOR/S6k was inactivated by chronic treatment with rapamycin, rimonabant treatment was no longer able to stimulate the gastric secretion of Nucb2/nesfatin-1.

CONCLUSION

The peripheral cannabinoid system regulates food intake through a mechanism that implies gastric production and release of Nucb2/Nesfatin-1, which is mediated by the mTOR/S6k pathway.

Key words: NUCB2/nesfatin-1; Stomach; Food intake; Cannabinoid receptor 1; mTOR

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Core tip: The peripheral pharmacological blockade of the cannabinoid receptor 1 (CB1) induces a decrease in food intake in rats by stimulating the gastric secretion, and consequently the circulating levels, of the anorexigenic peptide Nucb2/nesfatin-1. The present data show that gastric CB1 receptors modulate Nucb2/Nesfatin-1 production in the stomach at the intracellular level, which is mediated by the mTOR pathway.

Folgueira C, Barja-Fernandez S, Prado L, Al-Massadi O, Castelao C, Pena-Leon V, Gonzalez-Saenz P, Baltar J, Baamonde I, Leis R, Dieguez C, Pagotto U, Casanueva FF, Tovar SA, Nogueiras R, Seoane LM. Pharmacological inhibition of cannabinoid receptor 1 stimulates gastric release of nesfatin-1 *via* the mTOR pathway. *World J Gastroenterol* 2017; 23(35): 6403-6411 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6403.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6403

INTRODUCTION

Nucb2/nesfatin-1 is a 42 kDa peptide, first discovered as an anorexigenic factor that regulates energy balance. The product of the Nucb2 gene is a 396 amino acid secreted peptide that, after being processed by the enzyme prohormone convertase (PC-1/2), becomes the N-terminal fragment known as nesfatin-1[1]. Nucb2 is mainly expressed in the gastric mucosa and adipose tissue, although its expression has also been detected elsewhere, such as hypothalamus, brain stem, pancreas and testis[1-5]. In the gastrointestinal tract, Nucb2 is most highly expressed in the X/A cells from the oxyntic mucosa of the stomach, which also synthesizes ghrelin, the main peptide hormone in the gastrointestinal tract[4,6,7].

Despite being secreted by the same cells, Nucb2 and ghrelin have opposing biological functions and regulation. Central and peripheral administration of Nucb2 suppresses feeding behavior[1,8], while ghrelin stimulates appetite[9,10]. Interestingly, the fact that Nucb2 and ghrelin are produced in the same gastric cells but in different vesicles suggests that both proteins might experience differential regulation at the gastric level to maintain energy balance. Supporting this theory, Nucb2/nesfatin-1 production and regulation by nutritional status decreases during

fasting[1], while ghrelin levels are up-regulated[11]. Lastly, this decrease in Nucb2 production during fasting conditions is concomitant with inhibition of the mTOR/pS6k1 pathway, a sensor of the metabolic status of organisms[12]. Ghrelin secretion, meanwhile, is negatively associated with mTOR/pS6k1 pathway activity under fasting conditions[13].

The cannabinoid system plays an important role as an endogenous regulator of energy balance, acting at multiple levels. One of those mechanisms includes its interaction with gastric ghrelin production to regulate appetite and body weight[14]. More specifically, the blockade of the cannabinoid receptor CB1 decreases ghrelin expression in the stomach, and this effect was mediated by activation of the mTOR/pS6k1 pathway[14]. Taking into account the opposing functions and regulation of ghrelin and Nucb2/nesfatin at the gastric level, as well as their opposite relationship with the mTOR/S6K1 pathway, we sought to investigate whether the cannabinoid system might also regulate Nucb2/nesfatin-1 production in the stomach and whether the mTOR/pS6k1 intracellular pathway mediates this effect.

MATERIALS AND METHODS

Ethics statement

The authors of this manuscript declare that the animal work in this study was approved by the Animal Care Committee of Santiago de Compostela University (Santiago de Compostela, Spain) in accordance with our institutional guidelines and the European Union standards for the care and use of experimental animals. The approaches in the present manuscript were performed under the procedure 15005/2015/003 reviewed and approved by the Faculty Animal Committee at the University of Santiago de Compostela.

Animal and experimental design

Male Sprague-Dawley rats were housed in air-conditioned rooms (22-24 ℃) under a controlled light/dark cycle (12 h light, 12 h darkness) with free access to food and water. The animals were euthanatized, trunk blood was collected and immediately centrifuged, and the plasma was stored at -80 ℃ for biochemical measurements.

Effects of pharmacological blockade of CB1 on food intake, gastric Nucb2 production, gastric Nucb2 secretion and plasma Nucb2/nesfatin-1 levels

A group of overnight fasted adult male rats was treated with Rimonabant intraperitoneal (i.p.) at a dose of 3 mg/kg (drug or vehicle, a solution of saline-DMSO 70%). To corroborate the data, a second set of animals was treated with AM-281 (Tocris Cookson Inc., Ellisville, MO), a CB1 antagonist. Animals were assigned to one of two experimental groups: the first group of rats was fasted for 36 h before receiving either an i.p. injection of Rimonabant or AM281 (3 mg/kg) 1 h prior to euthanasia (Rimonabant or AM281 group). The second group was fasted for 36 h prior to receiving an injection of vehicle (DMSO) 1 h before euthanasia (Control group). After sacrifice, the stomach was surgically excised and the plasma collected.

An additional set of animals was utilized to perform the food intake study. A group of overnight fasted adult male rats was treated with Rimonabant (i.p.) at a dose of 3 mg/kg or vehicle (a solution of saline-DMSO 70%) and immediately after treatment allowed free access to chow diet. One hour after the injection, the amount of food eaten was recorded for each rat.

Effects of pharmacological blockade of CB1 on gastric Nucb2 production, gastric Nucb2 secretion and plasma Nucb2/Nesfatin-1 in animals with a pharmacological inhibition of the gastric mTOR signaling by rapamycin treatment

Adult male rats were assigned to one of two experimental groups: 36-hour fasted animals treated with rapamycin (1 mg/Kg i.p. for 6 d) (Rapa group); 36-h fasted animals treated with rapamycin (1 mg/Kg i.p. for 6 d) and rimonabant (3 mg/Kg i.p.) 1 h before euthanasia (rapa+rimo group). Rapamycin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, United States).

Experimental techniques

Tissue explant culture: Tissue explants were obtained from adult Sprague-Dawley rats. To obtain *ex vivo* tissue explants, after euthanasia the stomachs were rapidly excised and transported to the lab in Krebs-Ringer-HEPES buffer (NaCl, 125 mmol/L; KCl, 5 mmol/L; $MgSO_4$, 1.2 mmol/L; KH_2PO_4, 1.3 mmol/L; $CaCl_2$, 2 mmol/L; glucose, 6 mmol/L; HEPES, 25 mmol/L; pH 7.4). After removing the blood vessels and connective tissue, the stomach was washed with sterile Krebs-Ringer-HEPES buffer. The tissue explants were placed in six-well dishes containing 2.5 mL of Dulbecco's modified Eagle's medium supplemented with L-glutamine (200 mmol/L), penicillin (1000 U/mL) and streptomycin (100 μg/mL). After a pre-incubation period of 1 h at 37 ℃ under a humidified atmosphere of 95% air-5% CO_2, the media was aspirated, and 2.5 mL of fresh medium was added into each well. The culture medium was collected after 2 h and tissue was weighed[11]. The samples were stored at -80 ℃ until used for biochemical analysis or processed to sample concentration by ultracentrifugation units (Amicon Ultra 3-kDa cut off, Millipore, Billerica, United States) for Western blot analyses.

Protein extraction and Western blotting analyses

The fundus of the stomach was obtained after euthanasia from animal models and homogenizated using a TissueLyser II (Qiagen, Tokyo, Japan) in cold

RIPA buffer [containing 200 mmol/L Tris/HCl (pH 7.4), 130 mmol/L NaCl, 10% (v/v) glycerol, 0.1%(v/v) SDS, 1%(v/v) Triton X-100, 10 mmol/L $MgCl_2$] with anti-proteases and anti-phosphatases (Sigma-Aldrich; St. Louis, MO, United States). The tissue lysates were centrifuged for 10 min at 18000 *g* in a microfuge at 4 ℃. Then, 30 μg of stomach mucosa were separated in 10% sodium-dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes. Equal loading was confirmed by measuring the amount of β-actin in whole tissue protein extracts. The membranes were probed successively with primary antibodies and peroxide-conjugated secondary antibodies (Jackson Immunoresearch, Baltimore, United States). Specific antigen-antibody binding was visualized using a chemiluminescence method according to the manufacturer's instructions (Pierce ECL Western Blotting Sustrate, Thermo Scientific). Primary anti-nesfatin-1 (1-82) was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, United States) and anti- β-actin (A-5316) was purchased from Sigma Chemical Co., (St Louis, MO, United States).

Western blots were performed using independent samples from different rats belonging to each group. β-actin detection was performed for all western blots as a loading control. The Western blot data are expressed as the mean ± SEM of percentages normalized to β-actin levels (arbitrary units).

RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from the stomach mucosa of animals using TRIzol (Invitrogen, CA, United States), according to the manufacturer's recommendations. The extracted total RNA was purified with DNase treatment using a DNA-free kit as a template (Ambion, United States) to generate first-strand cDNAs using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, United States). Quantitative real-time PCR was performed using a StepOne Plus Instrument (Applied Biosystems) with specific Taqman qRT-PCR primers and probes. For analyses, the NUCB2 gene expression levels (Assay ID: Rn01510621_m1) were normalized to the mRNA expression levels of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 ($HPRT_1$) (Assay ID: Rn01527840_m1, TaqMan: Applied Biosystems) and are expressed relative to the average value of the control group.

Biochemical analysis

Variations in the Nucb2 mRNA expression were measured in the rat gastric mucosa by real-time PCR and immunoblotting was performed to test the Nucb2 protein content in the mucosa.

To measure plasma Nucb2/Nesfatin-1 levels, blood samples were collected in tubes containing EDTA (1 mg/mL blood) and immediately centrifuged. Gastric tissues explants obtained from the experimental animals were incubated as described below[11]. The secretome collected from the gastric explants were immediately processed for sample concentration (corrected per gram of tissue) by ultra-centrifugation units (Amicon Ultra UFC800324 and UFC500324 off, Millipore, Billerica, United States). Nucb2/Nesfatin-1 secretion was measured by ELISA in the secretome and plasma using reagents kits and methods provided by Phoenix Pharmaceuticals, Inc. (Burlingame, United States). The assay sensitivity limit was 0.1 ng/mL. The results are expressed as ng/mL of NUCB2/nesfatin-1.

Statistical analysis

The data are presented as the mean ± SEM. Statistical analyses were performed with GraphPad Prism 5 software. Analyses comparing different groups were performed using the non-parametric Mann-Whitney *U* test. The statistical significance is indicated as follows: $^aP < 0.05$ and $^bP < 0.01$.

RESULTS

Effects of pharmacological blockade of CB1 on food intake, gastric Nucb2 production, gastric Nucb2 secretion and plasma Nucb2/nesfatin-1 levels

Animals administered rimonabant (i.p.) showed a decrease in food intake after 1 h compared to control group (Figure 1A). Gastric mRNA levels of Nucb2 (Control: 1.02 ± 0.06 *vs* Rimonabant: 0.74 ± 0.06 arbitrary units, $^aP < 0.05$) (Figure 1B) and Nucb2 protein content in gastric mucosa (Control: 100 ± 6.81 *vs* Rimonabant: 76.23 ± 4.76, $^aP < 0.05$) (Figure 1C) were significantly reduced after the blockade of the peripheral CB1 receptors with rimonabant. The decreased content in Nucb2 in the gastric mucosa in the group of animals treated with rimonabant was associated with an increased secretion of Nucb2/Nesfatin-1 from the stomach, as found by Nucb2/Nesfatin-1 values measured in the secretome obtained from gastric explants (control 0.16 ± 0.06 *vs* rimonabant 0.71 ± 0.22, $^bP < 0.01$) (Figure 1D). The circulating levels of Nucb2/Nesfatin-1 measured in plasma were accordingly increased after rimonabant treatment (control 5.83 ± 0.39 ng/mL *vs* rimonabant 7.37 ± 0.4 ng/ml, $^aP < 0.05$) (Figure 1E).

In keeping with data from the rimonabant treatment, the i.p. injection of a CB1 antagonist (AM281) caused a decrease in mRNA levels of Nucb2 (Control: 1.02 ± 0.06 *vs* AM-281: 0.78 ± 0.06 arbitrary units, $^aP < 0.05$) (Figure 2A). Moreover, the measurement of Nucb2 secretion from gastric explants from animals treated with AM-281 showed a tendency towards an increase in gastric Nucb2 secretion, although it was not statistically significant (Figure 2B). In addition, circulating Nucb2/nesfatin-1 levels analyzed by ELISA showed a significant increase after AM281 treatment (Control: 3.39 ± 0.13

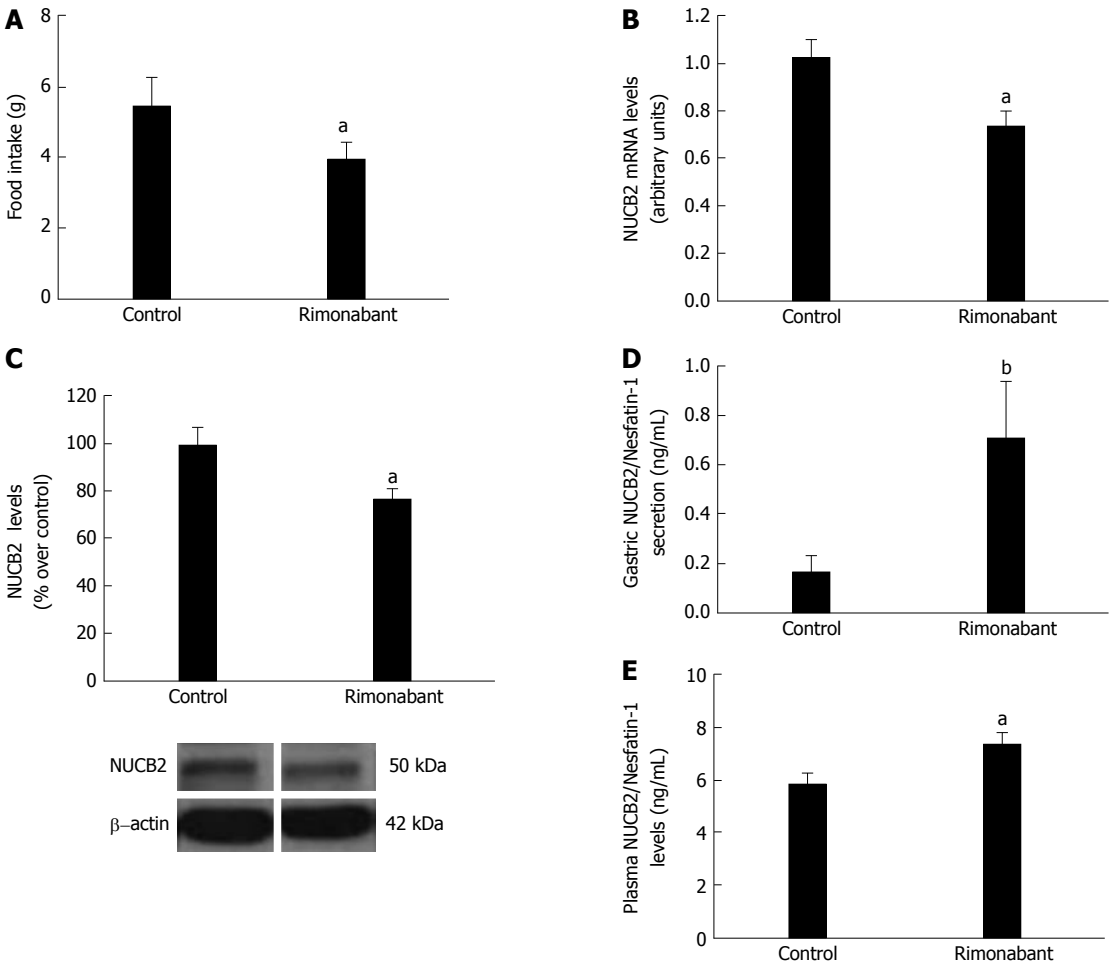


Figure 1 Effects of pharmacological blockade. Food Intake (A), mRNA expression levels measured by real-time PCR for Nucb2 (B), Nucb2 protein levels and representative Western blot from the mucosa; β-actin was used as a loading control (C). Dividing lines indicate splicings within the same gel; D: Nucb2/Nesfatin-1 secretion from tissue explant analyzed by ELISA; E: Plasma Nucb2/Nesfatin-1 levels obtained from 36-h fasted animals that received i.p. treatment with Rimonabant (3 mg/kg i.p.) (n = 9) or vehicle (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$.

ng/mL *vs* AM281: 4.46 ± 0.38 ng/mL, $^{a}P < 0.05$) (Figure 2C).

Effects of pharmacological blockade of CB1 on gastric Nucb2 production, gastric Nucb2 secretion and plasma Nucb2/Nesfatin-1 in animals with inhibited gastric mTOR signaling

We found that gastric CB1 blockade with rimonabant treatment in the fasting state induces activation of the mTOR/S6K1 pathway (vehicle: 100 ± 13.99 *vs* rimonabant: 198.11 ± 34.5; $P < 0.05$) (Figure 3A). Accordingly, in the present work we observed that AM281 administration to fasted animals induces activation of the mTOR/pS6K1 pathway, as observed by increased expression of pmTOR/mTOR in the gastric mucosa of AM281 treated animals compared to controls (vehicle, 100 ± 13.41 *vs* AM281, 154.13 ± 15.31, $P < 0.05$) (Figure 3C).

In light of mTOR pathway activation in response to gastric CB1 blockade with rimonabant, the next step was to determine whether this pathway might be mediating the effect of cannabinoids on Nucb2/nesfatin-1 production. To this end, we administered rimonabant to a group of animals subjected to chronic rapamycin treatment to establish mTOR pathway inactivation. We find that, contrary to the effects found in control rats, i.p. administration of rimonabant to animals previously treated with rapamycin did not affect gastric Nucb2 mRNA levels (Figure 4A), gastric Nucb2/Nesfatin-1 protein levels (Figure 4B), gastric secretion of Nucb2/Nesfatin-1 (Figure 4C) or circulating levels of Nucb2/Nesfatin-1 (Figure 4D).

DISCUSSION

Ghrelin and Nucb2/Nesfatin-1 are co-expressed in the same endocrine cells of the stomach, together with the cannabinoid receptor CB1[4]. In the present study we show, for the first time to our knowledge, that Nucb2/Nesfatin-1 regulation in the stomach is governed by the cannabinoid system in an inverse relation to the described in the bibliography for ghrelin[14]. First, we found that blockade of the endogenous CB1 receptor at peripheral level with two different antagonists, rimonabant and AM281, increases gastric Nucb2/Nesfatin-1 secretion, which is reflected by an increase

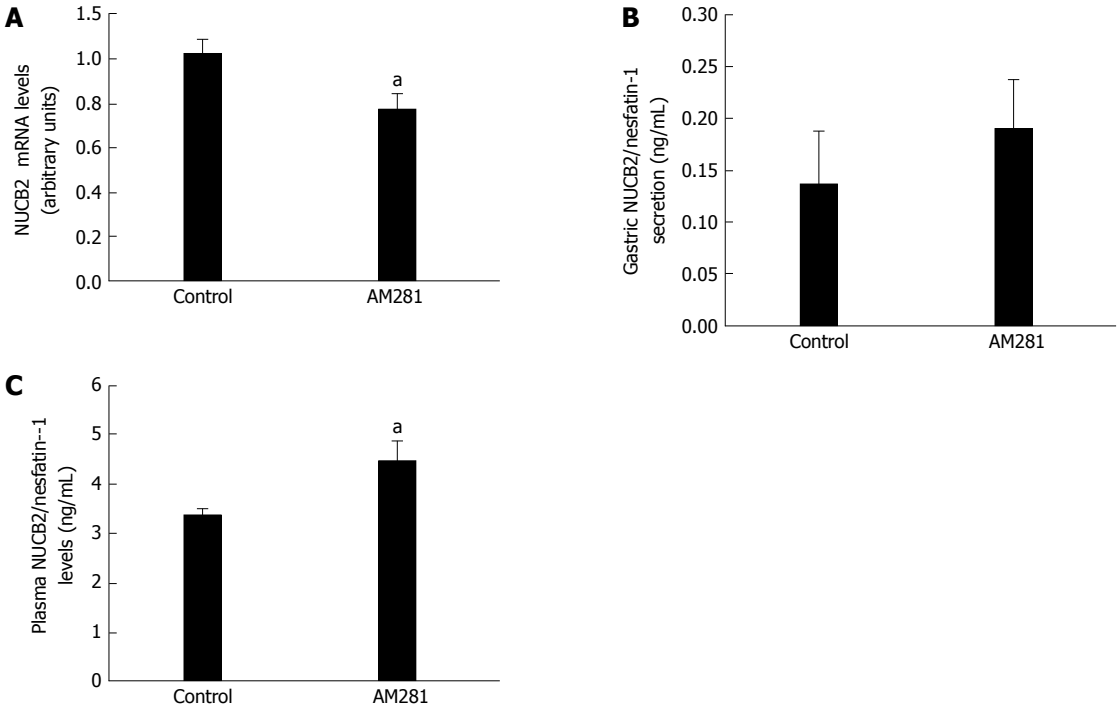


Figure 2 mRNA expression levels measured by real-time PCR for Nucb2 (A), nucb2/nesfatin-1 secretion from tissue explant analyzed by ELISA (B), plasma Nucb2/nesfatin-1 levels (C) obtained from 36-h fasted animals that received i.p. treatment with AM281 (3 mg/Kg i.p.) (*n* = 6) or vehicle (*n* = 6). $^{a}P < 0.05$ *vs* control.

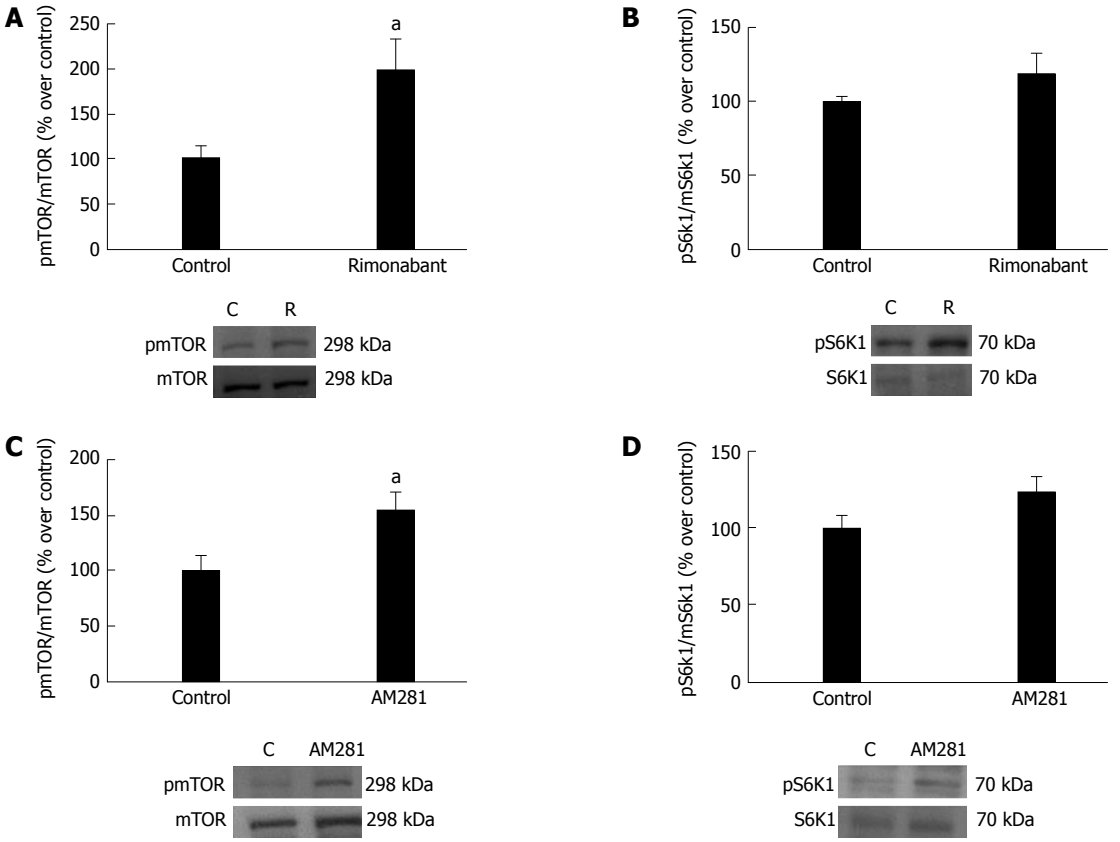


Figure 3 Measures of phospho-mTOR/mTOR levels (A) and phospho-S6K1/S6K1 (B) in gastric mucosa and representative Western blots from animals in the fasting state treated with i.p. rimonabant or vehicle (*n* = 9). Measures of phospho-mTOR/mTOR levels (C) and phospho-S6K1/S6K1 (D) in gastric mucosa and representative Western blots from animals in the fasting state treated with i.p. AM281 (*n* = 6) or vehicle (*n* = 6). β-actin was used as a loading control. Dividing lines indicate splicings within the same gel. The results are expressed as percentages over control, $^{a}P < 0.05$.

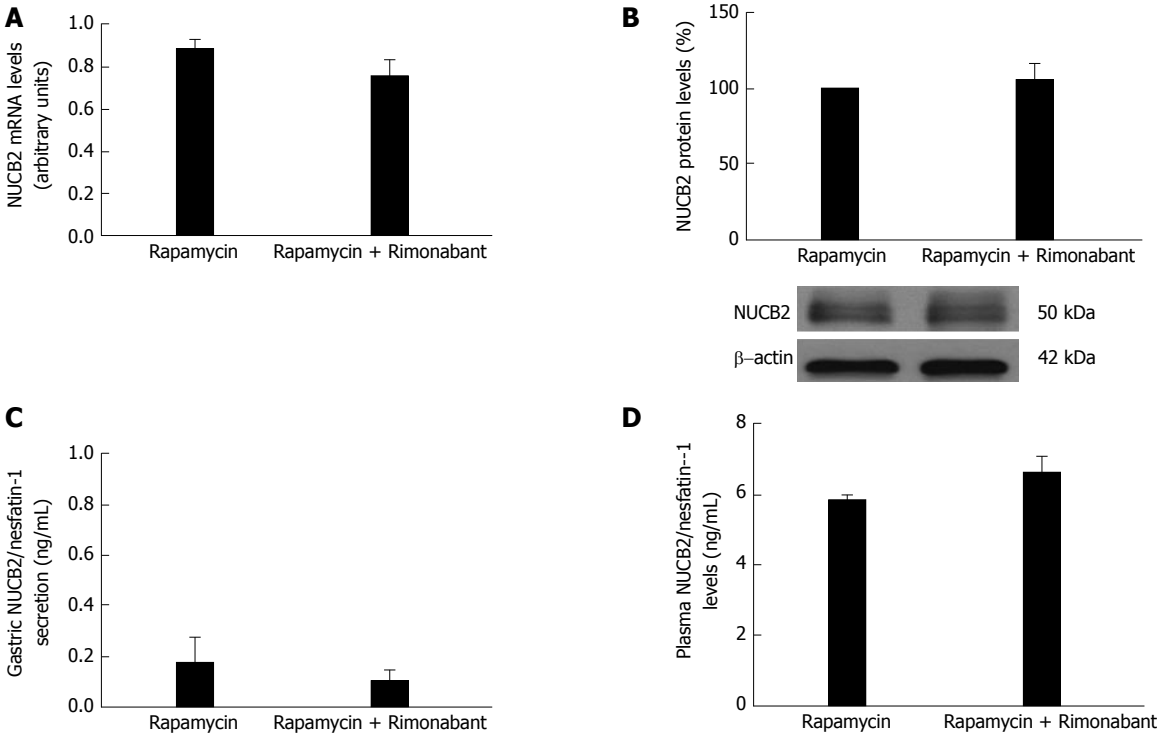


Figure 4 mRNA expression levels measured by real-time PCR for Nucb2 (A), Nucb2 protein levels and representative Western blot from the mucosa. β-actin was used as a loading control. Dividing lines indicate splicings within the same gel (B), Nucb2/Nesfatin-1 secretion from tissue explant analyzed by ELISA (C), plasma Nucb2/Nesfatin-1 levels (D) obtained from 36-h fasted animals treated with rimonabant i.p. (n = 9) and/or rapamycin i.p. chronically for 1 wk (n = 9).

in the circulating levels of this peptide. An anorexigenic effect of Nucb2/nesfatin-1 has been extensively described[4] animals treated with rimonabant showed a significant decrease in food intake. The anorexigenic effect after CB1 blockade might be attributed to the increased production of Nucb2/nesfatin-1 in the stomach. Moreover, it was previously shown that the peripheral blockade of CB1 with rimonabant resulted in decreased food intake that was mediated by a decrease in the production of the orexigenic ghrelin[14]. Altogether, the data in the present study shows that Nucb2/nesfatin-1 is regulated in the stomach in a way that is diametrically opposed to those previously described for ghrelin, suggesting an equilibrium at the gastric level between the anorexigenic Nucb2/nesfatin-1 and the orexigenic ghrelin that regulates food intake to maintain body weight.

The next step of the present work was to understand the intracellular mechanism mediating the effect of the cannabinoid system on gastric Nucb2/nesfatin-1. It was previously demonstrated that the regulation of ghrelin production in the stomach by the cannabinoid system is driven by the mTOR/S6K1 pathway[14]. In this sense, it was also reported that Nucb2/nesfatin-1 production in the stomach is modulated by mTOR signaling during nutritional changes[12]. Taking into account that ghrelin and Nucb2/nesfatin-1 are produced in the same cell type in the stomach, we wanted to determine whether the intracellular mTOR/S6K1 pathway may also be involved in the gastric regulation of Nucb2/nesfatin-1 by the cannabinoid system. As expected, pharmacological blockade of CB1 with rimonabant, or AM281, activated the mTOR/S6K1 pathway, which is a sensor of nutritional status.

In light of the present findings the blockade of the cannabinoid system by CB1 antagonism with rimonabant or AM281 exerts the activation of the mTOR pathway and the increase of the anorexigenic signal Nucb2/nesfatin-1 that induces satiation.

To further confirm our results and to more completely explore the mechanism mediating the effects of cannabinoid on Nucb2/nesfatin-1 production and release, we investigated whether injection of rimonabant would be effective in animals with mTOR/S6K1 pathway inactivation. In agreement with our hypothesis, peripheral injection of rimonabant did not affect gastric Nucb2/nesfatin-1 production or circulating levels in rats with an inactivated mTOR/S6K1 pathway by rapamycin treatment.

In summary, the main findings of this present research are as follows: (1) peripheral CB1 receptors modulate food intake and the data might suggest that these regulation is drive through a mechanism that implies gastric Nucb2/nesfatin-1 production; (2) gastric CB1 receptors modulate Nucb2/nesfatin-1 production in the stomach at the intracellular level mediated by the mTOR pathway; and (3) taking into account previous data with reference to cannabinoid regulation of ghrelin[14] and the present, we can conclude that Nucb2/nesfatin-1 and ghrelin are regulated at the gastric level by the cannabinoid system

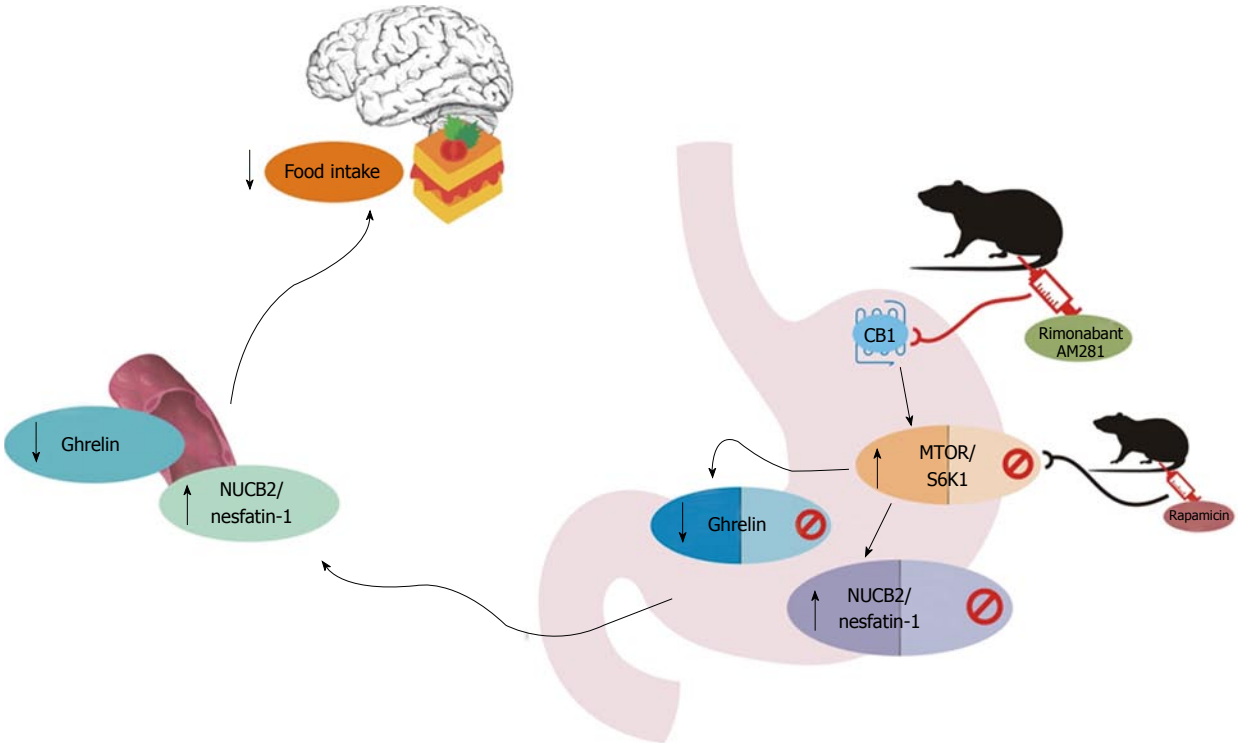


Figure 5 Graphical abstract describing the pathways of ghrelin and nucb2/nefastatin-1 and association with cannabinoid system and mTOR pathway regulating food intake.

in a diametrically oppositional manner to regulate the food intake and maintain energy balance (Figure 5).

Altogether, the data in the present paper show for the first time that the cannabinoid system, and more precisely CB1, regulates the nutrient sensor mTOR, consequently controlling the production of the anorexigenic hormone Nucb2/Nesfatin-1. Moreover, this newly described mechanism also provides clues to the balance of anorexigenic and orexigenic signals in the stomach that maintain energy homeostasis. Further work should focus on testing whether this gastric mechanism is affected under pathological conditions such as obesity or anorexia, which lead to an unhealthy body weight. Moreover, the possibility of modulating a peripheral target, such as Nucb2/Nesfatin-1, may represent a novel pharmacological therapy for obesity, thereby potentially avoiding the non-desirable off-target effects of many drugs.

COMMENTS

Background

Nowadays the more effective treatment against obesity is gastric surgery which suggests that gastrointestinal tract signals are crucial factors in the regulation of energy homeostasis. Although several works in the last years has been focused in the role of different gastric signals with a relevant role in body weight regulation the full mechanisms governing this actions has not been elucidated.

Research frontiers

The discovery of new mechanisms at gastric level represents the possibility of new therapeutic targets to treat obesity avoiding the collateral effects at central level that suppose a serious problem in the pharmacological design of drugs.

Innovations and breakthroughs

The gastric mechanism described in the present regulating the production by the stomach of one of the newest gastrointestinal hormone related with a decreasing effect in appetite was has been previously reported with an inhibitory effect in the release of the only known gastric hormone with orexigenic effects. This data highlight the existence of a gastric mechanism controlling appetite through the differential regulation of anorexigenic and orexigenic gastric signals.

Peer-review

This is an interesting manuscript on the regulation of food intake through a mechanism that implies gastric production and release of Nucb2/Nesfatin-1.

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ORIGINAL ARTICLE

Basic Study

Effects of aspirin and enoxaparin in a rat model of liver fibrosis

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Author contributions: All listed authors participated meaningfully in the study and have seen and approved the final manuscript; Li CJ and Shi XL designed the study; Li CJ and Yang ZH performed the animal experiments; Li CJ and Liu DL analyzed the data; Li CJ, Yang ZH, Shi XL and Liu DL wrote and revised the paper.

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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 East China Normal University.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

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Abstract

AIM

To examine the effects of aspirin and enoxaparin on liver function, coagulation index and histopathology in a rat model of liver fibrosis.

METHODS

Forty-five male Sprague-Dawley rats were randomly divided into the control group (n = 5) and model group (n = 40). Thioacetamide (TAA) was used to induce liver fibrosis in the model group. TAA-induced fibrotic rats received TAA continuously (n = 9), TAA + low-dose aspirin (n = 9), TAA + high-dose aspirin (n = 9) or TAA + enoxaparin (n = 9) for 4 wk. All rats were euthanized after 4 wk, and both hematoxylin-eosin and Masson staining were performed to observe pathological changes in liver tissue.

RESULTS

Liver fibrosis was assessed according to the METAVIR score. Compared with untreated cirrhotic controls, a significant improvement in fibrosis grade was observed in the low-dose aspirin, high-dose aspirin and enoxaparin treated groups, especially in the high-dose aspirin treated group. Alanine aminotransferase and total bilirubin were higher, albumin was lower and both prothrombin time and international normalized ratio

were prolonged in the four treatment groups compared to controls. No significant differences among the four groups were observed.

CONCLUSION
Aspirin and enoxaparin can alleviate liver fibrosis in this rat model.

Key words: Aspirin; Enoxaparin; Thioacetamide; Liver fibrosis; Rat

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Core tip: Several lines of evidence show that intra-hepatic thrombosis is associated with liver fibrosis. Based on these observations, we hypothesized that routine use of antithrombotic drugs may prevent liver fibrosis. In our study, we successfully established a rat model of liver fibrosis after 8 wk and successfully established a rat model of liver cirrhosis after 12 wk through the thioacetamide-induced method. Our study clearly shows that aspirin and enoxaparin can reduce liver fibrosis in rats. However, reporting the results in humans is more complicated because other aspects, such as renal function, need to be considered.

Li CJ, Yang ZH, Shi XL, Liu DL. Effects of aspirin and enoxaparin in a rat model of liver fibrosis. *World J Gastroenterol* 2017; 23(35): 6412-6419 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6412.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6412

INTRODUCTION

Liver fibrosis is a common liver disease posing a heavy burden on human health, and is a common cause of death. The mortality rate due to end-stage liver disease caused by fibrosis and subsequent severe liver cirrhosis is 20%-50%. In recent years, liver fibrosis has become a research hotspot, but its occurrence and development have not been fully elucidated. Liver fibrosis is closely related to the chronic inflammatory response of the liver, and inflammation leading to liver fibrosis has been studied in detail. In some cases, other pathways may also contribute to liver fibrosis in addition to inflammation.

There is evidence showing that intra-hepatic thrombosis is associated with liver fibrosis. Wanless *et al*[1] found that microthrombosis in the intrahepatic portal vein and hepatic venous system was associated with hepatic atrophy and fibrosis in transplanted livers. Papatheodoridis *et al*[2] showed that, in patients with chronic viral hepatitis, risk factors for thrombosis were strongly associated with liver fibrosis and cirrhosis. Thrombin, a serine protease activated by proteolytic cleavage of prothrombin, can directly activate stellate cells through protease-activated receptors. Many antithrombotic therapies, such as standard heparin, low molecular weight heparin, vitamin K antagonists and antiplatelet agents, can reduce the proliferation and activation of stellate cells and reduce the fibrotic regions in various animal models with liver fibrosis.

Based on these observations, we hypothesized that routine use of antithrombotic drugs may prevent liver fibrosis in humans. To test this hypothesis, we used thioacetamide (TAA) to establish a rat model of liver fibrosis and examined the effect of antithrombotic therapy with aspirin and enoxaparin in this model.

MATERIALS AND METHODS

Animals

Forty-five healthy and pure Sprague-Dawley (SD) male rats weighing 200-250 g were included in this study and were housed in a specific pathogen-free environment. The ambient temperature was maintained at 20-25 ℃ and humidity was maintained at 50%-70%. The animals were acclimated to their new environment for 2 wk prior to experimentation. The rats were treated in accordance with ethical requirements for laboratory animal care, and were weighed weekly to adjust the dose of TAA and drugs.

Drugs and reagents

TAA was purchased from Sigma (St Louis, MO, United States). Aspirin enteric-coated tablets were produced by Bayer Healthcare Limited Corporation and enoxaparin was produced by Sanofi-Aventis (Beijing, China) Pharmaceutical Limited Corporation.

Grouping and modeling

Preparation period: SD rats were randomly divided into two groups: the control group (Group A, n = 5) and the model group (n = 40). Hepatic fibrosis was induced by intraperitoneal injection of TAA at a dosage of 200 mg/kg body weight, given twice weekly for 8 wk. The control group received an intraperitoneal injection of saline. After 8 wk, four rats in the model group were sacrificed at random and liver tissue was obtained to verify the model.

Treatment period: The rats in the model group were subdivided into four groups: TAA group (Group B), TAA + low-dose aspirin group (Group C), TAA + high-dose aspirin group (Group D) and TAA + enoxaparin group (Group E), with 9 rats in each group. One milliliter of physiological saline was administered by gavage in Group A and B, 1 mL of aspirin (30 mg/kg) was administered by gavage in Group C, aspirin (300 mg/kg) was administered by gavage in Group D, and 400 L of enoxaparin (2 mg/kg) was injected subcutaneously in Group E. The frequency of administration in each group was once a day over a period of 4 wk.

During establishment of the model and the treatment

period, the weight of the rats was measured twice a week and drug dosage was adjusted according to body weight. The dosage selected for each intervention was based on the results of previous studies using these agents in various fibrotic diseases[3,4]. To prevent spontaneous degeneration of liver cirrhosis, TAA (200 mg/kg) injections were continued twice a week during the treatment phase of the study in Groups B-E.

General observation
The main indices observed were activity, death and weight.

Determination of liver biochemistry
Serum and plasma were collected before the experiment, and at wk 8, 10 and 12 after treatment. Serum alanine aminotransferase (ALT), albumin (ALB), total bilirubin (TBIL) and plasma prothrombin time (PT) as well as international normalized ratio (INR) were measured.

Determination of liver histopathology
Liver histopathology was determined by naked-eye observation and photography and by staining, microscopic observation and photography.

HE staining: Histopathological changes in the liver were observed, including necrosis of hepatocytes, the degree of fibrosis, the presence of regenerative nodules or pseudo-lobules, particularly the presence of thrombosis in sinusoidal or intrahepatic vessels, and the presence of luminal stenosis.

Masson staining: Liver collagen fibers were stained using Masson stain. The degree of fibrotic hyperplasia in sections was classified using the METAVIR liver fibrosis scoring system[5], which was blindly applied to each liver biopsy sample.

Statistical analysis
All data were statistically analyzed using SPSS 18.0 statistical software. The data were expressed as median, and the overall variance analysis was carried out using nonparametric Kruskal-Wallis H and Mann-Whitney *U* tests, as the data obtained were not consistent with normal distribution and variance was not homogeneous. *P*-values of < 0.05 were considered statistically significant.

RESULTS

General observations of rat status
At wk 12, healthy control rats had shiny hair and normal activities, with no mortality. The TAA group had disheveled hair and decreased activities; one rat died at wk 9 and another died at week 11, giving a mortality rate of 22.2%. Activity in the TAA + low-dose aspirin group, TAA + high-dose aspirin group and TAA + enoxaparin group decreased compared with the healthy control group, but was greater than that in the TAA group. The mortality rate in the TAA + low-dose aspirin group and TAA + high-dose aspirin group was 11.1%, with one death at wk 11 in each group. No deaths occurred in the TAA + enoxaparin group.

Compared with the healthy control group, body weight in rats in the model groups was lower at wk 8, 10 and 12 ($P < 0.05$; Table 1), but there was no statistically significant difference between them.

Biochemical changes in rats
Biochemical changes in the rats are shown in Table 2. At wk 8, ALT and TBIL were higher in the model group than in the control group ($P < 0.05$). ALB decreased, and both PT and INR were prolonged, but were not statistically significant. Along with the formation of liver fibrosis, liver function became abnormal.

At wk 8, 10 and 12, ALT and TBIL increased, ALB decreased, and both PT and INR were prolonged in the four model groups ($P < 0.05$). However, there were no significant differences between the groups.

General observations of rat liver
At wk 12, livers in the healthy control group were bright red, shiny and soft, with a sharp edge (Figure 1A). Livers in the TAA group were dark, dull and rough, and showed granular nodules, hard texture and a blunt edge (Figure 1B). Livers in the TAA + low-dose aspirin group (Figure 1C), TAA + high-dose aspirin group (Figure 1D) and TAA + enoxaparin group (Figure 1E) were slightly darker than in the healthy control group, but slightly softer than in the TAA group, and the granular nodules on the surface were not obvious compared to the TAA group. In particular, livers were improved in the TAA + high-dose aspirin group and TAA + enoxaparin group. TAA effectively induced liver fibrosis/cirrhosis in this rat model, while aspirin and enoxaparin reduced the degree of liver fibrosis/cirrhosis.

Rat liver histopathology using HE staining and Masson staining
At wk 8, conventional HE staining of liver sections from the model group (Figure 2A) showed that hepatocytes around the central vein in lobules were disordered and both steatosis and fat vacuoles were visible in the cytoplasm. By Masson staining, collagen fibers around the central vein and portal area demonstrated significant proliferation, had a cord-like extension to the liver lobule and did not form pseudo-lobules. The rat model of liver fibrosis was successfully established.

At wk 12, HE staining of liver sections from the healthy control group (Figure 2B) showed normal hepatocyte morphology, complete structure of liver lobules and a neat radiation-like arrangement of liver tissue around the central vein. Masson staining showed no obvious collagen fibrosis. HE staining of

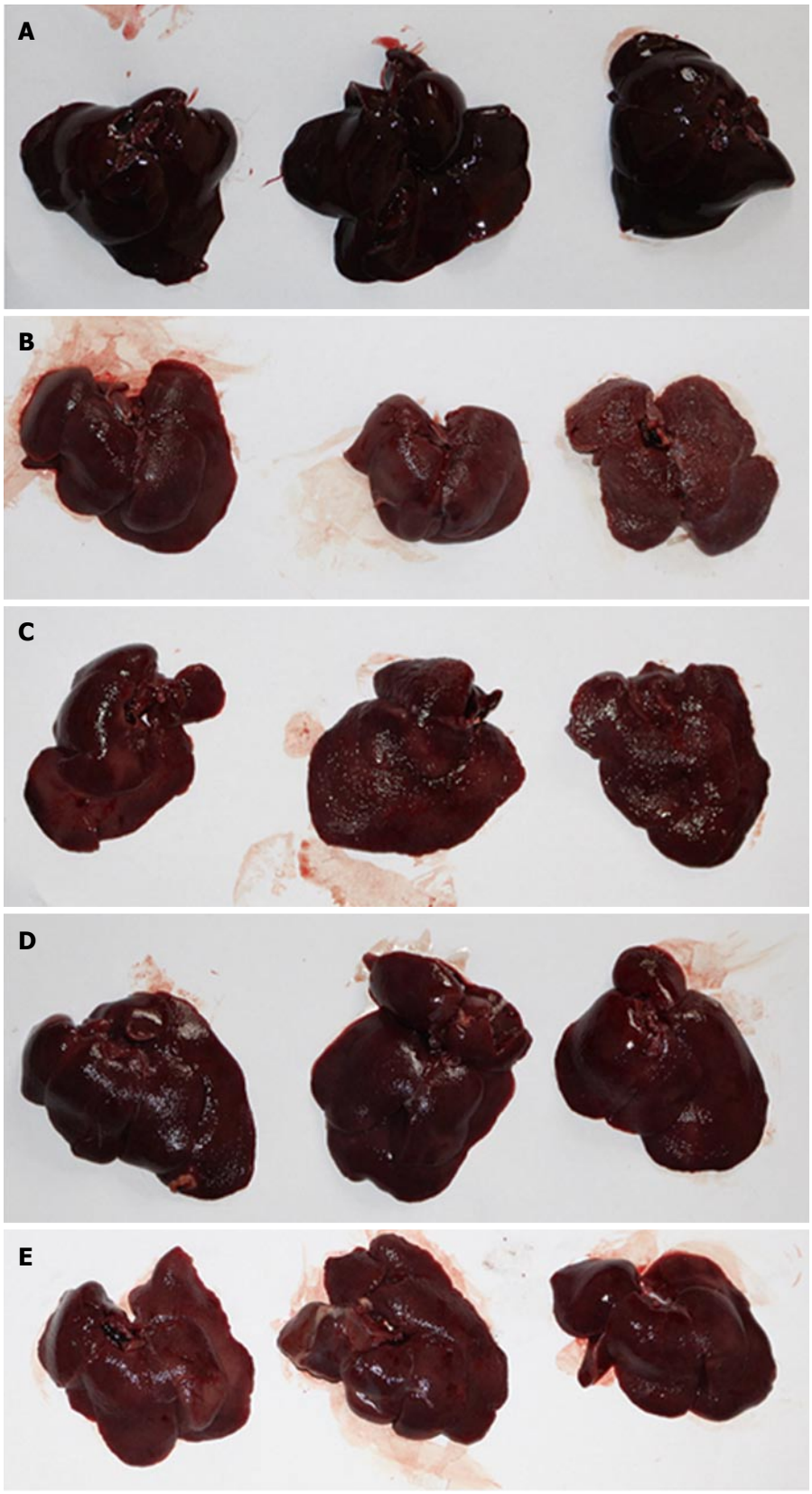


Figure 1 General observations of the rat model of liver fibrosis/cirrhosis. A: Healthy control group; B: TAA group; C: TAA + low-dose aspirin group; D: TAA + high-dose aspirin group; and E: TAA + enoxaparin group. TAA: Thioacetamide.

liver sections from the TAA group (Figure 2C) showed damaged liver lobule structure and abnormal structure of pseudo-lobules due to regeneration of hepatocytes. Masson staining revealed significant proliferation of collagen tissue and the formation of pseudo-lobules. HE staining of liver sections in the remaining

Table 1 Body weight in each group at wk 8, 10 and 12

Group	Body weighting		
	8 wk	10 wk	12 wk
Healthy control group, A	510	536	527
TAA group, B	394	401	454
TAA + low-dose aspirin group, C	381	403	403
TAA + high-dose aspirin group, D	381	364	408
TAA + enoxaparin group, E	385	409	415

TAA: Thioacetamide.

Table 2 Changes in alanine aminotransferase, albumin, total bilirubin, prothrombin time and International normalized ratio in each group

Group	Wk	ALT	ALB	TBIL	PT	INR
Healthy control group, A	8	35	29.1	2.95	9.2	-
	10	41.0	30.1	2.3	8.8	0.83
	12	40.0	30	2.8	9.3	0.88
TAA group, B	8	45.0	29	3.4	9.4	0.78
	10	49.5	29	3.4	9.4	0.89
	12	60.0	28.5	4.2	10.3	0.97
TAA + low-dose aspirin group, C	10	48.0	29.6	2.2	10.2	0.96
	12	54.5	28.5	4.4	10.5	0.99
TAA + high-dose aspirin group, D	10	60.5	29.7	3.5	9.45	0.895
	12	47.0	29.25	4.75	10.1	0.950
TAA + enoxaparin group, E	10	45.0	30	3.4	9.45	0.895
	12	47.0	28.7	4.8	10.2	0.96

ALB: Albumin; ALT: Alanine aminotransferase; INR: International normalized ratio; PT: Prothrombin time; TBIL: Total bilirubin; TAA: Thioacetamide.

groups (Figure 2D-F) showed disordered hepatocyte arrangement, but no obvious formation of pseudo-lobules. Masson staining exhibited different degrees of collagen fiber proliferation.

The staging and scoring of liver fibrosis in each group at wk 12 are shown in Table 3. The difference in fibrosis grading between the healthy control group and the other model groups was statistically significant ($P < 0.01$), indicating that the model was successfully established. The difference between the TAA group and the other drug-intervention groups was statistically significant ($P < 0.05$), indicating that the drug intervention was effective.

DISCUSSION

Liver fibrosis/cirrhosis is a common outcome of chronic liver disease due to various etiologies, with high morbidity and mortality. Treatment of advanced liver fibrosis/cirrhosis is very unsatisfactory and is currently limited to the treatment of its underlying etiologies and remission of its complications. Therefore, there is an urgent need to develop new antifibrotic treatments that can prevent, halt or even reverse liver fibrosis. Recent,

Table 3 Staging and scoring of liver fibrosis in each group at wk 12

Group	*n*	Liver fibrosis stage					Liver fibrosis score
		F0	F1	F2	F3	F4	
Healthy control group, A	5	5	0	0	0	0	0
TAA group, B	7	0	0	2	1	4[b]	4
TAA + low-dose aspirin group, C	9	0	1	5	3	0[b,c,e]	2
TAA + high-dose aspirin group, D	8	0	6	0	1	1[b,c]	1
TAA + enoxaparin group, E	8	0	0	5	3	0[b,c,e]	2

[b]$P < 0.01$ *vs* the healthy control group; [c]$P < 0.05$ *vs* the TAA group; [e]$P < 0.05$ *vs* the TAA + high-dose aspirin group. TAA: Thioacetamide.

evidence has shown that intrahepatic thrombosis is associated with liver fibrosis. Mancuso[6] proposed that chronic microvascular ischemia of the liver may be a milestone in the development of liver cirrhosis. Chronic liver damage can eventually lead to endothelial injury and microvascular thrombosis, triggering inappropriate hepatocyte proliferation and fibrosis. In order to validate this theory, we established a rat model of liver fibrosis induced by TAA, determined the presence or absence of thrombosis in rat fibrotic livers, and examined whether antithrombotic therapy with aspirin and enoxaparin could reverse liver fibrosis.

The establishment of this animal model of liver fibrosis is of great significance in understanding the pathogenesis and treatment strategy of liver fibrosis and cirrhosis. To date, animal models have been developed using mice, rats, rabbits and pigs to mimic the complex processes of liver fibrosis and cirrhosis. Liver fibrosis induced by TAA is a widely used model and TAA can be administered by oral intake or peritoneal injection; however, peritoneal injection provides more consistent results. TAA-induced liver fibrosis is very similar to human liver fibrosis in terms of changes in hemodynamics, morphological and biochemical metabolism changes[7].

TAA damages DNA, RNA and protein synthetase in hepatocytes, leading to disorders of metabolism and necrosis of hepatocytes. An obvious feature of this model relative to the carbon tetrachloride model is that its fibrosis remains stable for several weeks after TAA withdrawal. However, TAA is a weak carcinogen and is both toxic and volatile. Large nodular cirrhosis and portal hypertension developed in the present model at 12 wk. A significantly highly powered cycle may be required to induce fibrosis for longer periods. Approximately 40% of rats develop ascites. After 18 wk of TAA administration, rats may develop cholangiocarcinoma.

In the present study, the rat liver fibrosis model was successfully induced by TAA in the 8th week. ALT and TBIL in the model group were significantly higher than those in the control group ($P < 0.05$). ALB

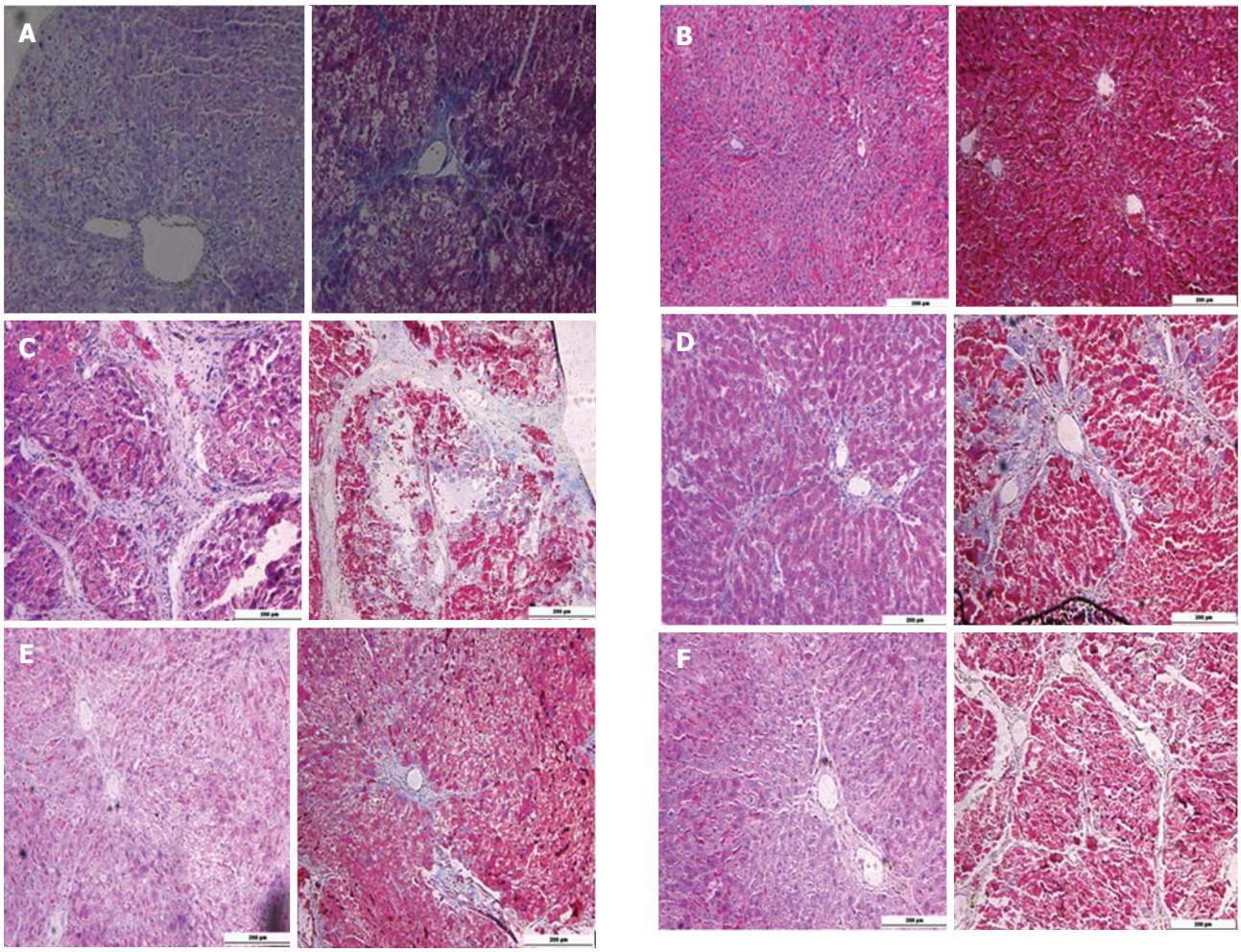


Figure 2 Liver histopathology of rat liver fibrosis/cirrhosis. A: Liver histopathology in the model group at wk 8; B: Liver histopathology in the healthy control group at wk 12; C: Liver histopathology in the TAA group at wk 12; D: Liver histopathology in the TAA + low-dose aspirin group at wk 12; E: Liver histopathology in the TAA + high-dose aspirin group at wk 12; and F: Liver histopathology in the TAA + enoxaparin group at wk 12. Left: HE × 100; Right: Masson × 100. TAA: Thioacetamide.

decreased, and both PT and INR were prolonged, but prolongation was not statistically significant. These findings indicated that following the formation of liver fibrosis, liver function became abnormal. At the 12th week, the rat model of liver cirrhosis was successfully established.

In 1995, Wanless *et al*[8] showed that both congestion and non-congestion in liver cirrhosis were present in the hepatic circulation during microthrombosis. By studying liver biopsy specimens from patients with chronic viral hepatitis, Papatheodoridis *et al*[2] in 2003 found that thrombosis-related factors were closely related to hepatitis staging and the degree of liver fibrosis, and antithrombosis drugs seemed to reverse liver fibrosis. It was suggested that microthrombosis may be involved in the formation of liver fibrosis in chronic liver disease, rather than the result of cirrhosis. In the present study, both HE staining and Masson staining showed that liver fibrosis in model rats was significant, and progressed to liver cirrhosis at 12 wk. However, no apparent microthrombosis was found, probably because HE staining and Masson staining were not sensitive enough to detect microthrombi. It is necessary to identify a more sensitive experimental method to observe microthrombosis in the liver.

Aspirin is regarded as an agent for preventing arterial thrombosis, but can also reduce the risk of venous thromboembolism[9]. The mechanism underlying the antifibrotic properties of aspirin may be related to its antithrombotic properties. Sinusoidal endothelial cell injury can also lead to increased platelet adhesion and a coagulation state. Indeed, such mechanisms have been implicated in injury repair and hepatic sinus obstruction syndrome after liver transplantation, and aspirin has been shown to reduce the risk of chemotherapy-associated sinusoidal lesions.

Aspirin also exhibits anti-inflammatory activity and inhibits nuclear factor kappa B (NFκB) transcript adhesion molecules in endothelial cells and vascular smooth muscle cells *in vitro*, thereby affecting macrophage and T lymphocyte adherence to these cells. Aspirin also reduces the levels of interleukin-6, monocyte chemoattractant protein-1, transforming growth factor-beta (TGF-β), tumor necrosis factoralpha and platelet-derived growth factor. Chavez *et al*[10] showed that in the liver fibrosis model induced by

carbon tetrachloride, aspirin partially inhibited the expression of TGF-β and prevented translocation of NFκB into the nucleus.

The anti-inflammatory effects of aspirin are achieved by endogenous anti-inflammatory lipids known as "aspirin-triggering lipoxins" (ATLs). ATLs are derived from arachidonic acid (converted by COX-2 acetylation), and even low-dose aspirin can induce ATLs[11]. Interestingly, ATL analogues reduced the level of TGF-β and reversed the fibrosis process in a mouse model of pulmonary fibrosis induced by bleomycin[12]. Thus, the effect of aspirin in reducing liver fibrosis is mediated by its antithrombotic and/or anti-inflammatory properties.

Some studies have attempted to determine whether aspirin or other antiplatelet drugs play a role in the progression of liver fibrosis. In a rat model of nonalcoholic fatty liver disease, Fujita *et al*[13] reported that antiplatelet drugs, such as aspirin, ticlopidine and cilostazol, significantly reduced liver fibrosis. Wanless *et al*[14] also proved that in a rabbit model of liver sinus fibrosis induced by cholesterol and diethylstilbestrol, dipyridamole inhibited platelet aggregation and reduced fibrosis in *vitro*. Yoshida *et al*[15] recently reported that platelet activation directly promoted liver fibrosis, and aspirin prevented the progression of liver fibrosis in animal models.

In addition to animal studies, Poujol-Robert *et al*[16] reported that daily low-dose aspirin administration reduced the progression of liver fibrosis in patients with recurrent hepatitis C after liver transplantation. Our study found that aspirin reduced liver fibrosis in rats and that high-dose aspirin (300 mg/kg) was superior to low-dose aspirin (30 mg/kg) in terms of antifibrotic effects.

Current experimental evidence suggests that coagulation factors, particularly thrombin, are closely related to liver fibrosis. Thrombin, the major effector of the coagulation cascade, causes a series of cellular effects through a family of G protein-coupled receptors known as protease-activated receptors. Through these receptors, thrombin stimulates the proliferation of hepatic stellate cells, which are important mediators of liver fibrogenesis. Thrombin receptors are expressed in the liver, and during the process of acute and chronic liver injury, the expression of thrombin receptors is up-regulated. Heparin, through its interaction with antithrombin, induces a conformational change in the antithrombin molecule, which greatly facilitates the interaction between antithrombin and targets of serine proteases such as thrombin, as well as the factors Xa, IXa, XIa and XIIa[17]. Low molecular weight heparin induces inhibition of the coagulation cascade, and its potential beneficial effects in liver fibrosis have been evaluated[18]. In our study, enoxaparin was shown to reduce liver fibrosis in rats.

In conclusion, we successfully established a rat model of liver fibrosis after 8 wk and successfully established a rat model of liver cirrhosis after 12 wk induced by TAA. Our study clearly shows that aspirin and enoxaparin can reduce liver fibrosis in rats. However, it is unclear whether the antifibrotic mechanisms of the two drugs are directly related to their anticoagulant effects in preventing thrombosis, or to other mechanisms, Further investigations are needed using anticoagulants as potential treatment for liver fibrosis/cirrhosis.

ACKNOWLEDGMENTS

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COMMENTS

Background

Liver fibrosis is a hotspot of research. It is closely related to the chronic inflammatory response of the liver, and inflammation leading to liver fibrosis has been studied in detail. At present, several lines of evidence show that intrahepatic thrombosis is associated with liver fibrosis. A variety of chronic liver damage can eventually lead to endothelial injury and microvascular thrombosis, triggering inappropriate hepatocyte proliferation and fibrosis. Based on these observations, they hypothesized that routine use of antithrombotic drugs may prevent liver fibrosis.

Research frontiers

In the past, there was widespread belief that patients with liver cirrhosis experienced hypocoagulability. In recent years, however, this belief has been challenged. Studies found that patients with cirrhosis had hypercoagulability and even had a thrombus in some cases. There is evidence that coagulation is an important factor in the progression of cirrhosis. It is important to discuss the relationship among coagulation, anticoagulant therapy and liver fibrosis/cirrhosis.

Innovations and breakthroughs

Reports of studies on antithrombotic therapies and antiplatelet agents reducing liver fibrosis in animals are rare. In this study, the authors evaluated if aspirin and enoxaparin are beneficial in a thioacetamide-induced rat model of liver fibrosis.

Applications

Aspirin and enoxaparin can reduce liver fibrosis in a thioacetamide-induced rat model. They are beneficial in humans with liver fibrosis as well.

Terminology

Liver fibrosis and subsequent liver cirrhosis are common outcomes of chronic liver disease caused by various etiologies, with high morbidity and mortality.

Peer-review

The manuscript is very interesting, describing a study using a rat model, but reporting the results in humans is more complicated because we should consider other aspects, such as renal function. The manuscript is well written.

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ORIGINAL ARTICLE

Retrospective Study

Different clinical presentations of metachronous pulmonary metastases after resection of pancreatic ductal adenocarcinoma: Retrospective study and review of the literature

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Abstract

AIM

To analyze pancreatic cancer patients who developed metachronous pulmonary metastases (MPM) as a first site of recurrence after the curative-intent surgery.

METHODS

One-hundred-fifty-nine consecutive pancreatic ductal adenocarcinoma (PDAC) patients who underwent radical pancreatic surgery between 2006 and 2013 were included in this retrospective analysis. The clinical data including age, sex, grade, primary tumor location, pTNM stage, lymph node infiltration, microangioinvasion, perineural invasion, lymphovascular invasion, the therapy administered, and follow-up were all obtained from medical records. Further analysis covered only patients with metachronous metastases. Clinical and histopathological data (age, sex, grade, primary tumor location, pTNM stage, lymph node infiltration, microangioinvasion, perineural invasion, lymphovascular invasion, the therapy administered and follow-up) of patients with metachronous non-pulmonary metastases and patients with metachronous pulmonary metastases were statistically assessed. Disease-free survival (DFS) from pancreas resection until metastases onset and overall survival (OS) were calculated. Wilcoxon test, χ^2 test and survival functions computed by the Kaplan-Meier method were used. Statistical significance was evaluated by the log-rank test using SPSS. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Metachronous pulmonary metastases were observed in 20 (16.9%) and were operable in 3 (2.5%) of PDAC patients after a prior curative-intent surgery. Patients with isolated pulmonary metastases (oligometastases and multiple metastases) had estimated prior DFS and OS of 35.4 and 81.4 mo, respectively, and those with metachronous pulmonary metastases accompanied by other metastases had prior DFS and OS of 17.3 and 23.4 mo, respectively. Patients with non-pulmonary metastases had prior DFS and OS of 9.4 and 15.8 mo, respectively. Different clinical scenarios according to the presentation of MPM were observed and patients could be divided to three subgroups with different prognosis which could be used for the selection of treatment strategy: isolated pulmonary oligometastases, isolated multiple pulmonary metastases and pulmonary metastases accompanied by other metastases.

CONCLUSION

Surgery should be considered for all patients with isolated pulmonary oligometastases, but the risk of intervention has to be individually weighted for each patient.

Key words: Pancreatic cancer; Pulmonary metastases; Metachronous metastases; Surgical resection; Overall survival; Chemotherapy

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Core tip: Metachronous pulmonary metastases represent the most frequent extraabdominal site of recurrence following radical resection for pancreas cancer. Three different clinical scenarios according to the presentations of metachronous pulmonary metastases were observed in our cohort: isolated oligometastases, multiple pulmonary metastases and pulmonary metastases accompanied by other metastases. Patients with isolated pulmonary metastases have the longest disease-free survival and overall survival. Surgery should be considered for all patients with isolated oligometastases.

Lovecek M, Skalicky P, Chudacek J, Szkorupa M, Svebisova H, Lemstrova R, Ehrmann J, Melichar B, Yogeswara T, Klos D, Vrba R, Havlik R, Mohelnikova-Duchonova B. Different clinical presentations of metachronous pulmonary metastases after resection of pancreatic ductal adenocarcinoma: Retrospective study and review of the literature. *World J Gastroenterol* 2017; 23(35): 6420-6428 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6420.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6420

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignant disease with an increasing incidence[1]. Even though there is a long latency period between the emergence of a malignant cell clone and the death from metastatic disease, the majority of patients still present with either locally advanced or metastatic disease[2]. Only 15%-20% of patients have localized disease which may be considered surgically resectable at the time of diagnosis. An additional 20%-30% of patients have border-line resectable or locally advanced disease among whom one to two thirds could be potential candidates for curative surgery following a neoadjuvant therapy[3,4]. Finally, around 50% of patients have a metastatic disease at the time of diagnosis. The prognosis of PDAC patients after curative surgery is still relatively poor with the 5-year survival rate ranging between 15%-35%[5-7]. Up to 30% of PDAC patients die within the first year after radical surgery due to the disease progression and the mortality rate increases to around 60% by the end of the second year[8,9].

Although the most frequent site of metastases

in PDAC is the liver[10], among long-term PDAC survivors the most common site of metastases is the lung[5]. Since the pulmonary metastases usually occur in the context of general disease progression, chemotherapy is considered to represent the standard treatment option. However, with regard of PDAC long-term survivors, there is a subgroup of patients with metachronous pulmonary metastases being the only site of the disease recurrence, presenting an option for surgical treatment. The literature concerning the use of surgical therapy in the treatment of metachronous isolated pulmonary PDAC metastases is still very limited. Isolated late pulmonary metastases in PDAC have been reported as single cases[11-14], case series[15], and retrospective cohort studies[7,16-21]. Katz *et al*[5] in a study focusing on long-term survivors reported the highest rate (22%) of pulmonary metastases. Most recently, some retrospective analyses tried to define criteria based on the report derived of Thomford *et al*[22], including a disease-free survival (DFS) of more than 20 mo after the primary surgery, pulmonary metastases $\leqslant$ 16 mm, primary stage Ⅰ, good performance status and a technically operable tumor, for patients in whom the surgical resection of isolated metastases would be meaningful[18,22,23]. However, due to the low prevalence of metachronous pulmonary metastases reliable data regarding the clinical outcome and indications for the optimal surgical or medical treatment are currently limited.

The aim of the present study was to retrospectively analyze a homogeneous cohort of PDAC patients who underwent a surgery with curative intent between the years 2006 and 2013 in our center. The primary objective was the identification of patients with metachronous pulmonary metastases as the first site of a disease recurrence after surgery and the assessment of prior DFS, and an overall survival (OS) in this subgroup of PDAC patients compared to the patients with non-pulmonary metastases. Different clinical scenarios according to a type of pulmonary metastases could also be discerned.

MATERIALS AND METHODS

Patients

A cohort of 159 consecutive PDAC patients who underwent radical surgery between the years 2006 and 2013 in our center was included in this retrospective analysis. Histopathological diagnosis of PDAC was performed according to the previous standard classification, and all specimens were evaluated before the introduction of the Leeds protocol. The clinical data including age, sex, grade, primary tumor location, pTNM stage, lymph node infiltration, microangioinvasion, perineural invasion, lymphovascular invasion, the therapy administered, and follow-up were all obtained from medical records or from the Czech National Oncological Registry.

Disease free survival (DFS) was defined as the time between the date of surgery and the date of diagnosis of new disease recurrence. Criteria for new recurrence were applied according to previous studies[5,19]. The patients were followed in 3-mo intervals with clinical examination including CA 19-9 level assessment, and 6-mo intervals with cross-sectioning computed tomography (CT), or positron emission tomography (PET/CT) in case elevation CA 19-9 was noted[24,25]. Oligometastatic disease was defined as limited systemic metastatic tumors in which local ablative therapy could be curative[26-30]. As the study was focused to metachronous metastatic PDAC, patients without recurrence, who died in postoperative period (90 d), had R2 resections and those with incomplete data were excluded from DFS analysis. The Thomford criteria for pulmonary metastasectomy including controlled primary lesion, no extrapulmonary metastases present, metastases limited to one lung, patient being able to tolerate surgery were applied[22]. Consensus from a multidisciplinary team had to be obtained before surgery. OS was defined as the time between the date of primary surgery and death. Surviving patients were censored at the last follow-up on 31st December 2016.

Further analysis covered only patients with metachronous metastases. Clinical and histopathological data (age, sex, grade, primary tumor location, pTNM stage, lymph node infiltration, microangioinvasion, perineural invasion, lymphovascular invasion, the therapy administered and follow-up) of patients with metachronous non-pulmonary metastases and patients with metachronous pulmonary metastases (MPM) were statistically assessed. Patients with MPM were initially divided into two groups; metachronous isolated pulmonary metastases, and metachronous pulmonary metastases with metastases to other organs.

Statistical analysis

Wilcoxon test and χ^2 test were used for analysis of age and other parameters. The survival functions were computed by the Kaplan-Meier method and statistical significance was evaluated by the log-rank test using SPSS. A *P*-value of less than 0.05 was considered statistically significant.

This study has been approved by the institutional Review Board at the University Hospital Olomouc.

RESULTS

Study population

The study population consisted of 159 patients with resectable PDAC, *i.e.*, no evidence of clinically detectable metastases, cM0 (Supplementary Table 1). R0 and R1 resection rate was not formally assessed as the samples have been evaluated before the introduction of the Leeds protocol into clinical practice at our institution, and the results concerning

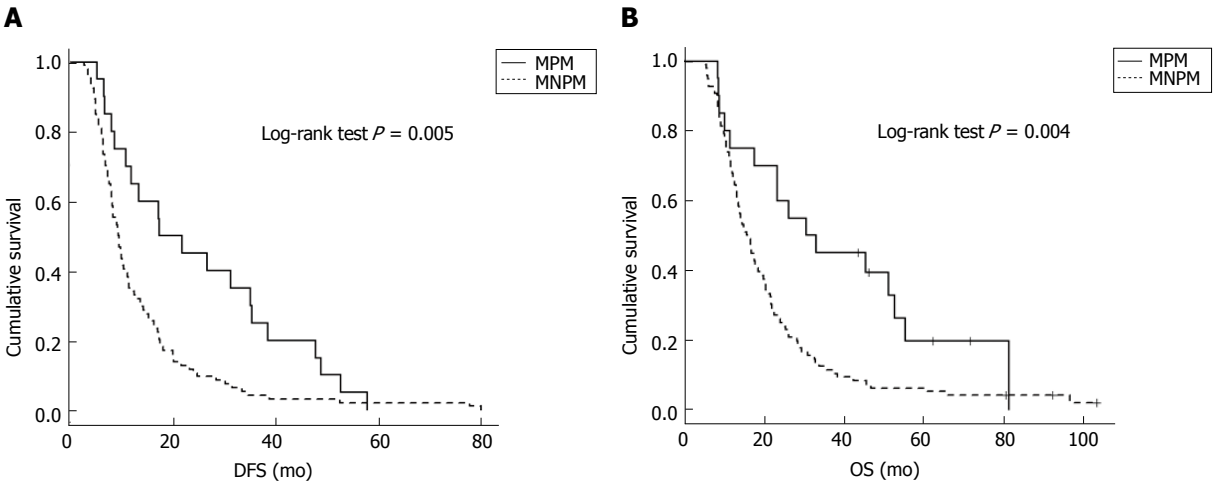


Figure 1 Prior disease-free survival (A) and overall survival (B) in patients with metachronous pulmonary metastases and metachronous non-pulmonary metastases. MPM: Metachronous pulmonary metastases; MNPM: Metachronous non-pulmonary metastases; DFS: Disease-free survival; OS: Overall survival.

Table 1 Prior disease-free survival and overall survival in patients with metachronous metastases

	n	DFS	OS
Non-pulmonary	96	9.48	16.19
Metachronous pulmonary metastases	20	19.58	31.81
Isolated pulmonary	5	35.44	81.36
Pulmonary and other metastases	15	17.26	23.37
Metastatic cohort	117	10.03	16.90
Entire cohort	159		17.59

DFS: Disease-free survival; OS: Overall survival.

R0 resection could have been overestimated.Two patients (1.3%) had neoadjuvant therapy consisting of concomitant radiotherapy with continuous 5-fluorouracil administration. Adjuvant chemotherapy consisting of nucleoside analogues (gemcitabine and 5-fluorouracil) was administered in 111 (69.8%) patients. Forty-one patients (25.8%) were excluded from further analysis due to no recurrence (n = 24), incomplete data (n = 14), postoperative death (n = 2) or R2 resection (n = 1). In total 1-, 2-, 3- and 5-years survival rates in entire cohort were 66.7%, 36.6%, 28.1% and 18.7%, respectively. The perineural invasion and lymph node infiltration were frequently identified (66.7% and 53.5%), while lymphovascular and microangioinvasion were identified less often (32.7% and 18.2%). The median DFS was 10 mo, and the median OS in entire cohort reached 17.6 mo. The most common sites of metastases within the first year included liver (57%), locoregional recurrence (37%), lymph nodes (22%), peritoneum (18%), and lung (10%), with multiple sites involved in 51%. In the second year, most frequent were locoregional (45%), lymph nodes (40%), liver (20%), and lung (20%), with involvement of multiple sites in 40%. In most patients who subsequently developed pulmonary metastases the primary procedures were head resections or total pancreatectomies with the predominant tumor location in pancreatic head (88.7%). Splenic vein obstructions were not identified. Further analysis was focused on patients who developed metachronous metastases.

Data of patients with metachronous metastases included in the further analysis are summarized in Supplementary Table 2. Most patients with isolated pulmonary metastases were diagnosed using PET/CT scan triggered by CA 19-9 elevation during the follow up. In total 20 patients (17%) had metachronous pulmonary metastases, almost all diagnosed with PET/CT scans. Adjuvant chemotherapy after primary pancreatic resection was used in 85% of MPM patients compared to 74% of non-pulmonary metastases group. Perineural invasion was higher in MPM patients, but the difference was not statistically significant (P = 0.057). Statistically significant differences between patients with metachronous pulmonary and non-pulmonary metastases were evident in the sex (P = 0.012) and survival rate (P = 0.004). Figure 1 displays prior DFS and OS in both subgroups, with statistically significant differences (P = 0.005 and 0.004, respectively).

Metachronous pulmonary metastases patients

In five patients (25%), all females, metastases were isolated to the lung. Pulmonary metastases along with multiple metastases to other organs were detected in 15 cases (75%). Figure 2 displays DFS and OS in both groups. Analysis is difficult due to very small numbers, but OS prolongation is of borderline statistical significance (P = 0.054). While the estimated median OS is similar in patients with non-pulmonary metastases and the entire cohort, the median OS is double in MPM (Table 1).

In the subgroup of metachronous isolated metastases (n = 5) estimated medians of DFS and OS are 35.4 and 81.4 mo, respectively (Table 1). There were two patients with multiple bilateral lesions and three patients with isolated oligometastatic recurrence.The patients with multiple bilateral lesions were treated with systemic chemotherapy (5-fluorouracil combined

Table 2 Previous reports of metachronous pulmonary metastases

Study	IPM	DFS	MPMR	SMT	SMU	OSPMD
Van den Broeck *et al*[16] RA 1998-2005	15	17.8	nr	nr	nr	nr
[1]Katz *et al*[5] nr 1990-2002	50	nr	nr	nr	nr	nr
[2]Arnaoutakis *et al*[17] RCCS 2000-2009	31	34	9	51	23	nr
[1]Thomas *et al*[18] RA 1992-2010	7	52.4	7	nr	nr	nr
[2]Wangjam *et al*[7] RA 1998-2007	28	12.7	nr	nr	nr	8.5
Downs-Canner *et al*[20] RA 2000-2010	49	14.9	5	44.4	29.9	nr
Yamashita *et al*[19] RCCS 2003-2012	14	nr	2	70	nr	nr
Kruger *et al*[21] RA 2002-2015	22	10.5	nr	nr	nr	25.5
Decoster *et al*[36] RCCS 2007-2013	22	nr	3	nr	nr	nr
Deeb *et al*[15] Cserie	5		0			
Brieau *et al*[14] CR	2		2			
Kotoulas *et al*[12] CR	1		1			
Moon *et al*[11] CR	1		1			
Sanjeevi *et al*[13] CR	1		1			
Kitasato *et al*[33] CR + national cases	1	156	1+20[3]			
Nakajima *et al*[23] CR + national cases			17[3]			
Present study Cserie 2006-2013	5	34	2	12+	nr	12+

[1,2]Same institution; [3]Mostly same cases. IPM: Isolated pulmonary metastases; DFS: Median of disease free survival; MPMR: Resected metachronous pulmonary metastases; SMT: Median survival of MPM treated; SMU: Median survival of MPM untreated; OSPMD: OS after diagnose of MPM; RA: Retrospective analysis; RCCD: Retrospective case control study; Cserie: Case serie; CR: Case report.

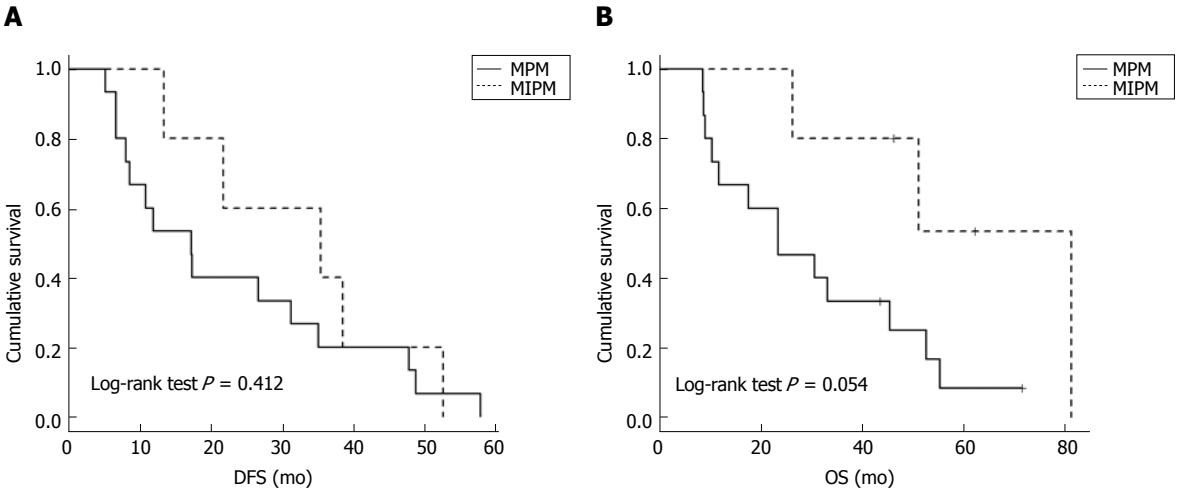


Figure 2 Prior disease-free survival (A) and overall survival (B) in patients with metachronous isolated pulmonary *vs* metachronous pulmonary and other metastases patients. MPM: Metachronous pulmonary metastases; MIPM: Metachronous isolated pulmonary metastases; DFS: Disease-free survival; OS: Overall survival.

with oxaliplatin or gemcitabine). In one patient thoracoscopic biopsy identified PDAC metastases 22 mo after initial pancreatic resection. The lesions were relatively stable and progressed slowly during the sequential lines of systemic chemotherapy, but brain metastases developed 80 mo after primary resection and the patient died 82.5 mo after resection. No intraabdominal recurrence was detected. The second patient was treated with gemcitabine and died of progressive disease 50.5 mo after the initial pancreatic resection.

Patients with metachronous isolated oligometastases (n = 3) could all be considered candidates for surgery. One patient was treated outside of our institution with gemcitabine and subsequently developed liver metastases and died 12.5 mo after diagnosis of MPM. Two patients in this group underwent uncomplicated pulmonary resections for isolated oligometastatic recurrence that manifested 34 and 52 mo after primary pancreatic resections and PDAC metastases were histologically verified. As of December 2016, these patients are still alive without recurrence with a second disease free interval after pulmonary resection of 11+ and 13+ mo, respectively.

In further analysis of patients with pulmonary metastases along with metastases to other organs (n = 15) there were 3 patients with pulmonary metastases and extrapulmonary metastases restricted to retroperitoneal paraaortal lymph nodes. These patients had DFS similar to patients with isolated pulmonary metastases (median 35 mo) and significantly longer OS (median 45 mo) compared to

the patients with pulmonary metastases accompanied by metastases to other organs (median DFS and OS of 13.5 mo and 19.6 mo, respectively). However, the data are very limited because of small numbers of patients.

The shortest DFS (9.5 mo) and OS (16.2 mo) were observed in patients with metachronous non-pulmonary metastases where the first location of metastatic disease were liver, peritoneum, locoregional, or lymph nodes.

DISCUSSION

PDAC may metastasize to any organ[10,26,31-34] by hematogenous, lymphogenic, and perineural route or by direct intracavitary spread[10,26]. Pulmonary metastases as the first site of disease recurrence after resections for PDAC can present as isolated oligometastases, isolated multiple metastases or in association to metastatic spread to other organs. The incidence of PDAC in Central Europe is high, and, in fact, the incidence in the Czech Republic is currently the highest in the world. To the best of our knowledge, the present study is the first large homogenous cohort of PDAC describing these differences on Central European population.

Patients with PDAC after primary radical multi-disciplinary treatment (surgical resection followed by adjuvant therapy) have a very high risk of distant recurrence. One-hundred-thirty-two patients (83.1%) in the present cohort developed metastases.

Even though metachronous pulmonary metastases after pancreatic resection are the most frequent extraabdominal site of PDAC recurrence, this presentation of recurrent disease is relatively rare, with the reported incidence of MPM as first site being between 3%-22%[5,7,16-19,21]. The highest rate of 22% was reported by Katz *et al*[5] in a highly selected cohort of patients of whom 77% had neoadjuvant therapy administered before primary resections. In the present study isolated pulmonary recurrence was observed in 4.2% of all cases of recurrent PDAC and 3.1% of entire cohort, in accordance with 3% rate of MPM reported previously by Arnaoutakis *et al*[17]. The present study indicates a low rate in unselected patients after radical primary treatment not exceeding 5%. Interestingly, isolated metachronous pulmonary metastases developed only in women, and female predominance was statistically significant in comparison of MPM and non-pulmonary metastatic patients.

Kamisawa *et al*[10] in an autopsy series in an unselected cohort of patients treated with chemotherapy, radiation, palliative or radical procedures focusing on hematogenous PDAC metastases reported a prevalence of clinically detectable pulmonary metastases in an absence of liver metastases in 12.3%. Hematogenous spread to the lungs was considered mostly for primary location in the tail and splenic vein obstruction. In the present cohort the location of primary tumor in the tail was only in 5% and no case with splenic vein obstruction was noted. In addition, remarkably high rate of perineural invasion in tumor analysis was reported in patients with MPM, but the difference was not statistically significant in comparison with non-pulmonary metastatic patients and, therefore, thus needs to be evaluated in larger cohort or meta-analysis.

Due to the low prevalence of MPM, the clinical course and optimal surgical or medical therapy are currently poorly defined. Early diagnosis of MPM in long-term survivors can bring the best therapeutic result and the highest chance for prolonged OS or even salvage therapy with curative intent. The rate of postoperative systemic therapy after primary resection in patients with pulmonary metastases varies between 85% in the present study to 100%[21]. MPM develop later than intraabdominal metastases and are the most frequent extraabdominal site across the studies[17-20]. In case of isolated pulmonary metastases the age at primary diagnosis is lower than in other groups (44-66 years, median 57).

MPM can present as three different clinical scenarios, in contrast to patients with non-pulmonary metastases as evidenced in the present cohort. Metachronous isolated pulmonary oligometastases with up to 10 small lesions in one lung are eligible for surgical therapy[7,22,23,26,29]. If the patient is fit for pulmonary resection, this represents the best therapeutic option with the potential of cure and longest OS (Table 1). In the present cohort only three cases were operable according to the previously established criteria[7,22,23]. One patient was managed with gemcitabine chemotherapy without surgery. Subsequent liver metastases developed and patient died 12.5 mo after diagnosis of MPM. Only two patients were radically operated and both are currently alive more than one year without recurrence. In cases of metachronous isolated multiple pulmonary metastases local therapy including surgery or radiotherapy has no rationale and systemic therapy is only potential option. Long-term survival was observed in both patients (50.5 and 82.5 mo), but these patients ultimately died of progressive disease or development of subsequent metastases typical for primary pulmonary carcinomas, in one case the brain metastases. The third scenario included metachronous pulmonary with metastases to other organs. In this group a very small subgroup of patients with MPM and retroperitoneal paraaortal lymph node metastases with DFS comparable with group of isolated MPM was identified. Prior DFS and OS in those patients were longer than in patients with local or regional recurrence.

In accordance with prior reports MPM patients in the present study had significantly longer DFS and OS compared to patients with non-pulmonary metastases. In patients with isolated pulmonary metastases median DFS ranges between 10.5 and 52.4 mo and OS between 23 and 92.3 mo[16-18,20,21]. The longest DFS of 156 mo after distal pancreatectomy was reported in

one case by Kitasato *et al*[35]. In the present study we observed median DFS of 34 mo in isolated pulmonary metastatic patients with an estimated median OS of 81.4 mo. Arnaoutakis *et al*[17], Thomas *et al*[18] and Downs-Canner *et al*[20] demonstrated in a total of 21 patients the feasibility and safety of surgical resection for MPM with significantly improved survival in comparison with non-surgical therapy. Reports from Japan include at least 23 cases with resected MPM of PDAC with long-term survival[19,23,35]. The most recent European study by Decoster *et al*[36] reported 22 MPM patients, 3 of whom were treated surgically, and indicated a female predominance. Our study also showed massive predominance of women in isolated pulmonary metastases. Female predominance in both studies is interesting, but there are currently no evidence based facts to explain this association. An effect of sex hormone could be one of the potential mechanisms, but without support by *in vitro* or *in vivo* data remains only speculative. Future studies should investigate whether MPM cases are characterized by specific molecular pathogenesis and biology that could explain this particular metastatic pattern.

The most obvious limitation of the present study is the low number of patients with MPM, but due to the rarity of this presentation, our study is comparable to the earlier published retrospective analyses (Table 2). Even though some of the results cannot be statistically significant because of the small number of patients, together with the previously published data on other small cohorts the findings seem to be highly relevant for clinical practice. Another limitation is the evaluation of resection margins. Because the present study is of retrospective nature, resection margins could not be reevaluated according to the currently used Leeds protocol due to a technically completely different method of resection specimen evaluation. On the other hand, the present study is unique because of the origin of the study population. To date, most studies were published on American PDAC population[16-20], with two studies originating from Japanese PDAC centers (with most of the patients overlapping between both studies)[23,35]. The present study on Central European population together with three similar retrospective analyses from Western Europe[16,21,36] published during last year are important to confirm the results previously reported elsewhere.

MPM, especially cases with isolated pulmonary metastases, are rare, and, obviously, prospective trials addressing the optimal management of these patients would be difficult to organize. Thus, for the foreseeable future retrospective analyses like the present report would remain the only source of information to guide the management of these patients.

Most importantly, in patients with metastatic PDAC only systemic therapy is considered in general practice. Despite some improvements resulting from the introduction of new regimens such as the combination of oxaliplatin, irinotecan, folinic acid and 5-fluorouracil or the combination of gemcitabine with nab-paclitaxel, the efficacy of systemic therapy is still very limited. The results of trials with targeted agents so far have been disappointing[37-39]. Contrary to the prevalent opinion in the medical and surgical oncology community, present data indicate that there is a subgroup of patients with metastatic PDAC who would benefit from surgical therapy. Future studies should identify clinical parameters and molecular biomarkers that would select this population and help with therapeutic decisions.

In conclusion, metachronous pulmonary metastases as first and only site of recurrence after resection for PDAC develop later and have better prognosis than other presentations of metastatic disease. OS is longer than in patients with other sites of metastases. Three different clinical scenarios according to the type of pulmonary oligometases with different prognosis and distinct therapeutic strategy were noted. The resection of pulmonary metastases was safe, with zero mortality and low morbidity. Patients who underwent pulmonary metastasectomy had longer survival compared to patients treated with chemotherapy or symptomatic therapy. Thus, surgery should be considered for all patients with solitary pulmonary metastases, but this has to be carefully weighted for each individual patient. Early diagnosis of recurrence may be viewed as precondition for successful management in these cases. Careful follow up with short (quarterly) interval and flexible PET/CT scanning can lead to early identification of recurrence.

COMMENTS

Background

Metachronous pulmonary metastases (MPM) represent the most frequent extraabdominal site of pancreatic ductal adenocarcinoma (PDAC) recurrence. The aim of this study was to analyze PDAC patients who underwent a curative-intent surgery with focus on MPM as a first site of recurrence and to assess disease free survival prior to the diagnosis of metastases and the overall survival.

Research frontiers

The manuscript addresses the very rare and clinically important entity of metachronous pulmonary metastases as a first site of pancreatic cancer recurrence with the need of an individualized therapeutic approach as the surgery significantly prolongs overall survival of patients. In particular, the data suggest that surgery should be considered for all patients with isolated pulmonary metastases, but the risk of intervention has to be individually weighted for each patient.

Innovations and breakthroughs

Metachronous pulmonary metastases as first and only site of recurrence after resection for PDAC develop later and have better prognosis than other presentations of metastatic disease. Overall survival is longer than in patients with other sites of metastases. Three different clinical scenarios according to the type of pulmonary oligometases with different prognosis and distinct therapeutic strategy were noted. The resection of pulmonary metastases was safe, with zero mortality and low morbidity. Patients who underwent pulmonary metastasectomy had longer survival compared to patients treated with chemotherapy or symptomatic therapy. Thus, surgery should be considered

for all patients with solitary pulmonary metastases, but this has to be carefully weighted for each individual patient.

Applications

The data suggested that metachronous pulmonary metastases of pancreatic cancer need an individualized therapeutic approach

Terminology

Metachronous metastases were defined as those cases in which lesions were observed later on or within 3 mo after the after curative-intent surgery.

Peer-review

The authors studied a special subgroup of PDAC patients who suffered from metachronous pulmonary metastasis after surgery in a Central European population. They identified three different patterns of metachronous pulmonary metastasis and found much better prognosis in such subgroup compared to non-pulmonary metastatic patients. This clinical finding is important and valuable to clinical practice.

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ORIGINAL ARTICLE

Retrospective Study

Evaluation of novel slim biopsy forceps for diagnosis of biliary strictures: Single-institutional study of consecutive 360 cases (with video)

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Institutional review board statement: This study was reviewed and approved by our institutional review board (Tokyo Medical University No. 3516).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Tokyo Medical University.

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Abstract

AIM

To evaluate the feasibility and reliability of endoscopic transpapillary bile duct biopsy for the diagnosis of biliary strictures.

METHODS

A total of 360 patients (241 men) who underwent endoscopic retrograde cholangiopancreatography for biliary strictures with biopsy from April 2012 to

March 2016 at Tokyo Medical University Hospital were retrospectively reviewed. This study was approved by our Institutional Review Board (No. 3516). Informed consent was obtained from all individual participants included in this study. The biopsy specimens were obtained using a novel slim biopsy forceps (Radial Jaw 4P, Boston Scientific, Boston, MA, United States).

RESULTS

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 69.6%, 100%, 100%, 59.1%, and 78.8%, respectively. The sensitivity was 75.6% in bile duct cancer, 64% in pancreatic cancer, 61.1% in gallbladder cancer, and 57.1% in metastasis. In bile duct cancer, a lower sensitivity was observed for perihilar bile duct stricture (68.7%) than for distal bile duct stricture (83.1%). In terms of the stricture lengths of pancreatic cancer, gallbladder cancer, and metastasis, a longer stenosis resulted in a better sensitivity. In particular, there was a significant difference between pancreatic cancer and gallbladder cancer ($P < 0.05$). One major complication was perforation of the extrahepatic bile duct with bile leakage.

CONCLUSION

Endoscopic transpapillary biopsy alone using novel slim biopsy forceps is feasible and reliable, but restrictive. Biopsy should be performed in consideration of the stricture level, stricture length, and cancer type.

Key words: Endoscopic transpapillary biopsy; Biopsy forceps; Biliary stricture; Bile duct cancer; Endoscopic retrograde cholangiography

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Core tip: Various radiological imaging procedures have been established as first-line modalities for detecting biliary strictures. However, a definitive diagnosis of biliary strictures can only be established by histocytological examination. At present, there are several histocytological sampling techniques such as aspiration cytology, brush cytology, aspiration needle biopsy, and forceps biopsy. However, the optimal sampling technique remains controversial. In this study, we found that transpapillary biopsy was feasible and reliable for diagnosing biliary strictures and should be performed in consideration of the stricture level, stricture length, and cancer type.

Yamamoto K, Tsuchiya T, Itoi T, Tsuji S, Tanaka R, Tonozuka R, Honjo M, Mukai S, Kamada K, Fujita M, Asai Y, Matsunami Y, Nagakawa Y, Yamaguchi H, Sofuni A. Evaluation of novel slim biopsy forceps for diagnosis of biliary strictures: Single-institutional study of consecutive 360 cases (with video). *World J Gastroenterol* 2017; 23(35): 6429-6436 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6429.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6429

INTRODUCTION

To date, transabdominal ultrasonography (TAUS), computed tomography (CT), magnetic resonance imaging (MRI), and magnetic resonance cholangiopancreatography (MRCP) have been established as the first-line modalities for detecting biliary strictures, being minimally invasive modalities. However, a definitive diagnosis of biliary strictures can only be established by histocytological examination, which is crucial for making a decision regarding the further management of this disease entity.

Endoscopic retrograde cholangiopancreatography (ERCP) is the most widely used close-up examination method using direct cholangiography for evaluating biliary strictures which allows transpapillary histocytological sampling from the strictures by aspiration cytology, brush cytology, and endobiliary forceps biopsy. Several studies have examined whether combining tissue sampling techniques can improve the diagnostic accuracy for biliary strictures during ERCP[1-4]. Theoretically, core tissue sampling using biopsy forceps can provide more sufficient information for diagnosing biliary strictures than cytology. However, as conventional biopsy forceps has been used in gastrointestinal tract diseases and has a thick and hard shaft, transpapillary insertion *via* the accessory channel of the duodenoscope is difficult.

Recently, a novel biopsy forceps which has a thin and soft shaft has been developed. Herein, we evaluated this novel slim biopsy forceps for the diagnosis of biliary strictures.

MATERIALS AND METHODS

A total of 360 patients (241 men) who underwent ERCP for biliary strictures with biopsy from April 2012 to March 2016 at Tokyo Medical University Hospital were retrospectively reviewed.

Patients were excluded in the following cases: (1) prior histological confirmation of malignancy; (2) postoperative biliary strictures; (3) ampullary tumor; and (4) less than 6 mo of follow-up in patients with negative malignant results. The final diagnosis was established by histopathological examination of tissues obtained endoscopically or surgically. If the histopathological diagnosis was negative for carcinoma, a clinical diagnosis was made from the clinical course for over 6 mo or more and several radiological image findings from various imaging modalities such as TAUS, CT, MRI, MRCP, and endoscopic ultrasonography (EUS). If biopsy forceps failed to achieve a definitive diagnosis and biliary strictures or filling defects were still indeterminate even with the use of various radiological modalities, peroral cholangioscopy was performed for a definitive diagnosis.

ERCP was performed using the standard technique with a duodenoscope. When biliary strictures were

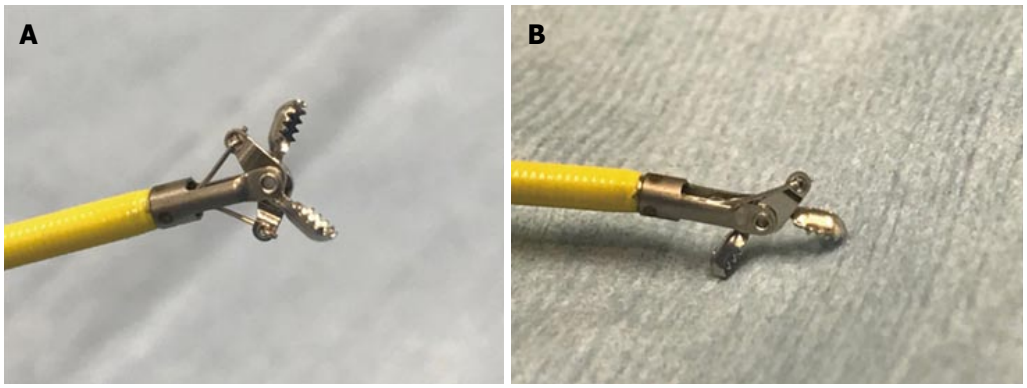


Figure 1 Biopsy forceps. A: Radial Jaw 4P; B: Swing function.

Table 1 Patient characteristics

Patient characteristics	
Number of patients	360
Age [mean ± SD (range), yr]	71 ± 11.8 (17-95)
Sex, male/female	241/119
Disease (number of patients)	
Bile duct cancer	132
Pancreatic cancer	86
Gallbladder cancer	18
Metastasis	14
Benign stricture	110

identified under radiographic guidance, endoscopic sphincterotomy (EST) was performed before tissue sampling. The biopsy specimens were obtained using a novel slim biopsy forceps (Radial Jaw 4P, Boston Scientific, Boston, MA, United States) (Figure 1A, video 1). The forceps was transpapillary advanced to the distal end of the stricture alongside the guidewire. The obtained specimen was immediately fixed in 10% formalin. All procedures were performed by operators with experience of more than 100 ERCP cases per year.

This study was approved by our Institutional Review Board (No. 3516) and informed consent was obtained from all individual participants included in this study.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were calculated. Statistical analyses were performed using StatMate Ⅲ (ATMS, Tokyo, Japan). A *P*-value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

The characteristics of the patients are shown in Table 1. Their median age was 71 ± 11.8 years (range: 17-95 years). The diseases were bile duct cancer in 132 patients, pancreatic cancer in 86, gallbladder cancer in 18, metastasis of other cancers in 14, and benign biliary stricture in 110. Among the patients with metastases, the primary sites were as follows: colon cancer in 3 patients, gastric cancer in 2, pancreatic cancer in 2, lung cancer in 2, breast cancer in 2, ovarian cancer in 2, and cholangiocellular carcinoma in 1.

Sample collection rate

The biopsy forceps could be inserted *via* the papilla after EST, and the specimens evaluated were collected from all 360 patients (technical success: 100%), with 820 bites in total (range: 1-6 bites). The mean number of biopsies was 2.28 ± 0.91 (Table 2).

Overall results

The sensitivity, specificity, PPV, NPV, and accuracy were 69.6%, 100%, 100%, 59.1%, and 78.8%, respectively (Table 2). The sensitivity was 75.6% in bile duct cancer, 64% in pancreatic cancer, 61.1% in gallbladder cancer, and 57.1% in metastasis (Table 3).

Type of cancers

The examination results for bile duct cancer are shown in Table 4. According to the stricture level of bile duct cancer, the sensitivity was 68.7% in the perihilar bile duct and 83.1% in the distal bile duct; 95% in the middle and 76.7% in the lower. Furthermore, comparing the type of stricture with space that the biopsy forceps could open with the type of stricture without space in the lower bile duct (Figure 2), the sensitivity with an open space was 84.6% and that without an open space was 64.7%.

The examination results for extrinsic neoplasms are shown in Table 5. As for pancreatic cancer according to the stricture level, the sensitivity was 45.5% in the middle bile duct and 66.7% in the lower bile duct. In addition, according to the localization of the primary tumor, the sensitivity was 68.4% in pancreatic head cancer and 30% in pancreatic body cancer. As for gallbladder cancer according to the stricture level,

Table 2 Overall diagnostic ability in all cases (*n* = 360)

Sample collection rate	
Total number of biopsies	820
Mean number of biopsies, ± SD (range)	2.28 ± 0.91 (1-6)
Overall results	
Sensitivity	69.6%
Specificity	100%
PPV	100%
NPV	59.1%
Accuracy	78.8%

PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Overall sensitivity for malignancy

Diagnosis	*n*	Sensitivity
Bile duct cancer	100/132	75.6%
Pancreatic cancer	55/86	64.0%
Gallbladder cancer	11/18	61.1%
Metastasis	8/14	57.1%

Table 4 Sensitivity of biopsy forceps in bile duct cancer

	n	Sensitivity
Total	100/132	75.60%
Site of stricture		
Perihilar bile duct	46/67	68.7%
Distal bile duct	54/65	83.1%
Middle	21/22	95%
Lower	33/43	76.7%
(Open space +	22/26	84.6%
(Open space −	11/17	64.7%

Table 5 Sensitivity of biopsy forceps in extrinsic neoplasms

	n	Sensitivity
Pancreatic cancer	55/86	64.0%
Site of stricture		
Middle bile duct	5/11	45.5%
Lower bile duct	50/75	66.7%
Localization of tumor		
Head	52/76	68.4%
Body	3/10	30.0%
Gallbladder cancer	11/18	61.1%
Site of stricture		
Perihilar bile duct	6/11	54.5%
Distal bile duct	5/7	71.4%
Metastasis	8/14	57.1%
Site of stricture		
Perihilar bile duct	4/6	66.7%
Distal bile duct	4/8	50.0%

Table 6 Mean stricture lengths in extrinsic neoplasms

	Positive (mm)	Negative (mm)	*P* value
Pancreatic cancer	16.9	13.5	< 0.05
Gallbladder cancer	22	13.4	< 0.05
Metastasis	17.1	13.2	NS

NS: Not significant.

the sensitivity was 54.5% in the perihilar bile duct and 71.4% in the distal bile duct. As for metastasis according to the stricture level, the sensitivity was 66.7% in the perihilar bile duct and 50% in the distal bile duct.

Length of stricture in cancers

Apart from the stricture level, the mean stricture lengths were 16.9 mm, 22 mm, and 17.1 mm in the biopsy-positive group and 13.5 mm, 13.4 mm, and 13.2 mm in the biopsy-negative group for pancreatic cancer, gallbladder cancer, and metastasis, respectively (Table 6). The biopsy-positive group tended to have a longer stricture length than the biopsy-negative group. In particular, there was a significant difference between pancreatic cancer and gallbladder cancer ($P < 0.05$).

Procedure-related adverse events

There were no procedure-related adverse events, although there was 1 patient with perihilar bile duct cancer in whom perforation of the distal bile duct with bile leakage was observed. The condition was improved only by temporary endoscopic nasobiliary drainage tube placement.

DISCUSSION

In this study, we found that transpapillary biopsy using a novel slim biopsy forceps could be easily and safely performed and was effective for diagnosing biliary strictures.

At present, there are several cytological and histological sampling techniques such as aspiration cytology, brush cytology, aspiration needle biopsy, and forceps biopsy. However, the optimal sampling technique remains controversial. The ideal technique for obtaining tissue for a definitive diagnosis of malignant biliary strictures should be simple, safe, and effective with a high sensitivity and specificity.

Although bile cytology is easy, its accuracy range is very low (6%-32%)[5,6]. Mohandas *et al*[7]. described that bile cytology with dilation of biliary strictures up to 10-Fr during ERCP enhanced its sensitivity by 63% compared with the 27% sensitivity of bile cytology without dilation. Regarding brush cytology, although this can be undertaken relatively easily and safely in a short time, its sensitivity is still considered to be low (30%-57%)[1-3]. Farrell *et al*[8] reported that the sensitivity of brush cytology increased by 85% after stricture dilation and endoscopic needle aspiration compared with the 57% sensitivity of brush cytology alone. In contrast, de Bellis *et al*[9] suggested that

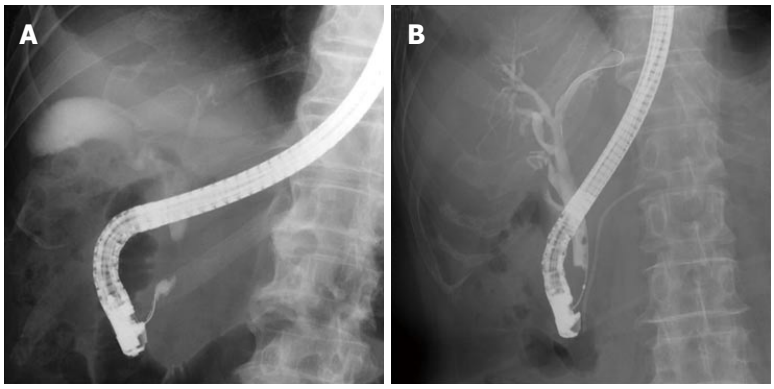


Figure 2 Fluoroscopic images during endoscopic retrograde cholangiopancreatography. A: Stricture with an open space; B: Stricture without an open space.

Table 7 Characteristics of studies evaluating bile duct biopsies

Study	Year	Sample size (*n*)	Sensitivity, %
Pugliese *et al*[1]	1995	94	53
Ponchon *et al*[3]	1995	233	43
Howell *et al*[10]	1996	28	15
Sugiyama *et al*[11]	1996	52	81
Jailwala *et al*[2]	2000	133	37
Rösch *et al*[12]	2004	50	36
Kitajima *et al*[13]	2007	60	65
Weber *et al*[14]	2008	58	53
Our case	2016	360	70

stricture dilation did not improve the sensitivity of brush cytology for cancer detection. Hence, the efficacy of stricture dilation for diagnosis is still controversial.

On the other hand, transpapillary forceps biopsy has not been widely used because it is thought to be technically difficult, resulting in a waste of time. Moreover, its sensitivity is very variable (15%-81%)[1-3,10-14] (Table 7). In 2002, de Bellis *et al*[9] summarized 502 previously reported cases and found that the sensitivity and specificity of transpapillary forceps biopsy were 56% (43%-81%) and 97% (90%-100%), respectively. Their report showed that the diagnostic performance of forceps biopsy was higher than that of other tissue sampling methods[15,16]. Furthermore, forceps biopsy is useful not only for the diagnosis of malignant/benign tumors but also for the selection of therapeutic methods (*e.g.*, chemotherapy) based on the histological types of cancer, and the judgment of the superficial intraductal spread of bile duct cancer.

In the present study, we used a novel slim biopsy forceps (Radial Jaw 4P, Boston Scientific) (Figure 1A). The Radial Jaw series are the world's first disposable biopsy forceps released in 1991. The first type of Radial Jaw had a diameter of 2.2 mm and had tiny teeth around the cup. After repeated improvements, the present Radial Jaw 4P was released in 2011. Radial Jaw 4P has a thinner shaft of 1.8 mm, a wire with a sheath to pass through the scope smoothly, and an open width/angle of 5.4 mm/150°. In addition, its thinner cup increases sampling capacity and its swing function helps achieve tangential biopsy (Figure 1B). Sufficient tissue samples for a definitive diagnosis, which include the mucosal cancer or invasive cancer below the bile duct epithelium, can be obtained using this forceps (Figure 3). Hence, the present Radial Jaw 4P may be more appropriate for performing optimal biopsy. In fact, in the present study, this novel forceps could collect adequate specimens for evaluation from all of the patients. Thus, our data showed a higher sensitivity than previous studies of bile duct biopsies (Table 7), suggesting that this novel slim biopsy forceps appears suitable as a tissue sampling device for the diagnosis of biliary strictures.

Regarding bile duct cancer, a lower sensitivity was observed for the perihilar bile duct stricture than for the distal bile duct stricture. This may be due to the narrow and smooth stricture and the distance from the papilla, preventing the biopsy forceps from being able to sufficiently open the stricture and precisely hitting the tumor. In contrast, a better sensitivity was observed for the distal bile duct stricture, particularly in the middle bile duct. For the lower bile duct, a better sensitivity was observed for the stricture with an open space than for the stricture without an open space. In the stricture without an open space for a biopsy forceps to open sufficiently, adequate and accurate biopsy tissue samplings from the target site may be slightly difficult.

Thus far, some reports have shown that the combination of brush cytology and forceps biopsy improved sensitivity[3,8,10]. However, a recent systematic review and a meta-analysis have suggested that the combination of brush cytology and forceps biopsy only modestly increased sensitivity[17]. These combination techniques may be ideal because the tumor is usually exposed to the bile duct surface, allowing brush cytology to obtain cells from the entire lumen surface in bile duct cancer, particularly in severe strictures of the perihilar bile duct and lower bile duct without an open space. On the other hand, the sensitivity of biopsy in pancreatic cancer was lower than that in

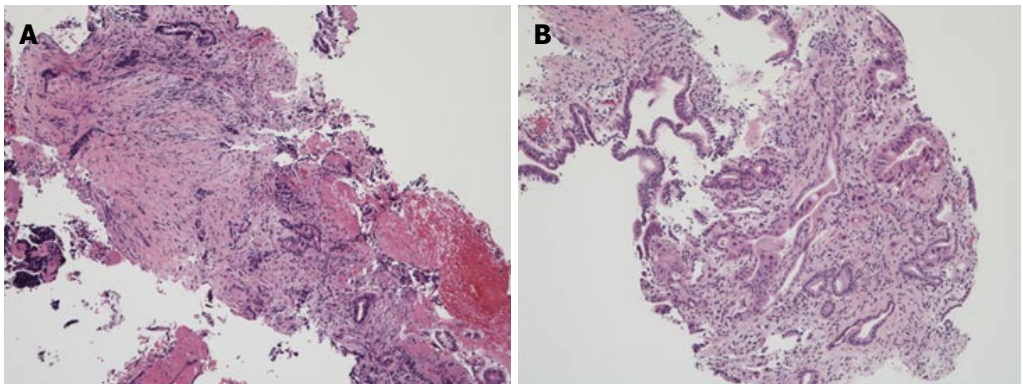


Figure 3 Biopsy specimens obtained using Radial Jaw 4P. The biopsy specimen obtained was sufficient and included adenocarcinoma. A: Bile duct cancer; B: Pancreatic cancer (HE. stain, × 10).

bile duct cancer similarly to previous reports[1,3,11,18]. Theoretically, tumor cells from the surface of the bile duct in pancreatic cancer appear to be fewer than tumor cells in bile duct cancer. Thus, sensitivity in pancreatic cancer as well as in gallbladder cancer and metastases, namely, "extrinsic neoplasms", appears to be limited even with the use of additional brush cytology in combination with biopsy forceps. Interestingly, some reports[3,19] have also demonstrated that improvement of sensitivity cannot be expected with the combination of aspiration cytology and brush cytology for cancers with a bile duct stricture by extrinsic exclusion.

Furthermore, the sensitivity for a stricture in pancreatic body cancer was lower than that for a stricture in pancreatic head cancer. This is the reason why the bile duct stricture in pancreatic body cancer, which even shows a biliary stricture that is far from the bile duct, occurs not by direct invasive reaction but as a secondary fibrous cicatricial stricture by stromal reaction. In addition, many pancreatic body cancers occur in the middle bile duct stricture, resulting in a lower sensitivity for the middle bile duct stricture.

In the present study, we showed that a longer stricture had a better sensitivity for diagnosis. This suggests an increase in the positive diagnostic rate of forceps biopsy because the contact area with the bile duct of extrinsic cancer becomes wider according to the stricture length. Hence, to increase the diagnostic accuracy, multiple biopsies appear necessary for the diagnosis of a shorter biliary stricture due to extrinsic cancers.

Recently, EUS-FNA has been introduced for evaluating biliary strictures[12,20]. EUS-FNA may be a preferable method for tissue sampling of neoplasms of extrinsic origin such as pancreatic cancer, gallbladder cancer, and metastasis after obtaining negative or nondiagnostic ERCP tissue sampling results. On the other hand, ERCP-based tissue sampling may be better for bile duct cancer of biliary epithelial origin.

Pancreatitis and cholangitis are generally observed, although their incidence is low and their severity is mild in most cases requiring only conservative treatment[2,21]. Although rare, bile duct perforation can occur during forceps biopsy[1]. Repeated biopsies at the same site may account for the perforation. In the present study, 1 case of perforation of the extrahepatic bile duct with bile leakage occurred during forceps biopsy in a patient with perihilar bile duct cancer. Although Radial Jaw 4P is a soft biopsy forceps, care should be taken during its usage.

Various tips in performing forceps biopsy have been reported. Of interest is the study of Tamada *et al*[22] which suggested factors for a successful bile duct biopsy under percutaneous transhepatic cholangioscopy (PTCS) from the viewpoint of the growth type of bile duct cancers. They recommended that 2 biopsy samples should be obtained from the mass in polypoid-type bile duct cancers and from the margin in stenotic-type bile duct cancers with a dilated vessel. Moreover, 3 biopsy samples should be obtained from the margin of the stricture in stenotic-type bile duct cancers without a dilated vessel, and biopsy specimens should be obtained from within the stricture in pancreatic cancer. Hence, transpapillary bile duct biopsy is performed under fluoroscopic guidance and is not similar to the biopsy performed under direct vision by PTCS. Thus, more than 3 biopsy specimens may be necessary and the biopsy sites, such as the margin of the stricture or within the stricture, should be considered according to the types of cancers such as cancers of biliary epithelial or extrinsic origin.

The limitations of this study were its retrospective nature, the lack of a control group, and the inclusion of a single-center experience.

In conclusion, endoscopic transpapillary biopsy using a novel slim biopsy forceps is feasible and reliable, but is a restrictive method for tissue sampling of malignant biliary strictures. When performing the biopsy, it is necessary to consider the stricture levels, stricture lengths, and types of cancers such as cancers of biliary epithelial or extrinsic origin to improve the diagnosis of malignant biliary strictures.

COMMENTS

Background

There are several cytological and histological sampling techniques for a definitive diagnosis of malignant biliary strictures. However, the optimal sampling technique remains controversial. The ideal technique should be simple, safe, and effective with a high sensitivity and specificity.

Research frontiers

Transpapillary forceps biopsy has not been widely used because it is thought to be technically difficult, resulting in a waste of time. Moreover, its sensitivity is very variable (15%-81%). In the present study, a novel slim biopsy forceps could collect adequate specimens for evaluation from all of the patients and our data showed a higher sensitivity (70%) than previous studies of bile duct biopsies.

Innovations and breakthroughs

It is necessary to consider the stricture levels, stricture lengths, and types of cancers such as cancers of biliary epithelial or extrinsic origin to improve the diagnosis of malignant biliary strictures.

Applications

Endoscopic transpapillary biopsy using a novel slim biopsy forceps is feasible and reliable.

Terminology

Endoscopic transpapillary biopsy for diagnosis of biliary strictures should be performed in consideration of the stricture level, stricture length, and cancer type.

Peer-review

The paper deals with the problem of obtaining reliable biopsies from lesions in the biliary tree. Evidently the methods described here yields a progress in this field. The paper is well written. This is good study exploring the efficacy and diagnostic value of a new slim forceps by Boston. The manuscript is generally interesting and offers useful clinical information to those who carry out endoscopic retrograde cholangiopancreatography.

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ORIGINAL ARTICLE

Retrospective Study

Evaluation of the relationship between hepatocellular carcinoma location and transarterial chemoembolization efficacy

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Institutional review board statement: The study was reviewed and approved by the Nippon Medical School Institutional Review Board.

Informed consent statement: Patients were not required to give informed consent for this study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Nippon Medical School.

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Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at genji@nms.ac.jp. Participants gave informed consent for data sharing.

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Abstract

AIM

To evaluate the relationship between the location of hepatocellular carcinoma (HCC) and the efficacy of transarterial chemoembolization (TACE).

METHODS

We evaluated 115 patients (127 nodules), excluding recurrent nodules, treated with TACE between January 2011 and June 2014. TACE efficacy was evaluated according to mRECIST. The HCC location coefficient was calculated as the distance from the central portal portion to the HCC center (mm)/liver diameter (mm) on multiplanar reconstruction images rendered (MPR) to visualize bifurcation of the right and left branches of the portal vein and HCC center. The HCC location coefficient was compared between complete response (CR) and non-CR groups in Child-Pugh grade A and B patients.

RESULTS

The median location coefficient of HCC among all nodules, the right lobe, and the medial segment was significantly higher in the CR group than in the non-CR group in the Child-Pugh grade A patients (0.82 *vs* 0.62, $P < 0.001$; 0.71 *vs* 0.59, $P < 0.01$; 0.81 *vs* 0.49, $P < 0.05$, respectively). However, there was no significant difference in the median location coefficient of the HCC in the lateral segment between in the CR and in the non-CR groups (0.67 *vs* 0.65, $P > 0.05$). On the other hand, in the Child-Pugh grade B patients, the HCC median location coefficient in each lobe and segment was not significantly different between in the CR and in the non-CR groups.

CONCLUSION

Improved TACE efficacy may be obtained for HCC in the peripheral zone of the right lobe and the medial segment in Child-Pugh grade A patients.

Key words: Hepatocellular carcinoma location; Transarterial chemoembolization efficacy; Child-Pugh; Modified response evaluation criteria in solid tumors; Central zone; Peripheral zone

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Core tip: The relationship between hepatocellular carcinoma (HCC) location and transcatheter arterial chemoembolization (TACE) efficacy was evaluated. In Child-Pugh A, the median location coefficient of HCC among all nodules, right lobe, and medial segment was significantly higher in the complete response (CR) group than in the non-CR group, with no significant differences in the lateral segment. In Child-Pugh B, the median location coefficient of HCC in each lobe and segment was not significantly different between the two groups. Therefore, improved TACE efficacy may be obtained for HCC in the peripheral zone of the right lobe and medial segment in Child-Pugh A patients.

Miki I, Murata S, Uchiyama F, Yasui D, Ueda T, Sugihara F, Saito H, Yamaguchi H, Murakami R, Kawamoto C, Uchida E, Kumita SI. Evaluation of the relationship between hepatocellular carcinoma location and transarterial chemoembolization efficacy. *World J Gastroenterol* 2017; 23(35): 6437-6447 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6437.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6437

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases worldwide, and it is a lethal tumor whose prognosis largely depends on tumor stage at diagnosis and patient access to radical treatment[1,2]. Management of HCC has been standardized according to clinical staging systems, such as the Barcelona Clinical Liver Cancer (BCLC) classification, in which patients are stratified by tumor stage and underlying liver disease. According to the BCLC staging system, transarterial chemoembolization (TACE) is the current standard of care for BCLC stage B patients. Stage B is defined as an intermediate-stage disease[3] that involves highly heterogeneous patients who have Child-Pugh grade A or B liver function with four or more tumors irrespective of size or two to three tumors larger than 3 cm in diameter, in the absence of cancer-related symptoms, macrovascular invasion, or extrahepatic spread[4]. Several studies and well-designed randomized trials have shown that TACE has a positive effect on patient outcome and survival[5-7]. Although many studies have reported on several prognostic factors for stage-B HCC patients, tumor number, maximal tumor diameter, Child-Pugh score, and tumor response to TACE are fundamentally strong predictors of survival in patients with initially unresectable HCC[8,9]. Complete necrosis following repeated TACE was a significant independent predictor of favorable survival after the first TACE[10].

Most HCC patients have some degree of cirrhosis. Regardless of etiology, compared with noncirrhotic livers, cirrhotic livers show right-lobe and medial-segment atrophy and lateral-segment and caudate-lobe hypertrophy[11]. This pattern suggests that microcirculatory changes play a role in the development of cirrhosis within segments. Indeed, an *in vivo* microscopic study[12] demonstrated differences in the blood flow in central and peripheral regions, indicating a direct and indirect reduction in the blood flow to the periphery of the main lobe.

Histopathological investigations of HCCs that were resected after TACE have shown that the most viable tissue is located at the periphery of the tumor[13]. The efficacy of TACE is limited by the dual blood supply (artery and portal vein) of liver tumors and collateral

arterial supply after TACE, which make it difficult to achieve complete necrosis of tumor tissue because of insufficient tumor ischemia. However, complete necrosis is sometimes obtained in the long-term. Therefore, before performing this procedure, it is very important for the physician to predict whether TACE will be effective, particularly while considering other therapeutic modalities.

Thus, the aim of this retrospective study was to evaluate the relationship between the location of the HCC, in particular, the location close to the peripheral or hepatic portal portion of the liver, and the efficacy of TACE.

MATERIALS AND METHODS

This retrospective study was conducted with the approval of our institutional review board. The requirement for informed consent was waived.

Patients

Between January 2011 and June 2014, 777 patients with HCC were treated with TACE in our institution. The eligibility criteria for the study were as follows: (1) diagnosis of HCC based on histologic findings, findings on dynamic contrast-enhanced multi-detector computed tomography (MDCT), or magnetic resonance imaging (MRI) performed in our institution in accordance with the American Association for the Study of Liver Disease[14] and/or findings of cirrhosis; (2) no indications for hepatic resection, percutaneous ethanol injection therapy, liver transplantation, or thermal ablation therapy; (3) presence of a bidimensionally measurable hepatic lesion; (4) adequate liver function (serum total bilirubin level < 2.0 mg/dL and Child-Pugh grade A or B); (5) adequate bone marrow function (leukocyte count > 3000 cells/mm^3 and platelet count > 50000 cells/mm^3); (6) adequate renal function (serum creatinine concentration $\leqslant$ 1.2 mg/dL); (7) no ascites; (8) absence of vascular invasion; (9) technical success of TACE; and (10) MDCT or MRI performed before TACE and at least 6 mo after TACE.

The exclusion criteria were as follows: (1) recurrent HCC; (2) tumor size > 7 cm; (3) inadequate iodized oil accumulation in the target lesion because of large tumor size or anastomosis with vital vessels; (4) balloon-occluded TACE; and (5) drug-eluting bead TACE. Overall, 115 patients with 127 HCC nodules were included in our study.

TACE procedure

Experienced interventional radiologists performed all TACE procedures. After administering local anesthesia in all patients, we punctured the femoral artery using the Seldinger technique. A 4-French sheath (Super Sheath; Medikit, Miyazaki, Japan) was inserted via the femoral artery. After performing arterial portography and hepatic arteriography to confirm feeding arteries to HCC, we inserted a 2.0-French microcatheter (Gold Crest-MRT; Koshin Medical, Tokyo, Japan) into the feeding arteries. Then, an emulsion of anticancer drugs [cisplatin (50 mg/m^2, up to 100 mg) or epirubicin (30 mg/m^2)] and iodized oil [Lipiodol (the sum of the major axis of the tumor up to 15 mL) Ultra Fluid, Laboratoires Guerber, Aulnay-sous-Bois, France] was injected slowly under fluoroscopic guidance until the vascular bed of the target nodule was filled with the emulsion. We performed embolization with 2 mm pieces of gelatin sponge (Gelpart; Nippon Kayaku, Tokyo, Japan) following TACE.

Imaging evaluation of TACE efficacy

Dynamic contrast-enhanced CT or MRI was used to evaluate the effect of TACE 6 mo after and on a per nodule basis, according to the Modified Response Evaluation Criteria in Solid Tumors (mRECIST)[15]: complete response (CR) = disappearance of any intratumoral arterial enhancement in all target lesions; partial response (PR) = at least a 30% decrease in the sum of the diameter of viable target lesions; stable disease (SD) = any cases that did not qualify as either PR or progressive disease; progressive disease (PD) = an increase by at least 20% of the sum of the diameters of the viable target lesions. The evaluation was performed by two independent and blinded observers. After individual evaluation, the findings were disclosed, and any discrepancy in the findings was discussed by both the observers.

Measurement of HCC location

The location of the HCC was determined using the most recent dynamic contrast-enhanced MDCT (within 1 mo) before TACE. The MDCT row data of the arterial or portal phase of pre-TACE were transferred to a workstation (OsiriX 7.0; Pixmeo, Bernex, Switzerland). The location coefficient of HCC was defined as follows: multi-planar reconstructed (MPR) images were rendered to visualize the bifurcation of the right and left branches of the portal vein and the center of the HCC, which was defined as the intersection of the long axes with the short axes in the maximum cross-section of the target nodule. Next, we traced the straight line through the bifurcation of the right and left branches of the portal vein to the center of the target nodule and the peripheral surface of the liver using MPR images (Figure 1). The location coefficient of the HCC was determined as a ratio of the distance from an internal surface of the liver to the center of the HCC/diameter of the liver on the traced line. An increased location coefficient indicates a more peripheral nodule. The bifurcation was the baseline point of measurement of the HCC location because the bifurcation had few

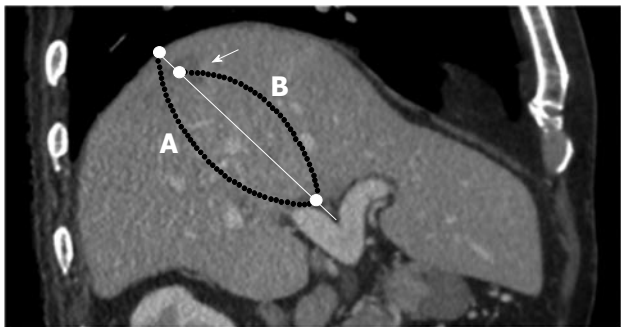


Figure 1 A 73-year-old man with hepatocellular carcinoma. An MPR image of the arterial phase shows a well-defined enhanced mass in segment 7 (arrowhead) and the bifurcation of the right and left branches of the portal vein. HCC location coefficient = distance from the medial surface of liver to the central HCC tumor (B)/diameter of the liver (A). MPR: Multi-planar reconstructed images; HCC: Hepatocellular carcinoma.

portal venous variations.

Evaluation of efficacy due to HCC location

The location coefficient of the HCC was compared between the CR group and non-CR group, including the PR, SD, and PD subgroups, among all nodules and each lobe or segment in Child Pugh A and B patients. The relationship between the efficacy of TACE and the location of the HCC was assessed.

Statistical analysis

All statistical analysis was performed using R 2.15.1 (CRAN: the Comprehensive R Archive Network at http://cran. R-project.org/). We compared the location coefficient of the HCC between the CR group and non-CR group using the Mann-Whitney *U* test. Moreover, receiver operating characteristic (ROC) curve analyses were used to determine the appropriate cut-off points of the location coefficient to predict the efficacy of TACE. In all comparisons, the results of the statistical analyses were considered significant at a *P*-value < 0.05.

RESULTS

The median patient age was 73 year (range, 49-92 year; 88 men and 27 women). The tumor size ranged from 5.0 mm to 56.7 mm (mean ± standard deviation: 14.00 ± 10.30) (Table 1). Other patient characteristics are presented in Table 1. Among the 127 HCC nodules, 81 achieved CR 6 mo after TACE according to the mRECIST criteria. Local recurrences were classified into the PR, SD, or PD subgroup in 46 nodules. Twenty-six nodules were classified as PR, 13 as SD, and 7 as PD (Table 2). The median recurrence time was 2 mo. Table 2 summarizes the efficacy of TACE for each segment of the liver where the HCC nodule presented in Child-Pugh grade A and B patients.

Table 1 Baseline characteristics (*n* = 115 patients, 127 nodules) *n* (%)

Characteristics	Values
Age	
Median (range)	73 (49-92)
Sex	
Male/Female	88 (76.5)/27 (23.5)
Etiology	
Hepatitis B/C/B + C	13 (11.3)/85 (73.9)/2 (1.7)
Alcohol	10 (8.7)
NASH	2 (1.7)
PBC	3 (2.6)
Child-Pugh class	
A/B	99 (86.1)/16 (13.9)
Location of target nodules	
Anterior segment	39 (30.7)
Posterior segment	37 (29.1)
Medial segment	27 (21.3)
Lateral segment	24 (18.9)
Size of target nodules (mm)[1]	14.00 ± 10.30 (5.0-56.7)
Chemotherapeutic drug	
Cisplatin/Epirubicin	87 (68.5)/40 (31.5)

[1]Values are continuous data and are presented as the means ± SD. Numbers in parentheses are the ranges. NASH: Non-alcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

patients.

Correlation between HCC location and efficacy

The median location coefficient of HCC was significantly higher in the CR group than the non-CR group among all 127 nodules (0.80 *vs* 0.63, $P < 0.001$). Among HCCs that presented in the right lobe and medial segment, the median location coefficient of HCC was also significantly higher in the CR group than in the non-CR group (0.81 *vs* 0.68, $P < 0.05$, 0.80 *vs* 0.49, $P < 0.01$, respectively). However, among HCCs in the lateral segment, there was no significant difference in the location coefficient of HCC between the CR group and non-CR group (0.74 *vs* 0.73, $P > 0.05$) (Figure 2 and Table 3).

HCC location and efficacy according to Child-Pugh grades

In the group classified as Child-Pugh grade A, the median location coefficient of HCC among all nodules in the right lobe and of those in the medial segment was also significantly higher in the CR group than in the non-CR group (0.82 *vs* 0.62, $P < 0.001$; 0.71 *vs* 0.59, $P < 0.01$; 0.81 *vs* 0.49, $P < 0.05$, respectively). However, there was no significant difference in the median location coefficient of HCC in the lateral segment between the CR group and non-CR group (0.67 *vs* 0.65, $P > 0.05$) (Table 3 and Figure 3). On the other hand, in the group classified as Child-Pugh grade B, the median location coefficient of HCC was not significantly different between the CR and non-CR

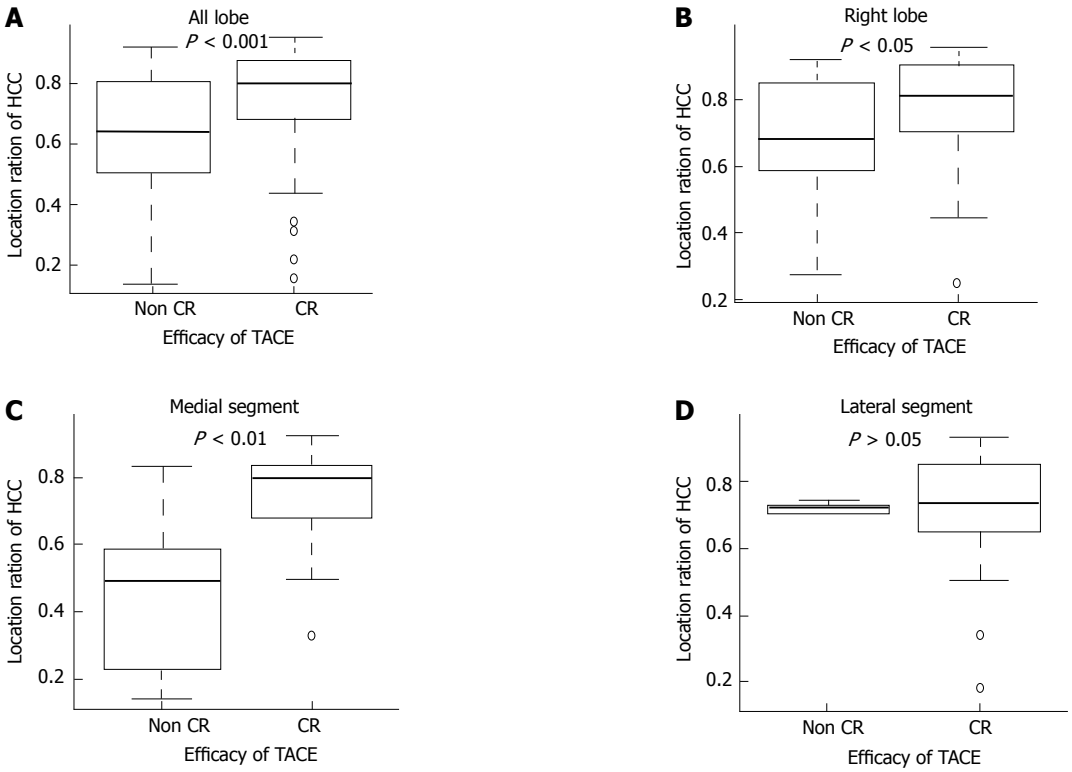


Figure 2 Relationship between transcatheter arterial chemoembolization efficacy and hepatocellular carcinoma location. A: All lobes; B: Right lobe; C: Medial segment; D: Lateral segment. The relationship between TACE efficacy and HCC location among all nodules and nodules that are present in the right lobe, medial segment, and lateral segment. TACE: Transcatheter arterial chemoembolization; HCC: Hepatocellular carcinoma; CR: complete response.

Table 2 Evaluation of efficacy 6 mo after transcatheter arterial chemoembolization according to the modified response evaluation criteria in solid tumors criteria

	CR	PR	SD	PD	Total
All nodules	81	26	13	7	127
	(63.8)	(20.5)	(10.2)	(5.5)	
Anterior segment	26	7	5	1	39
Posterior segment	22	8	3	4	37
Medial segment	14	8	4	1	27
Lateral segment	19	3	1	1	24
Patient with Child-Pugh	56	17	10	3	86
grade A	(65.1)	(19.8)	(11.6)	(3.5)	
Anterior segment	19	5	5	1	30
Posterior segment	14	5	1	1	21
Medial segment	9	6	3	0	18
Lateral segment	14	1	1	1	17
Patients with Child-Pugh	25	9	3	4	41
grade B	(61.0)	(22.0)	(7.3)	(9.7)	
Anterior segment	7	2	0	0	9
Posterior segment	8	3	2	3	16
Medial segment	5	2	1	1	9
Lateral segment	5	2	0	0	7

TACE: Transarterial chemoembolization; CR: Complete response; PR ± Partial response; SD: Stable disease; PD: Progressive disease.

groups for nodules in any lobe or segment (Figure 4).

Boundary line of HCC location

When we performed a ROC curve analysis for the location coefficient of HCC in all nodules, we found that the area under the curve (AUC) was 0.713 and that the cut-off value was 0.643, with a sensitivity of 56.5% and specificity of 85.2%. In the right lobe and medial segment, the ROC curve AUC was 0.678 and 0.852, respectively, and the cut-off value was 0.643 with a sensitivity of 50.0% and specificity of 89.6% and 0.674 with a sensitivity of 84.6% and specificity of 78.6%, respectively. In the group classified as Child-Pugh grade A, similar results were also obtained in all nodules, the right lobe, and medial segment; the AUC was 0.767, 0.753, and 0.827, respectively, and the cut-off value was 0.743, with a sensitivity of 80.0% and specificity of 69.6%, 0.643 with a sensitivity of 61.1% and specificity of 84.8%, and 0.716 with sensitivity of 88.9% and specificity of 77.8%, respectively (Figure 5).

DISCUSSION

In this retrospective study, we evaluated the relationship between the location of the HCC (in particular, the location close to the peripheral or hepatic portal portion of the liver) and the efficacy of TACE. Among all 127 nodules, the median location coefficient of the HCC was significantly higher in the CR group than in the non-CR group. Although the median location coefficient of HCC in the right lobe and medial segment was also significantly higher in the CR group than in the non-CR group, there was no significant difference in the location coefficient of HCC in the lateral segment.

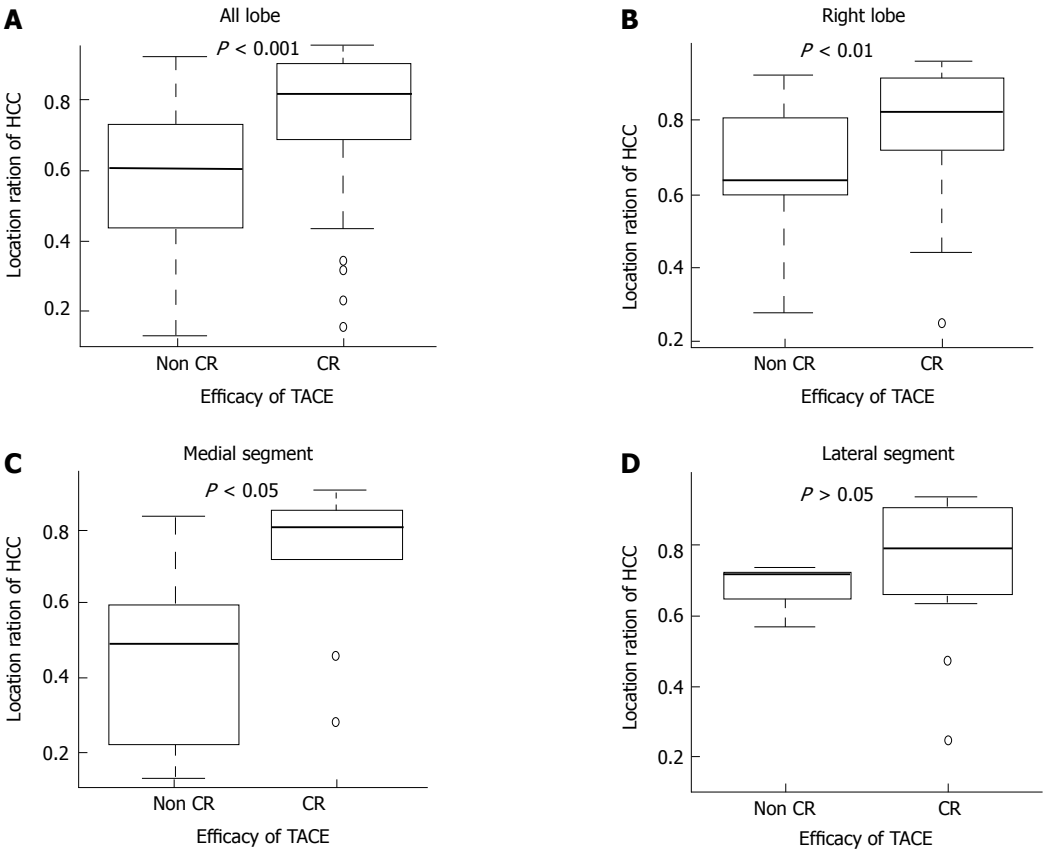


Figure 3 Results in the group classified as Child-Pugh grade A. A: All lobes; B: Right lobe; C: Medial segment; D: Lateral segment. Relationship between TACE efficacy and HCC location in the group classified as Child-Pugh grade A. TACE: Transcatheter arterial chemoembolization; HCC: Hepatocellular carcinoma; CR: Complete response.

Table 3 Hepatocellular carcinoma location coefficient in the complete response and non-complete response groups

	Non-CR group	CR group	*P* value
All lobes	0.63	0.8	< 0.001
Right lobe	0.68	0.81	< 0.05
Medial segment	0.49	0.8	< 0.01
Lateral segment	0.73	0.74	> 0.05
Patients with Child-Pugh grade A			
All lobes	0.62	0.82	< 0.001
Right lobe	0.59	0.71	< 0.01
Medial segment	0.49	0.81	< 0.05
Lateral segment	0.65	0.67	> 0.05
Patients with Child-Pugh grade B			
All lobes	0.73	0.75	> 0.05
Right lobe	0.84	0.79	> 0.05
Medial segment	0.43	0.69	> 0.05
Lateral segment	0.72	0.69	> 0.05

Values are expressed as the medians. HCC: Hepatocellular carcinoma; CR: Complete response.

These results suggested that the location of HCC may influence the efficacy of TACE for the treatment of HCC in the right lobe and medial segment. Moreover, considering the liver function represented by the Child-Pugh grade, a significant difference was observed between the location of HCC and the efficacy of TACE only in patients classified as Child-Pugh grade A. In the patients classified as Child-Pugh grade B, the median location coefficient of HCC was not significantly different between the CR group and the non-CR group in each lobe and segment. These results may represent conditions that have hepatic circulatory, anatomical, or biological significance.

TACE is a useful palliative treatment for HCC because the hepatic artery delivers > 99% of the blood supply to hepatic tumors. Compact lipiodol uptake results in complete necrosis in the resected tissues[16-19], and complete deposition of iodized oil in HCC lesions correlates with near-total necrosis[20-22]. However, HCCs showing a high uptake of iodized oil may continue to be partially fed by the collateral arteries or the portal vein[23]. Chan *et al*[24] reported that TACE induces a posttreatment surge of angiogenic factors, such as vascular endothelial growth factor (VEGF), which can occur as early as a few hours after TACE. This process may contribute to revascularization of the tumor, thus leading to the recurrence of HCC and reduced efficacy of TACE[25,26].

Generally, residual HCC tumor cells are observed in the peripheral region of the HCC. The reasons for

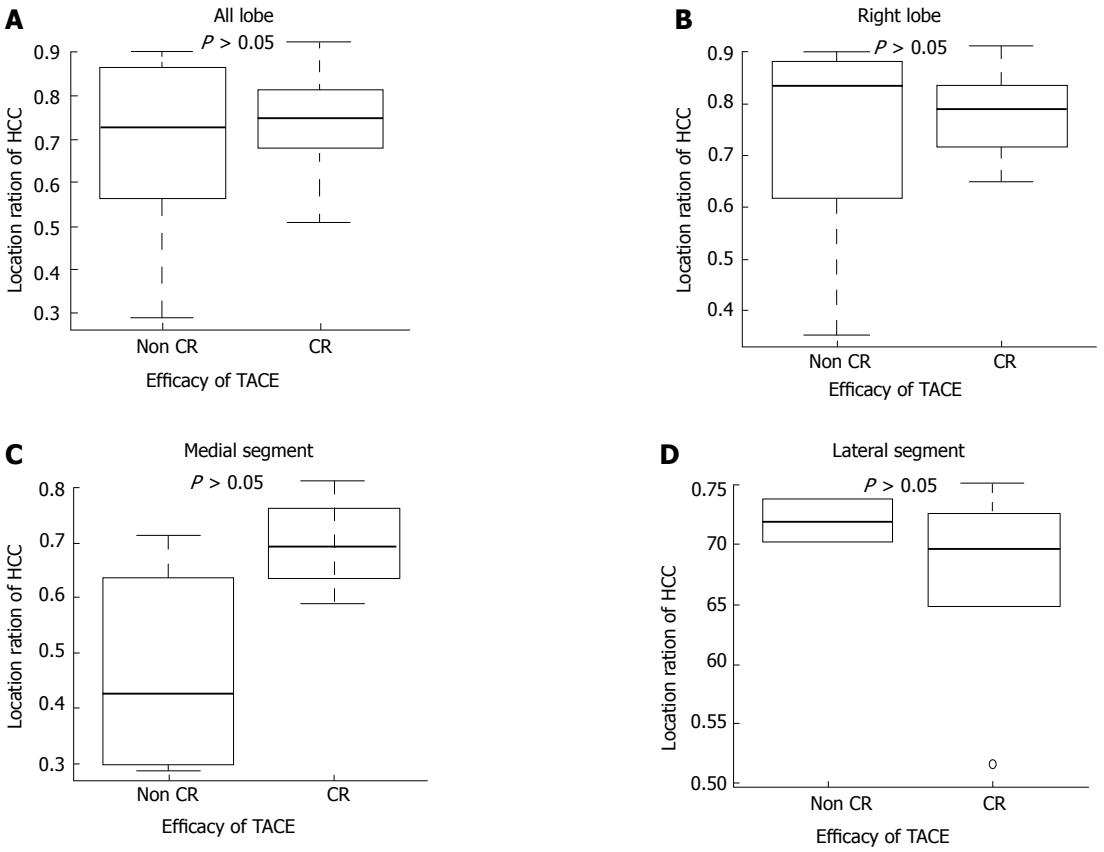


Figure 4 Results in the group classified as Child-Pugh grade B. A: All lobes; B: Right lobe; C: Medial segment; D: Lateral segment. Results of the relationship between TACE efficacy and HCC location in the group classified as Child-Pugh grade B. TACE: Transcatheter arterial chemoembolization; HCC: Hepatocellular carcinoma CR: Complete response.

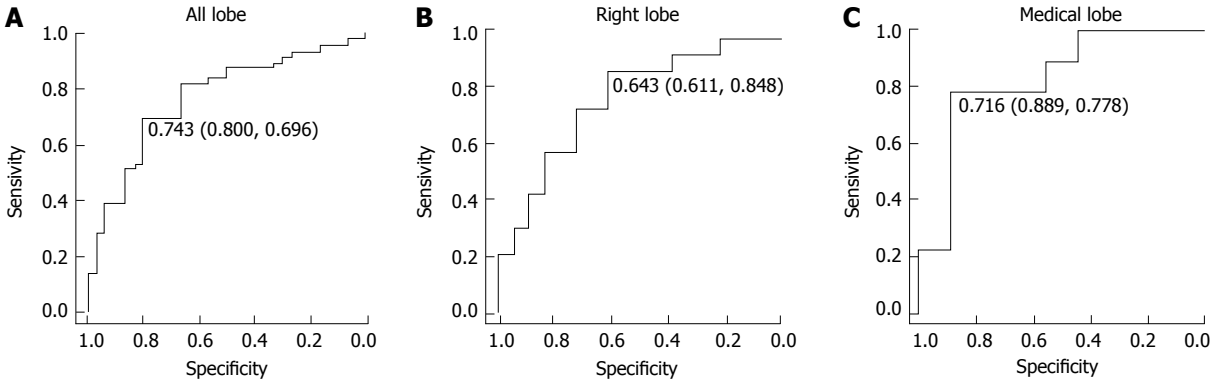


Figure 5 Receiver operating characteristic curve for hepatocellular carcinoma location. A: All lobes; B: Right lobe; C: Medial segment. ROC curve for HCC location as a predictor of TACE efficacy in all lobes, right lobe, and the medial segment in the group classed as Child-Pugh A. Values are the cut-off point (sensitivity and specificity). ROC: Receiver operating characteristic curve; HCC: Hepatocellular carcinoma; TACE: Transcatheter arterial chemoembolization.

this are complex and may be partially explained by the following: (1) incomplete embolization or partial recanalization of the hepatic artery after embolization or (2) formation of collateral circulation or the opening of communicating vessels as a microcirculatory reaction. Notably, residual viable tumor cells after TACE are exposed to an extremely hypoxic or even anoxic environment. Ischemic hypoxia is considered a physiological stimulus for angiogenesis, including endothelial cell proliferation[22,23]. Tumor cells induce neovascularization by producing VEGF, which in turn can nourish more tumor cells. Thus, neovascularization and VEGF expression may be important reasons for the survival of residual tumor cells after TACE.

There are several reasons why the median location coefficient of HCC in the right lobe and medial segment

was significantly higher in the CR group than in the non-CR group. An *in vivo* microscopic study[12] indicated a direct and indirect reduction in the blood flow to the periphery of the main lobe. Moreover, several reports have described functional and hemodynamic differences between the right and left lobes or between the peripheral (near the subcapsular area) and the central areas (near the porta hepatic) in the liver[27-30]. Okubo *et al*[31], in a study with gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced MRI, reported that the right lobe or central zone showed significantly more enhancement than the left lobe or peripheral zone. The hemodynamic differences between the peripheral and central zones and between the right and left lobes could cause the differences in hepatic enhancement. These reports suggested that vascularity around the HCC in the peripheral area is less than that in the central area, and TACE may induce much more ischemia in the HCC tissue in the peripheral area than in the central area because of protecting the formation of collateral circulation or the opening of communicating vessels.

We also evaluated why a significant difference was found only in the right lobe and medial segment and not in the lateral segment. The abovementioned reports suggested that there were differences between the right and left lobes. However, the medial segment is in the left lobe. We do not have a definitive answer to this question, but several phenomena may provide clues. First, thin anastomosis branches often present between the right hepatic and the middle hepatic arteries[32]. Second, cirrhotic livers associated with all etiologies demonstrate atrophy of the right lobe and medial segment as well as hypertrophy of the caudate lobe and lateral segment[11]. These phenomena suggest that differences in the frequency of revascularization between the central and peripheral areas also exist in the medial segment.

Our study demonstrated a significant difference between the location of HCC and the efficacy of TACE only in the Child-Pugh grade A patients. In Child-Pugh grade B patients, there was no relationship between any nodules, even in the right lobe and medial segment. These results suggested that hepatic hemodynamics may change depending on cirrhotic liver status. Indeed, qualitative analysis indicated that the parenchymal enhancement in the cirrhotic liver was significantly heterogeneous compared with that in the normal liver[33]. Moreover, the quantitative liver-spleen contrast ratio was significantly decreased with an elevation of the Child-Pugh score[31]. These reports suggest that hemodynamic features are different depending on cirrhotic liver status. In our study, considering a cut-off value of the location coefficient of HCC on the ROC curve, a boundary line that demonstrated a significant, effective difference in patients with Child-Pugh grade A was expected in the range of 0.6-0.7 in the right lobe and median segment. Considering these results and the changing hepatic hemodynamics that depend on cirrhotic liver status, we concluded that livers with advanced cirrhosis had atrophy of the peripheral area in the right lobe and medial segment. Consequently, there was no significant difference between the location of the HCC and the efficacy of TACE in Child-Pugh grade B patients.

A novel finding in our study was that the median location coefficient of HCC in the right lobe and medial segment was significantly higher in the CR group than in the non-CR group. Therefore, the coefficient could be used as an index when choosing an HCC treatment strategy. When HCC is located in the peripheral area in the right lobe or medial segment, TACE is much more suitable for treatment. However, when HCC is located in the central area in the right lobe or medial segment, an early combination of local ablation therapy (such as radiofrequency ablation and percutaneous ethanol injection) with TACE may be efficacious. Indeed, the synergistic effect of such an approach has already been demonstrated in both nonsurgical and recurrent HCC patients[34-36]. Therefore, the combination of local ablation therapy with TACE may be considered as a treatment of HCC in the central area.

There are several limitations in our study. First, there were very few HCC nodules in the caudate lobe in our study. If there were more HCCs in the caudate lobe, further studies could evaluate the difference in the efficacy of TACE in each lobe or segment in detail. Second, the baseline point of the center when measuring the HCC location was the bifurcation of the portal vein because of few portal vein variations in this study. The HCC location coefficient and the boundary line, demonstrating the difference in efficacy of TACE between the peripheral area and central area, may change when other baseline points, such as the bifurcation of the right and left branches of the hepatic artery, are defined. Therefore, further studies are necessary to confirm our findings.

In conclusion, our study suggested that improved efficacy of TACE may be obtained for treatment of HCC in the peripheral area of the right lobe and medial segment in Child-Pugh grade A patients.

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COMMENTS

Background

Transarterial chemoembolization (TACE) is the current standard of care for patients with Barcelona

clinical liver cancer stage B, which includes a highly heterogeneous group of patients. TACE has a positive effect on patient outcome and survival. Studies have shown that tumor number, maximal tumor diameter, Child-Pugh score, and tumor response to TACE are fundamentally strong predictors of survival in patients with initially unresectable hepatocellular carcinoma (HCC). Most HCC patients have some degree of cirrhosis. Microcirculatory changes play a role in the development of cirrhosis within segments. Histopathological investigations of HCCs that were resected after TACE have shown that the most viable tissue is located at the tumor periphery. Considering all these factors, it is very important for the physician to distinguish effective and ineffective TACE before performing this procedure. In this study, we evaluated the relationship between HCC location-in particular, a location close to the peripheral or hepatic portal portion of the liver-and TACE efficacy.

Research frontiers

Predicting whether TACE will be effective before performing this procedure is very important for the physician, especially while considering other therapeutic modalities. The results of this study evaluating the relationship between HCC location and TACE efficacy may prove to be an index when determining a HCC treatment strategy.

Innovations and breakthroughs

Several studies and well-designed randomized trials have shown that TACE has a positive effect on patient outcome and survival. However, no previous study has evaluated the relationship between the efficacy of this procedure and HCC. This retrospective study evaluated the relationship between the location of HCC and the efficacy of TACE and suggested that an improved efficacy of TACE may be obtained for treatment of HCC in the peripheral area of the right lobe and medial segment in patients with Child-Pugh grade A tumors. For patients with Child-Pugh grade B or HCC located in the central area or in the lateral segment, it might be difficult to obtain complete necrosis with TACE only.

Applications

This study suggests that improved TACE efficacy may be obtained for HCC in the peripheral zone of the right lobe and the medial segment in Child-Pugh grade A patients. When HCC is located in the central area in the right lobe or the medial segment, an early combination of local ablation therapy with TACE may be efficacious.

Terminology

TACE: A minimally invasive procedure performed in interventional radiology to reduce a tumor's blood supply. Child-Pugh score: A score to assess the prognosis of liver cirrhosis.

Peer-review

In this retrospective study, the authors evaluated the relationship between the location of the HCC (in particular, the location close to the peripheral or hepatic portal portion of the liver) and the efficacy of TACE. It is an interesting study on TACE for HCC. The results demonstrated a significant difference between the location of HCC and the efficacy of TACE only in the Child-Pugh grade A patients. When HCC is located in the peripheral area in the right lobe or medial segment, TACE is much more suitable for treatment. When HCC is located in the central area in the right lobe or medial segment, an early combination of local ablation therapy (such as radiofrequency ablation and percutaneous ethanol injection) with TACE may be efficacious. These findings will provide very important clinical guidance for the selection of TACE for HCC.

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ORIGINAL ARTICLE

Retrospective Study

Evaluation of recurrence in gastric carcinoma: Comparison of contrast-enhanced computed tomography and positron emission tomography/computed tomography

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Author contributions: Kim JH and Jeong YY made substantial contributions to conception and design and analysis of data; Heo SS, Kim JW, Min JJ and Kwon SY contributed to acquisition of data; Kang HK and Shin SS reviewed the literature.

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Informed consent statement: Informed consent was waived because of the retrospective nature of the study and the analysis used anonymous clinical data.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest in publishing this article.

Data sharing statement: No additional data are available.

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Abstract

AIM

To compare the value of contrast-enhanced abdominal computed tomography (CT) and fluorodeoxyglucose

(FDG) positron emission tomography/computed tomography (PET/CT) for detecting gastric carcinoma recurrence.

METHODS

We retrospectively examined data from 2475 patients who underwent both contrast-enhanced abdominal CT and FDG PET/CT for the surveillance of gastric carcinoma curative resection. Patients had an interval of less than 1 mo between their CT and PET/CT scans. Sixty patients who had recurrence were enrolled. Among 1896 patients who did not have recurrence, 60 were selected by simple random sampling. All CT and PET/CT images were reviewed retrospectively by two reviewers blinded to all clinical and pathologic information except curative resection due to gastric carcinoma.

RESULTS

The pathological stage of the recurrence group was statistically significantly higher than that of the control group ($P < 0.001$). In the 60 patients who had recurrence, there were 79 recurrent lesions. Forty-four patients had only one location of recurrence, 13 patients had two locations, and 3 patients had three. In the detection of patient-based overall recurrence, no statistically significant differences existed between the two modalities ($P = 0.096$). However, for peritoneal carcinomatosis, CT had a statistically significantly higher sensitivity compared to PET/CT (96% *vs* 50%, $P = 0.001$). Adenocarcinoma was the most common type of gastric carcinoma. On the pathology-based analysis, CT also had a statistically significantly higher sensitivity compared to PET/CT (98% *vs* 80%, $P = 0.035$).

CONCLUSION

Contrast-enhanced CT was superior to PET/CT in the detection of peritoneal carcinomatosis and pathologic type of adenocarcinoma.

Key words: Gastric carcinoma; Surgery; Contrast-enhanced abdominal computed tomography; Fluorodeoxyglucose positron emission tomograph/computed tomography; Surveillance; Recurrence

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Core tip: Contrast-enhanced abdominal computed tomography (CT) and positron emission tomography/computed tomography (PET/CT) are commonly used imaging methods for surveillance of recurrence after gastric cancer surgery. However, the ideal method for early detection of gastric carcinoma recurrence remains controversial. In this study, we compared the value of contrast-enhanced abdominal CT and PET/CT for detecting the recurrence of gastric carcinoma after curative resection. We found that contrast-enhanced CT was superior to PET/CT in the detection of peritoneal carcinomatosis and pathologic type of adenocarcinoma.

Kim JH, Heo SH, Kim JW, Shin SS, Min JJ, Kwon SY, Jeong YY, Kang HK. Evaluation of recurrence in gastric carcinoma: Comparison of contrast-enhanced computed tomography and positron emission tomography/computed tomography. *World J Gastroenterol* 2017; 23(35): 6448-6456 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6448.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6448

INTRODUCTION

Gastric carcinoma is a leading cause of cancer death worldwide[1]. In Asia, the incidence of gastric carcinoma is high. Its 5-year survival rate is 55%-66%, and a major cause of death after curative surgery for gastric carcinoma is recurrence with a substantial number of patients experiencing such recurrence[2-4]. Although the recurrence rate of early gastric cancer after curative resection is very low, advanced gastric cancer cases show a high recurrence rate after curative resection[5,6].

Several methods exist to detect the recurrence of gastric carcinoma after surgery, including tumor markers, endoscopy, and imaging studies[7]. Elevated tumor marker levels such as those of carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9) can be associated with tumor recurrence[8]. Non-systemically, endoscopy can be used to detect locoregional tumor recurrence[9]. Contrast-enhanced abdominal computed tomography (CT) and fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) are cross-sectional imaging modalities that can be used to detect locoregional and distant recurrence. However there is no specific guideline or indication for using CT or PET/CT in surveillance for recurrence after gastric cancer surgery.

Contrast-enhanced abdominal CT is a commonly used imaging method because it is widely available and relatively inexpensive[10]. Despite its widespread use, however, there have been controversies about the role of CT, compared to PET/CT, in evaluating the recurrence of gastric carcinoma after curative surgery. Previous reports showed that PET or PET/CT might be useful in the evaluation of the recurrent gastric cancer after gastric cancer surgery[10-12]. Additional use of PET/CT in contrast-enhanced CT studies could improve the detection of recurrence and provide other information such as an unexpected secondary malignancy[7]. On the other hand, PET or PET/CT might not be suitable for the detection and confirmation of recurrence after gastric cancer surgery[13,14]. Some reports have shown that contrast-enhanced CT and PET/CT have no substantial differences in their ability to detect gastric cancer recurrence after curative resection, except for detecting peritoneal carcinomatosis[7].

Thus, the ideal method for early detection of gastric carcinoma recurrence remains controversial[10,11,13-15]. Generally, previous studies reviewed images without

being blinded to the clinical findings and results of the imaging studies, or only evaluated PET or PET/CT for detecting recurrence after gastric surgery[10-12,14,16,17].

The aim of this study was to compare the efficacy of CT and PET/CT in detecting recurrence after curative gastric surgery, when using blinded clinical and imaging findings.

MATERIALS AND METHODS

Patient selection

This retrospective study was approved by the Institutional Review Board. Informed consent was waived because of the retrospective nature of the study. The data of 2475 patients who subsequently underwent both contrast-enhanced abdominal CT and PET/CT for the surveillance of gastric carcinoma recurrence after curative resection, from December 2011 to June 2015, were analyzed. Patients all had an interval of less than 1 mo between their CT and PET/CT scans. Of the 2475 patients, 519 who did not have a diagnosis validated by histopathological confirmation or subsequent imaging study least 6 mo were excluded. Sixty patients had recurrence confirmed by histopathologic evaluation or subsequent imaging study least 6 mo. Twenty eight patients had pathologic confirmation and 32 patients had imaging confirmation by serial contrast-enhanced CT, making a total of 60 patients. Radiologically, recurrence was defined to be present when a suspicious lesion showed the interval increment in size during serial imaging studies. In the remaining 1896 patients without recurrence, a control group of 60 patients was selected by simple random sampling (Figure 1). In case of patients who had recurrence and underwent both CT and PET/CT many times with intervals of less than 1 mo, the first CT or PET/CT images suspected to show recurrence were analyzed. In case of patients who had no recurrence and underwent both CT and PET/CT many times with intervals of less than 1 mo, the first CT and PET/CT images were analyzed.

Contrast-enhanced CT and FDG PET/CT scanning

Contrast-enhanced abdominal CT was performed with either 16 or 64-detector row CT scanner (LightSpeed 16 or LightSpeed VCT; GE Healthcare, Milwaukee, WI, United States) with the following parameters; 120 kV, 200-300 mA, slice thickness: 3.75 mm, slice increments: 3.75 mm, and pitch: 0.984:1. Automated tube current modulation (AutomA; GE Healthcaere, Milwaukee, WI, United States) was routinely used for all patients. AutomA was set between 100 and 300 mA with a noise index of 15. Single-phase (portal venous phase) contrast-enhanced CT was performed after intravenous injection of 100-150 mL of iopromide (Ultravist®; Schering, Berlin, Germany) at a rate of 2-3 mL/s. The total amount of injected contrast material was adjusted according to the body weight of the patients (2 mL/kg). Scanning was started 90 s after the intravenous injection, spanning from the liver dome to the symphysis pubis.

^{18}F-FDG PET/CT was performed with a Discovery ST PET/CT system (GE Healthcare), consisting of a bismuthgermanate full scanner and a 16-detector-row CT scanner. The patients fasted for at least 6 h prior to the intravenous administration of ^{18}F-FDG (7.4 MBq per body weight). At 60 min after ^{18}F-FDG administration, transmission data were acquired using low-dose CT (120 kV, automated from 10 to 130 mA, 50 cm field of view (FOV), a scan length of 40-50 s and a slice thickness of 3.75 mm, and a rotation time of 0.7 s), extending from the base of the skull to the proximal thighs. Immediately after CT acquisition, PET emission scans were acquired in the same anatomic locations with a 15.7 cm axial FOV acquired in the two-dimensional mode with 3 min/bed position. The CT data were used for attenuation correction. The images were reconstructed using a conventional iterative algorithm. A workstation (Xeleris) providing multi-planar reformatted images was also used for image display and analysis.

Image analysis

All CT and PET/CT images were reviewed retrospectively, and recurrence was defined by the consensus of two experienced abdominal radiologists (22 and 12 years of experience) and two experienced nuclear medicine physicians (20 and 12 years of experience). Reviewers were blinded to all clinical and pathological information about the patients except for their curative resection of gastric carcinoma.

We classified recurrent lesions into 5 categories: (1) Locoregional recurrence; (2) Lymph node recurrence; (3) Liver metastasis; (4) Peritoneal carcinomatosis; and (5) Other recurrence, for recurrence not included in categories 1-4.

In CT images, locoregional recurrence was defined as showing wall thickening with contrast enhancement of the anastomotic site and adjacent remnant stomach[18]. Lymph nodes more than 10 mm long in the short axis were considered lymph node recurrence. A nearly round shape, spiculated or indistinct borders, central necrosis, and marked or heterogeneous enhancement were integrated with the CT size criteria[19]. Liver metastasis was defined as a hypoattenuating lesion with peripheral rim enhancement[20]. Nodular-plaque or infiltrative soft tissue stranding in the mesentery or omentum, parietal peritoneal thickening and contrast enhancement with or without ascites was considered peritoneal carcinomatosis[21].

Focal hypermetabolic activity (> 3.0 SUV) in the anastomotic site and adjacent remnant stomach was considered locoregional recurrence[7]. Lymph nodes were graded as malignant or benign based on functional criteria (increased metabolism relative to the surrounding lymph nodes) independent of their size[22].

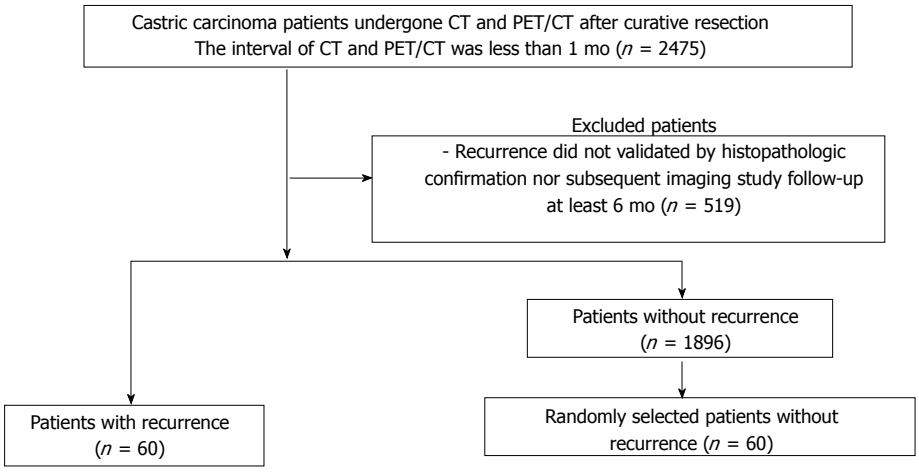


Figure 1 Flow diagram of study patients. CT: Computed tomography; PET/CT: Positron emission tomography/computed tomography.

Table 1 Patient characteristics *n* (%)

Characteristics		Recurrence group (*n* = 60)	Control group (*n* = 60)	*P* value
Age (yr)	Median	60.6	65	0.755
	Range	29-80	35-85	
Sex	Male	37 (62)	16 (27)	0.172
	Female	23 (38)	44 (73)	
Operation	Total gastrectomy	17 (28)	12 (20)	0.286
	Subtotal gastrectomy	43 (72)	48 (80)	
AJCC stage	Ⅰ A	0 (0)	33 (55)	< 0.001
	Ⅰ B	5 (8)	10 (17)	
	Ⅱ A	7 (12)	9 (15)	
	Ⅱ B	9 (15)	3 (5)	
	Ⅲ A	7 (12)	1 (2)	
	Ⅲ B	10 (16)	2 (3)	
	Ⅲ C	15 (25)	2 (3)	
	Ⅳ	7 (12)	0 (0)	
Pathology	Adenocarcinoma	51 (85)	55 (92)	0.454
	Signet ring cell carcinoma	5 (8)	2 (3)	
	Mucinous adenocarcinoma	4 (7)	3 (5)	
Interval between CT and PET/CT (d)	Median	5.9	2.8	< 0.001
	Range	0-28	0-9	

AJCC: American Joint Committee on Cancer (7th edition); CT: Computed tomography; PET/CT: Positron emission tomography/computed tomography.

Focal hypermetabolic activity greater than adjacent normal liver was considered liver metastasis[23]. Diffuse hypermetabolism spreading throughout the abdominopelvic cavity, obscuring visceral outlines and randomly located discrete foci of uptake anteriorly within the abdomen or dependently within the pelvis and unrelated to solid viscera or nodal stations was considered peritoneal carcinomatosis[19].

Statistical analysis

Data from the reviewers were analyzed separately. The characteristics of patients with and without recurrence were compared using Student's *t* test for noncategorical variables and the χ^2 test for categorical variables. Sensitivity, specificity, and accuracy were calculated. McNemar's test was conducted for comparing the diagnostic efficacy of contrast-enhanced abdominal CT and PET/CT in patients-, lesion- and pathological type-based analysis. A *P*-value of < 0.05 was considered to indicate a significant difference.

RESULTS

Patient characteristics

Table 1 details the patient characteristics. The pathological stage of the recurrence group was statistically significantly higher than that of the control group ($P < 0.001$). In the control group, the stage of 33 patients (55%) was Ia and none of the patients was IV. In the recurrence group, none of the patients was Ia and seven patients were. These patients did not have distant metastasis in their preoperative clinical stage. After the curative surgery, five patients had distant lymph node metastasis and two patients had peritoneal metastasis as revealed by peritoneal washing cytology. The median interval between contrast-enhanced CT and PET/CT was statistically significantly longer in the recurrence group than in the control group ($P < 0.001$). Other patient characteristics did not differ.

Characteristics of recurrence

In the 60 patients who had recurrence, there were 79 recurrent lesions (Table 2). Forty-four patients had only one location of recurrence, 13 patients had two locations, and 3 patients had three. Locations of the other recurrence sites ($n = 18$) were intestines ($n =$

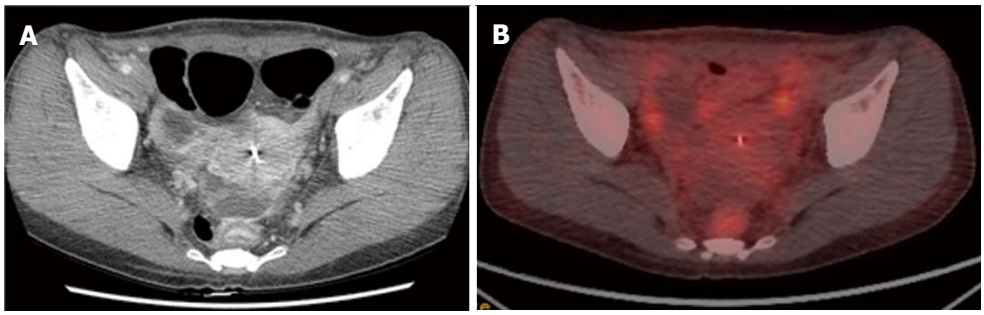


Figure 2 Peritoneal carcinomatosis detected only on contrast-enhanced computed tomography in a 46-year-old woman who underwent gastrectomy for advanced gastric cancer 17 mo prior. A: Contrast-enhanced abdominal computed tomography shows enhanced peritoneal thickening (arrow) with ascites. Peritoneal metastasis was confirmed by histological analysis of ascitic fluid. B: Positron emission tomography/computed tomography image at the same level without evident hypermetabolism in the peritoneum.

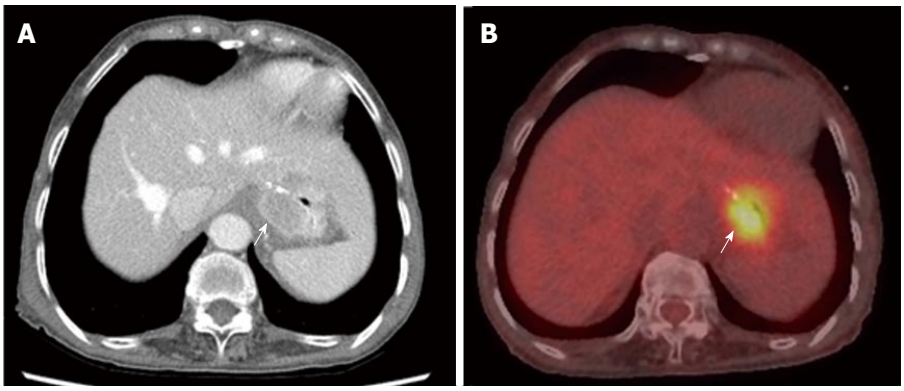


Figure 3 Locoregional recurrence detected on both contrast-enhanced computed tomography and positron emission tomography/computed tomography in a 76-year-old woman who underwent gastrectomy for advanced gastric cancer 21 mo prior. A: Contrast-enhanced abdominal computed tomography shows enhanced wall thickening (arrow) at the anastomotic site; B: Positron emission tomography/computed tomography image at the same level shows hypermetabolism at the anastomotic site.

Table 2 Locations of recurrent lesions

Location	Number of lesions *n* (%)
Locoregional recurrence	10 (13)
Lymph node recurrence	24 (30)
Liver metastasis	3 (4)
Peritoneal carcinomatosis	24 (30)
Other recurrence[1]	18 (23)
Total	79 (100)

[1]Other recurrence included intestine, bone, ovary, *etc.*

7), bone (n = 5), ovaries (n = 3), spleen (n = 2), and pancreas (n = 1).

Detection of recurrence in contrast-enhanced CT and PET/CT

For the detection of patient-based overall recurrence, CT had a sensitivity of 97%, a specificity of 97%, and an accuracy of 97%, whereas PET/CT had a sensitivity of 82%, a specificity of 95%, and an accuracy of 88%. These differences were not statistically significant (P = 0.096; Table 3). Two patients with recurrence did not have it detected by CT, and 12 patients who had recurrence did not have it detected by PET/CT.

For the detection of lesion-based overall recurrence, CT had a sensitivity of 86%, a specificity of 98%, and an accuracy of 97%, whereas PET/CT had a sensitivity of 76%, a specificity of 98%, and an accuracy of 96%; these differences were not statistically significant (P = 0.089). For peritoneal carcinomatosis, CT had a sensitivity of 96%, a specificity of 100%, and an accuracy of 99%, whereas PET/CT had a sensitivity of 50%, a specificity of 100%, and an accuracy of 90%, a statistically significant difference (P = 0.001; Figure 2). For detecting locoregional, and lymph node recurrences, and liver metastasis, there were no statistically significant differences between CT and PET/CT (Table 3, Figures 3 and 4). For detecting bone metastasis, CT had a sensitivity of 20%, a specificity of 100%, and an accuracy of 97%, whereas PET/CT had a sensitivity of 100%, a specificity of 100%, and an accuracy of 100%, these differences were not statistically significant (P = 0.125; Figure 5).Two patients who had locoregional recurrence with inadequate bowel distension were missed on CT. Another two patients with locoregional recurrence were missed on PET/CT, and their

Table 3 Comparison of contrast-enhanced computed tomography and positron emission tomography/computed tomography in the detection of recurrence

Site	Imaging	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)	*P* value
Overall	CT	97 (58/60)	97 (58/60)	97 (58/60)	97 (58/60)	97 (114/120)	0.096
	PET/CT	82 (49/60)	95 (57/60)	94 (49/52)	84 (57/68)	88 (106/120)	
Locoregional	CT	80 (8/10)	100 (110/110)	100 (8/8)	98 (112/110)	98 (118/120)	1.000
	PET/CT	80 (8/10)	99 (109/110)	89 (8/9)	98 (109/111)	98 (117/120)	
Lymph node	CT	92 (22/24)	99 (95/96)	96 (22/23)	98 (95/97)	98 (117/120)	1.000
	PET/CT	88 (21/24)	99 (95/96)	95 (21/22)	97 (95/98)	97 (116/120)	
Liver	CT	67 (2/3)	96 (112/117)	29 (2/7)	99 (112/113)	95 (114/120)	0.688
	PET/CT	100 (3/3)	98 (115/117)	60 (3/5)	100 (115/115)	98 (118/120)	
Peritoneal carcinomatosis	CT	96 (23/24)	100 (96/96)	100 (23/23)	99 (96/97)	99 (119/120)	0.001
	PET/CT	50 (12/24)	100 (96/96)	100 (12/12)	89 (96/108)	90 (108/120)	
Total lesion	CT	86 (68/79)	98 (511/521)	87 (68/78)	98 (511/522)	97 (579/600)	0.089
	PET/CT	76 (60/79)	98 (513/521)	88 (60/68)	96 (513/532)	96 (573/600)	

Numbers in parentheses are raw data. CT: Computed tomography; PET/CT: Positron emission tomography/computed tomography.

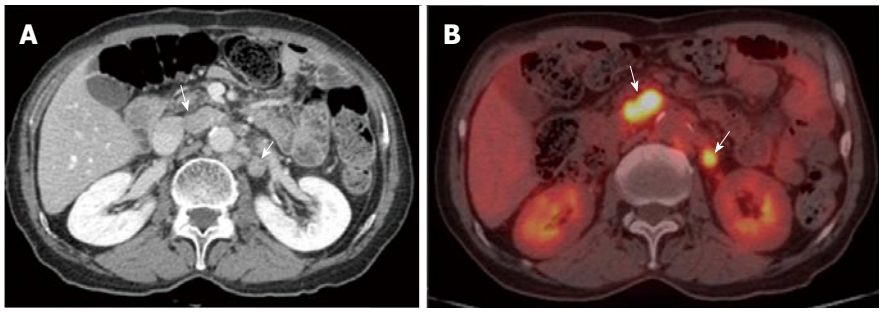


Figure 4 Lymph node recurrence detected on both contrast-enhanced computed tomography and positron emission tomography/computed tomography in a 73-year-old man who underwent gastrectomy 18 mo prior. A: Contrast-enhanced abdominal computed tomography shows enlarged lymph nodes at the aortocaval and para-aortic spaces; B: Positron emission tomography/computed tomography image at the same level shows hypermetabolism at the aortocaval and para-aortic spaces.

recurrences were concealed by physiological uptake of remnant stomach. Two patients who had lymph node recurrence of less than 10 mm in the short axis were missed on CT, but were detected on PET/CT. Three patients who had lymph node recurrence without significant hypermetabolism were missed on PET/CT. One hepatic metastasis (about 1.5 cm) was not detected on CT and the metastatic tumor had no attenuation difference compared to the adjacent liver. One patient with peritoneal carcinomatosis was considered as having peritonitis on CT, because he also had a very large abdominal abscess. Twelve patients who had peritoneal carcinomatosis without significant hypermetabolism were missed on PET/CT.

Adenocarcinoma was the most common type of gastric carcinoma (Table 1). On the pathological type-based analysis, CT had a sensitivity of 98%, a specificity of 95%, and an accuracy of 96% for detecting recurrence of adenocarcinoma, whereas PET/CT had a sensitivity of 80%, a specificity of 95%, and an accuracy of 88%; these differences were not statistically significant (P = 0.035; Table 4). For detecting the recurrence of signet ring cell carcinoma and mucinous adenocarcinoma patients, CT and PET/CT had no significant differences.

DISCUSSION

The recurrence rate of early gastric cancer after curative resection is very low and PET/CT is not commonly used for these patients. However advanced gastric cancer cases show a high recurrence rate. Contrast-enhanced abdominal CT and PET/CT are commonly used imaging methods, but the ideal method for early detection of gastric carcinoma recurrence remains controversial.

In terms of the patient characteristics, the pathological stages of the recurrence group were statistically significantly higher than those of the control group. In the recurrence group, the stages of 33 patients (55%) were Ia, none was Ia, and seven patients were IV. The median time interval between the contrast-enhanced CT and PET/CT was also statistically significantly longer in the recurrence group than in the control group. One of the inclusion criteria for our study was an interval shorter than 1 mo between the CT and PET/CT examination. Most of the patients in the control group were prescribed both CT and PET/CT around the same day for their regular follow ups. All

Table 4 Comparison of contrast-enhanced computed tomography and positron emission tomography/computed tomography in the detection of recurrence according to pathological type

Type	Imaging	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)	*P* value
Overall	CT	97 (58/60)	97 (58/60)	97 (58/60)	97 (58/60)	97 (114/120)	0.096
	PET/CT	82 (49/60)	95 (57/60)	94 (49/52)	84 (57/68)	88 (106/120)	
Adenocarcinoma	CT	98 (50/51)	95 (52/55)	94 (50/53)	98 (52/53)	96 (102/106)	0.035
	PET/CT	80 (41/51)	95 (52/55)	93 (41/44)	84 (52/62)	88 (93/106)	
Signet ring cell carcinoma	CT	100 (5/5)	100 (2/2)	100 (5/5)	100 (2/2)	100 (7/7)	1
	PET/CT	80 (4/5)	100 (2/2)	100 (4/4)	67 (2/3)	86 (6/7)	
Mucinous adenocarcinoma	CT	75 (3/4)	100 (3/3)	100 (3/3)	75 (3/4)	86 (6/7)	1
	PET/CT	100 (4/4)	100 (3/3)	100 (4/4)	100 (3/3)	100 (7/7)	

Numbers in parentheses are raw data. CT: Computed tomography; PET/CT: Positron emission tomography/computed tomography.

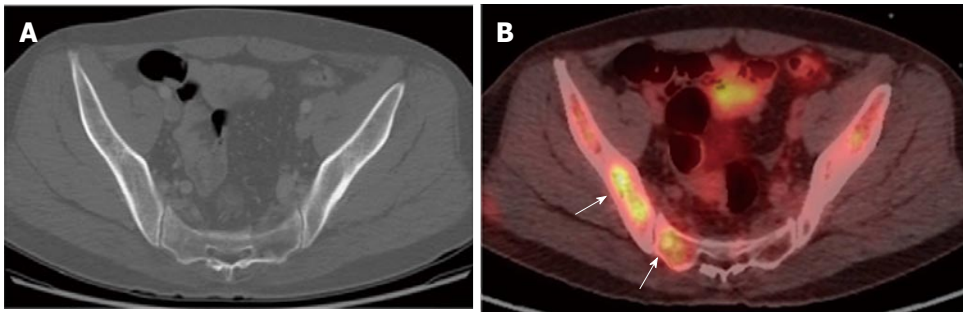


Figure 5 Bone metastasis detected only on positron emission tomography/computed tomography in a 47-year-old man who underwent gastrectomy for advanced gastric cancer 16 mo prior. A: Contrast-enhanced abdominal computed tomography showing the absence of bone lesions; B: Positron emission tomography/computed tomography image at the same level showing multiple hypermetabolic lesions in the pelvic bones and sacrum.

patients underwent only CT, and were only sometimes prescribed both CT and PET/CT for regular follow up. However, when recurrence was suspected in a regular follow-up CT in the recurrence group, an additional PET/CT was prescribed to increase confidence or evaluate the recurrent lesion. Our institution is a tertiary referral hospital, and appointments for imaging studies were therefore delayed.

For detecting locoregional and lymph node recurrence, the diagnostic performance of CT and PET/CT was not significantly different in our study, which is in agreement with previous studies[7,13]. Locoregional recurrence manifests as localized bowel wall thickening of the anastomotic site or remnant stomach on CT. However, inadequate bowel distension, surgical plication, bowel adhesion, and stomal polypoid hypertrophic gastritis can lower the specificity of the CT[23]. In PET/CT, focal hypermetabolism of the anastomotic site or remnant stomach was considered locoregional recurrence; however, physiological uptake in the bowel can compromise the diagnostic performance of PET-CT[7]. These disadvantages of both contrast-enhanced CT and PET/CT contribute to decreased diagnostic performance in the detection of recurrent gastric cancer. In our study, some locoregional recurrences were missed on CT with inadequate bowel distension and some were missed on PET/CT with physiological uptake in the stomach.

In this study, PET/CT was more sensitive than CT in detecting hepatic metastasis (100% *vs* 66%), but the number of patients who had hepatic metastases was too small to show statistical significance. Kinkel *et al*[16] reported that PET was the most sensitive method for the detection of hepatic metastases from gastrointestinal-tract cancer. However, other studies showed no significant difference between CT and PET/CT in detecting hepatic metastases[7,13].

CT was more sensitive for peritoneal carcinomatosis compared with PET/CT in this study. Previous studies showed similar results[7,11,13]. The detection of peritoneal carcinomatosis is difficult in cross-sectional images because the lesions are usually small (below 1 cm) and often flat. On PET/CT, physiological peritoneal FDG uptake due to involuntary muscle activity during scanning can hide underlying peritoneal metastases[14]. Therefore, patients who suspected to have peritoneal carcinomatosis in contrast-enhanced abdominal CT may not see any benefit in any additional PET/CT due to its moderate sensitivity.

For bone metastasis, CT had a detection sensitivity of 20% compared to 100% for PET/CT in our study. However, this difference was not statistically significant. Previous research showed that, PET/CT was more sensitive in detecting bone metastasis than CT mostly because it provides functional information[24]. However, the number of patients with bone metastasis enrolled in this study was too small to show statistical significance.

In our study, CT was more sensitive than PET/CT in detecting the recurrence of adenocarcinoma, contrary to the findings of previous studies[7,25]. Considering the high proportion of patients with a pathological type of adenocarcinoma was 88.6% in this study, CT efficacy may be superior to that of PET/CT. There have been reports that FDG avidity depends on histological type. Mucinous adenocarcinoma and signet ring cell carcinoma have low FDG avidity[25]. Although the number of mucinous and signet right cell carcinoma cases was not large, there was no statistically significant difference in detecting recurrence.

This study had two limitations. The first was its retrospective study design; thus, the indications for PET/CT were not defined and it was not possible to standardize the methods of follow-up imaging studies. This could cause selection bias, because patients who had not undergone CT or PET/CT were excluded. Secondly, not all the recurrent lesions were diagnosed histopathologically. Deeply located lymph node metastasis is difficult to histopathologically confirm in clinical practice. Practically, however, a clinical follow-up is a useful diagnostic method to confirm recurrence. It was regarded as a negative lesion when there was no change between images obtained with at least a 6 mo interval, as in a previous study[13]. But 6 mo of imaging follow up may not be enough to confirm the absence of recurrence.

In conclusion, the overall diagnostic performance of contrast-enhanced CT and PET/CT in patient- and lesion-based analysis was not significantly different in our study. However, contrast-enhanced CT was superior to PET/CT in the detection of peritoneal carcinomatosis. In patients with the most common pathological type of adenocarcinoma, contrast-enhanced CT might be more sensitive in detecting recurrence than PET/CT. Contrast-enhanced CT could thus be the primary method of surveillance for the recurrence of gastric carcinoma after curative resection.

COMMENTS

Background

Gastric carcinoma is still a leading cause of cancer death worldwide. Surgical resection is the potentially curative treatment for gastric cancer. Contrast-enhanced abdominal computed tomography (CT) and positron emission tomography/computed tomography (PET/CT) are commonly used imaging methods for surveillance of recurrence after gastric cancer surgery. But ideal method for early detection of gastric carcinoma recurrence remains controversial.

Research frontiers

Contrast-enhanced CT was superior to PET/CT in the detection of peritoneal carcinomatosis and pathologic type of adenocarcinoma. Contrast-enhanced CT could thus be the primary method of surveillance for the recurrence of gastric carcinoma after curative resection.

Innovations and breakthroughs

Contrast-enhanced abdominal CT and PET/CT are commonly used imaging methods for surveillance of recurrence after gastric cancer surgery. But there have been controversies about the role of CT, compared to PET/CT, in evaluating the recurrence of gastric carcinoma after curative surgery. We found contrast-enhanced CT was superior to PET/CT in the detection of peritoneal carcinomatosis and pathologic type of adenocarcinoma

Applications

Contrast-enhanced CT could be the primary method of surveillance for the recurrence of gastric carcinoma after curative resection.

Peer-review

This manuscript is very interesting and should be published in priority after minimal revision has been made. It is a retrospective study comparing the value of contrast enhanced abdominal CT and the fluorodeoxyglucose. Positron emission tomography/computed tomography for detecting recurrence of gastric carcinoma after curative resection.

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ORIGINAL ARTICLE

Retrospective Study

Duodenum and ventral pancreas preserving subtotal pancreatectomy for low-grade malignant neoplasms of the pancreas: An alternative procedure to total pancreatectomy for low-grade pancreatic neoplasms

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Abstract

AIM

To describe the indications, technique and outcomes of the novel surgical procedure of duodenum and ventral pancreas preserving subtotal pancreatectomy (DVPPSP).

METHODS

Data collected retrospectively from 43 patients who underwent DVPPSP and TP between 2009 and 2015 in our single centre were analysed. For enrolment, only patients with low-grade pancreatic neoplasms, such as pancreatic neuroendocrine tumors, intraductal papillary mucinous neoplasms (IPMNs), and solid pseudo-papillary tumors, were included. Ten DVPPSP (group 1) and 13 TP (group 2) patients were selected in this study.

RESULTS

There were no significant differences in age, gender, comorbidities, preoperative symptoms, American Society of Anesthesiologists score or indications for surgery between the two groups. The most common indication was IPMN for DVPPSP and TP (60% *vs* 85%, $P = 0.411$). Compared with the TP group, the DVPPSP group had

comparable postoperative morbidities (P = 0.405) and mortalities (both nil), but significantly shorter operative time (232 ± 19.6 min *vs* 335 ± 32.3 min, P < 0.001). DVPPSP preserved better long-term pancreatic function with less supplementary therapy (P < 0.001) and better quality of life (QoL) after surgery, including better scores in social (P = 0.042) and global health (P = 0.047) on functional scales and less appetite loss (P = 0.049) on the symptom scale.

CONCLUSION

DVPPSP is a feasible and safe procedure that could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas; DVPPSP had better metabolic function and QoL after surgery.

Key words: Low-grade malignant neoplasm; Ventral pancreas preserving; Subtotal pancreatectomy; Quality of life

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Core tip: Although total pancreatectomy (TP) can be performed safely, the long-term metabolic complications prevent its application. Herein, we report our experience of duodenum and ventral pancreas preserving subtotal pancreatectomy (DVPPSP), which could be an alternative procedure to TP for low-grade pancreatic neoplasms, with a focus on the surgical indications, techniques and outcomes. Our results indicate that DVPPSP is a feasible and safe procedure that could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas, with a better metabolic function and quality of life after surgery.

Wang X, Tan CL, Song HY, Yao Q, Liu XB. Duodenum and ventral pancreas preserving subtotal pancreatectomy for low-grade malignant neoplasms of the pancreas: An alternative procedure to total pancreatectomy for low-grade pancreatic neoplasms. *World J Gastroenterol* 2017; 23(35): 6457-6466 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6457.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6457

INTRODUCTION

Parenchyma-sparing procedures are increasingly performed for low-grade pancreatic neoplasms, such as intraductal papillary mucinous neoplasms (IPMNs), neuroendocrine tumors (NETs), and pseudo-papillary tumors (SPTs)[1,2]. These procedures preserve the pancreatic parenchyma and decrease the risk of long-term endocrine and exocrine dysfunction[3,4]. However, according to different reports, in these low-grade diseases, the percentages of total pancreatectomy (TP) range from 5% to 28% because these diseases frequently involve the entire gland[5-7]. The indications for primary elective TP are typically these pancreatic diseases. Although TP could be performed safely in many centres, the long-term metabolic complications that include brittle diabetes and unmanageable steatorrhea prevent its application. Additionally, wide resection of the total pancreas and duodenum after TP could lead to a reduced quality of life (QoL), which makes patients anguished. The conventional parenchyma-sparing procedures, such as enucleation and central pancreatectomy, are unable to substitute TP for lesions involving the head, body and tail of the pancreas.

As a function preserving procedure, Thayer *et al*[8] reported a complete dorsal pancreatectomy with preservation of the ventral pancreas (the proximal duodenum was resected for ischemia) in 2002. Duodenum-preserving pancreatic head resection was introduced into surgical practice in 1972. This surgical method has been well developed by Doctor Beger *et al*[9] in past decades. The rationale to apply a duodenum-preserving pancreatic resection is to avoid resection of the gastric antrum, to preserve the duodenum and the extrahepatic biliary duct for low-grade lesions. This procedure has low short-term postoperative morbidity and mortality, and maintenance of the endocrine and exocrine functions in the long-term after surgery. A significant superiority of the duodenum-preserving procedure regarding overall improvement of QoL was reported[10]. Herein, we report our experience of duodenum and ventral pancreas preserving subtotal pancreatectomy (DVPPSP), which could be an alternative procedure to TP for selected patients with low-grade pancreatic neoplasms. The aim of our present study was mainly to describe the indications, operative technique and outcomes of the novel surgical procedure compared with TP.

MATERIALS AND METHODS

From January 2009 to January 2015, patients who underwent DVPPSP for low-grade pancreatic neoplasms (such as NETs, IPMNs and SPTs) were identified in our institution. To evaluate the outcomes of DVPPSP compared with TP, patients who underwent TP for low-grade pancreatic neoplasms were also identified during this period. Surgeries for pancreatic ductal adenocarcinoma and chronic pancreatitis determined on postoperative pathology were excluded. The selected patients were divided into two groups as follows: DVPPSP group (group 1) and TP group (group 2). The groups were analysed regarding age, gender, type of comorbidities, body mass index (BMI), American Society of Anesthesiologists score (ASA), operative time, blood transfusion and postoperative data (histology of pancreatic disease, postoperative

mortality and morbidity, postoperative length of hospital stay). The postoperative mortality rate included all deaths within 30 d after surgery. The morbidity rate included all complications following surgery until discharge. Major postoperative morbidities were defined and graded using the criteria recommended by the International Study Group of Pancreatic Surgery, including postoperative pancreatic fistula, delayed gastric emptying, and postpancreatectomy haemorrhage[11-13]. Biliary leak was defined as drainage of any volume of fluid from percutaneous drains or a wound consistent with bile, which was defined by a bilirubin concentration greater than the serum concentration. Enteric leaks were identified using radiographic contrast imaging. Fluid collection in the intra-abdominal or pleural regions was determined via radiographic imaging, which was differentiated from abscess by positive microbial cultures. Pneumonia was diagnosed based on chest X-ray changes following antibiotic therapy.

Follow-up examinations were collected from the store of medical information centre in the hospital and from the results of follow-up by letters and telephone at the time of this study. The endocrine and exocrine controls were assessed by episodes of hyperglycaemia/day, daily dosage of insulin, pancreatic enzyme replacement. The QoL of patients were evaluated with the European Organization for Research and Treatment of Cancer Quality of life Questionnaire (EORTC QLQ-C30) version 3.0. The questionnaires were requested to be filled in by the patients themselves via mailing or completing in the clinic. The patients who had been dead or had tumor recurrence during the study were excluded from the analysis of QoL. This study was approved by the Ethics Committee of Sichuan University.

Surgical indication

DVPPSP was an alternative operative technique to TP of low-grade pancreatic tumor arising from the body and tail region but across the neck region of the pancreas (Figure 1). Contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), or/and endoscopic ultrasound (EUS) were routinely performed to evaluate the lesions. Furthermore, DVPPSP was finally considered after intraoperative evaluation of the lesion, which confirmed negative for neoplasm of ventral pancreas based on palpation and intraoperative ultrasonography. Frozen sectioning was routinely performed to achieve negative margins in the present study. If invasive carcinoma was found in the frozen section of the stump, the DVPPSP was immediately converted to TP with formal lymphadenectomy.

Surgical technique

The surgical procedures were performed by one group of experienced surgeons in our department of pancreatic surgery. We performed a complete Kocher manoeuvre to mobilize the duodenum, the pancreatic head and uncinate process from the posterior connective tissue membrane. There was a call for intraoperative ultrasound to confirm that the ventral pancreas was negative for neoplasm. Then, the neck and body of the pancreas was freed from the common hepatic artery, portal vein, and superior mesenteric vein. The gastrocolic trunk was divided and ligated at its confluence. Then, we dissected and taped the gastroduodenal artery (GDA). The anterior superior pancreaticoduodenal artery (ASPD) and other small branches of GDA in front of the cephalic pancreas could be resected to expose the common bile duct (CBD) at the upper edge of the pancreas and to completely resect the neoplasm. We preserved the GDA and the posterior superior pancreaticoduodenal artery (PSPD) (Figure 2). The transection of the cephalic side of the pancreas was begun at the edge of the neoplasm and was continued caudally. The posterior transection plane was along the anterior side of the CBD (Figure 2). Inferiorly, we dissected and exposed the CBD from the resecting pancreas toward the major papilla to achieve complete resection of the neoplasm.



Figure 1 Preoperative computed tomography view of a 26-year-old female patient with a diagnosis of pseudo-papillary tumors. The figure shows that the lesion occupied the body and neck region with necrosis of the tail on preoperative computed tomography imaging. Normal pancreatic tissue is observed in the ventral pancreas region.

If the neoplasm involved the entire dorsal pancreas, such as IPMN that involved the whole dorsal duct (including the Santorini duct) but ventral duct (Figure 3), a complete dorsal pancreatectomy was needed to achieve radical resection of the tumor. The embryological fusion plane between the ventral and the dorsal pancreas was confirmed by autopsy[14] and was described as a clear fibrous septum[8]. These anatomical characteristics made the complete dorsal pancreatectomy procedure theoretically feasible. However, the plane was difficult to identify in our study during surgery. Instead, we continued pancreatic transection and exposed the whole distal CBD (except its last centimetre) to achieve a complete dorsal pancreatectomy and we the confluence of the CBD with the ventral duct. The dorsal pancreatic duct was

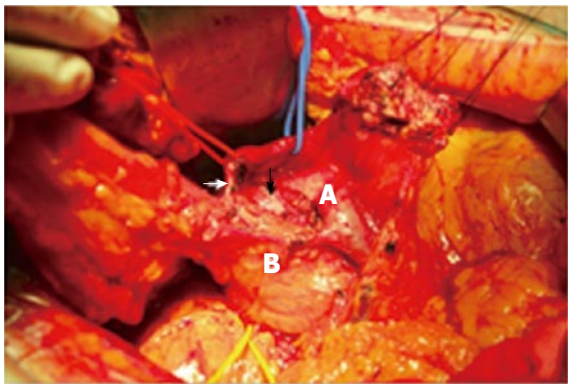


Figure 2 Intraoperative view of duodenum and ventral pancreas preserving subtotal pancreatectomy. A: Superior mesenteric vein; B: Ventral pancreas. Black arrow: intrapancreatic bile duct; White arrow: gastroduodenal artery.

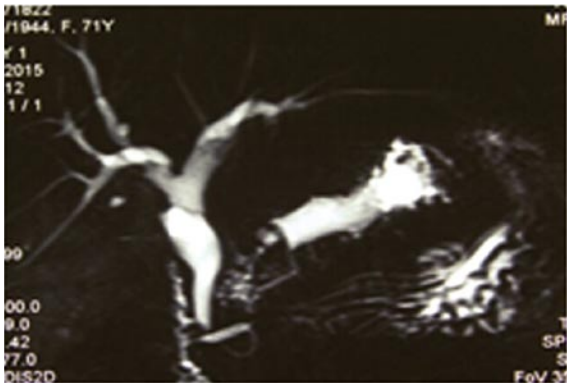


Figure 3 Preoperative magnetic resonance imaging view of a 71-year-old female patient with diagnosis of MD-IPMN. The figure shows that the lesion involves the whole dorsal duct (including Santorini duct). The diameter of ventral pancreatic duct is normal on the MRCP image.

divided and ligated 3 mm away from the duodenal lumen and was free of neoplasia on frozen section examination. The anterior inferior pancreaticoduodenal artery (AIPD) could be included in the resection site if necessary. Then, we identified the main pancreatic duct (Wirsung duct) in the stump of the residual pancreas and ligated this duct. The stump was negative for tumor on frozen sectioning, which was routinely performed in the present study.

Parenchyma sutures and tissue sealant were used in selected cases. Bipolar cautery, ultrasound scalpel and the suture technique using 4-0 absorbable sutures (Ethicon, Inc) were used for haemostasis and pancreatostasis. It was worth noting that bipolar cautery was not performed in the deep areas near the Wirsung duct or other important structures (CBD or major vessels) due to presumed heat injury. Parenchymal sutures and tissue sealant were used for cases in which haemostasis and pancreatostasis were not satisfied exclusively using bipolar cautery and ultrasound scalpel. Finally, we routinely placed at least one drain adjacent to the cut surface.

Drain amylase analysis was performed from postoperative day one to three and then every other day. For patients without a fistula, we progressively withdrew drainage starting on postoperative day three and finally removed the drain on postoperative day five. Postoperative octreotide was routinely used for seven days to decrease the secretion of the pancreas. We routinely performed abdominal EUS before finally removing the drain. The long-term follow-up should be performed after DVPPSP, even if the stump is negative.

Statistical analysis

The Pearson χ^2 test was used for categorical variables. Fisher's exact test was used in cases with a variable count < 5. For continuous variables, Student's *t* test was used for normally distributed variables and the Wilcoxon rank-sum test was used for non-normally distributed variables. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS version 18.0 software on a personal computer (SPSS Inc., Chicago, IL, United States).

RESULTS

DVPPSP was performed for 10 patients, and TP was performed for 33 patients between January 2009 and January 2015 in our department. Patients with chronic pancreatitis, pancreatic cancers (PC) and pancreatic metastases were excluded in this study. Of these, twenty patients who underwent TP, including 2 with chronic pancreatitis, 16 with PC, and 2 with pancreatic metastases from renal cell cancer, were excluded in our present study. Finally, ten DVPPSP (group 1) and 13 TP (group 2) patients were selected in this study.

The preoperative characteristics of the patients undergoing DVPPSP and TP are summarized in Table 1. There were no significant differences in age, gender, comorbidities, preoperative symptoms, ASA score or indications for surgery between the two groups. The median age was 49 in the DVPPSP group and 56 in the TP group. Four male patients (40%) were in the DVPPSP group, and 7 males (54%) were in the TP group. In the DVPPSP and TP groups, the majority of patients were symptomatic (100% *vs* 85%), and abdominal pain (40% *vs* 55%) was the most common symptom. The ASA III was given to 3 patients in DVPPSP and TP group. The pathologic features of the lesions in the DVPPSP and TP groups are shown in Table 1. The most common indication for DVPPSP or TP was IPMN (6/10, 60% *vs* 11/13, 85%). All the 6 IPMNs who underwent DVPPSP were main duct type (MD-IPMN). The 11 IPMNs in the TP group comprised 9 MD-IPMN, 1 mixed-type (MT-IPMN) and 1 multifocal type. According to the 2010 WHO classification, one out of 6 IPMN in the DVPPSP group and 5 out of 11 IPMN in the TP group were detected as invasive carcinoma (16.7% *vs* 45.5%, $P = 0.333$). The 2 NETs in the DVPPSP group and the 1 NET in the TP group

Table 1 Preoperative characteristics and final pathologic features of 23 patients in the duodenum and ventral pancreas preserving subtotal pancreatectomy and total pancreatectomy groups *n* (%)

Patient characteristics	DVPPST (*n* = 10)	TP (*n* = 13)	*P* vaule
Age (range, yr)	49 ± 16.9 (24-71)	56 ± 10.1 (40-70)	0.256
Gender (%)			0.680
Male	4 (40)	7 (54)	
Female	6 (60)	6 (46)	
Major comorbidity			
CAD	0	1	1.000
COPD	1	2	1.000
DM	2	3	1.000
Hypertension	3	3	1.000
Preoperative symptoms			
Incidental	0	2 (15)	0.486
Symptomatic (%)	10 (100)	11 (85)	
Abdominal pain	4 (40)	6 (55)	1.000
Pancreatitis	1	4	0.339
Weight loss	1	4	0.339
Gastrointestinal symptoms	4	3	0.65
ASA score			1.000
Ⅰ / Ⅱ	7	10	
Ⅲ	3	3	
Pathology (%)			0.411
IPMN	6 (60)	11 (85)	
Low-grade moderate-grade dysplasia	2	1	0.515
High-grade dysplasia	3	4	0.644
Invasive carcinoma	1 (16.7)	5 (45.5)	0.333
Neuroendocrine tumor	2	1	
G 2	2	1	1.000
SPT	2	1	
Total invasive malignancy	1 (10)	5 (38.5)	0.179

CAD: Coronary artery disease; COPD: Chronic obstructive pulmonary disease; DM: Diabetes mellitus; IPMN: Intraductal papillary mucinous neoplasm; SPT: Pseudo-papillary tumor; DVPPSP: Duodenum and ventral pancreas preserving subtotal pancreatectomy.

were classified as G2.

The intra-operative and postoperative short-term outcomes of the two groups are summarized in Table 2. None of the parameters listed were significantly different between the two groups except the operative time, which was significantly shorter in the DVPPSP group compared with the TP group (232 min *vs* 335 min, $P < 0.001$). The total morbidities were 20% (2/10) and 38% (5/13) in the DVPPSP and TP group, respectively. There was no significant difference in the post-operative morbidity between the two groups, including delay gastric emptying (DGE) and biliary fistula. Clinical pancreatic fistula (grade B or C) was not found in either group. There were no reoperations or readmissions in either group. The mortalities were nil in both groups. Although there was no significant difference, the post-operative hospital stay days (LOS)

Table 2 Intraoperative and postoperative outcomes of 23 patients after duodenum and ventral pancreas preserving subtotal pancreatectomy and total pancreatectomy

Operative characteristics	DVPPSP (*n* = 10)	TP (*n* = 13)	*P* vaule
Intraoperative transfusion (U)	0.20 ± 0.63 (0-2)	0.54 ± 1.05 (0-3)	0.379
Operative time (min)	232 ± 19.6 (215-255)	335 ± 32.3 (280-400)	< 0.001
Postoperative characteristics			
DGE	0	1	1.000
Biliary fistula	0	1	1.000
Pancreatic fistula (B and C)	0	0	–
Overall morbidity	2	5	0.405
Major morbidity (≥ grade 3)	1	2	1
Reoperation	0	0	–
Readmission (30 d)	0	0	–
LOS	9.7 ± 3.8 (7-20)	11.7 ± 5.2 (8-26)	0.318
Mortality	0	0	–
Recurrence[1](%)	2 (20%)	5 (38.5%)	0.405
IPMN	2/6	5/11	1.000

[1]The recurrences of 2 patients in the DVPPSP group and 5 patients in the TP group occurred after surgery for IPMN. DGE: Delayed gastric emptying; LOS: Length of postoperative stay; DVPPSP: Duodenum and ventral pancreas preserving subtotal pancreatectomy.

appeared to be slightly shorter in the DVPPSP group (9.7 d *vs* 11.7 d, $P = 0.318$). The mean follow-up lengths were 26.6 and 33.9 months in the DVPPSP and TP groups, respectively. There were 2 out of 10 patients in the DVPPSP group and 5 out of 13 patients in the TP group proven to have recurrences (20% *vs* 38.5%, $P = 0.405$) (Table 2). Two out of 6 IPMNs in the DVPPSP group were shown to have recurrence at 28 and 36 mo after surgery separately, although negative margins were detected by intra-operative frozen sections. A total of 5 out of 11 IPMNs in the TP group were shown to have recurrence at 16, 26, 30, 34, 50 mo after surgery separately (2/6, 33.3% *vs* 5/11, 45.5%, $P = 1.000$).

The differences in replacement therapy are summarized in Table 3. The mean follow-up lengths were 26.6 and 33.9 mo in the DVPPSP and TP groups, respectively. There were significant differences in replacement therapy between the two groups. The mean enzyme replacements were 0.9 cps/d *vs* 9.2 cps/d ($P < 0.001$) in the DVPPSP and TP groups, respectively. The total insulin doses were 6.4 U/d *vs* 29.7 U/d ($P < 0.001$). No patients had hyperglycaemia after insulin treatment in the DVPPSP group, whereas five patients (38%) in the TP group still had hyperglycaemia occasionally ($P = 0.046$). There were still 2 patients (20%) having diarrhea and steatorrhea occasionally after replacement treatment in the DVPPSP group, compared with 4 patients (31%) in the TP group ($P = 0.660$). The long term

Table 3 Postoperative condition of supplementary therapy of 23 patients after duodenum and ventral pancreas preserving subtotal pancreatectomy and total pancreatectomy

Parameters	DPPSP (n = 10)	TP (n = 13)	P vaule
Enzymes replacement (cps/d)	0.9 ± 2.0	9.2 ± 2.2	< 0.001
Total insulin dose (U/d)	6.4 ± 14.0	29.7 ± 9.3	< 0.001
Diarrhea or steatorrhea	2	4	0.660
Hyperglycemia	0	5	0.046

TP: Total pancreatectomy; DVPPSP: Duodenum and ventral pancreas preserving subtotal pancreatectomy.

Table 4 Postoperative condition of diabetes of 10 patients in the duodenum and ventral pancreas preserving subtotal pancreatectomy group

Parameters	DPPSP (n = 10)
New-onset diabetes	0 / 7
Worsen diabetes[1]	1/3
Diet to oral medication	–
Diet to insulin	–
Oral medication to insulin[1]	1/3

[1]There was 3 out of 10 patients in DVPPSP group who had diabetes before surgery and were treated by oral medicine. DVPPSP: Duodenum and ventral pancreas preserving subtotal pancreatectomy.

Table 5 Quality of lives after duodenum and ventral pancreas preserving subtotal pancreatectomy and total pancreatectomy

Parameters	DVPPSP (n = 8)	TP (n = 8)	P vaule
Functional scale			
Physical	95.0 ± 9.3 (80-100)	97.5 ± 7.1 (80-100)	0.554
Working ability	100	100	–
Cognitive	100	100	–
Emotional	98.4 ± 2.9 (93.8-100)	92.2 ± 8.0 (75-100)	0.057
Social	95.8 ± 7.7 (83.3-100)	85.4 ± 10.7 (66.7-100)	0.042
Global QoL	96.9 ± 6.2 (83.3-100)	88.5 ± 8.9 (75-100)	0.047
Symptom scale			
Fatigue	4.2 ± 8.3 (0-22.2)	11.1 ± 11.7 (0-33.3)	0.196
Nausea and vomiting	0	6.3 ± 12.4 (0-33.3)	0.197
Pain	0	0	–
Appetite loss	0	20.8 ± 24.8 (0-66.7)	0.049
Dyspnea	0	0	–
Sleep disturbance	4.2 ± 11.8 (0-33.3)	12.5 ± 17.2 (0-33.3)	0.281
Constipation	0	4.2 ± 11.8 (0-33.3)	0.351
Diarrhea	4.2 ± 11.8 (0-33.3)	12.5 ± 24.8 (0-66.7)	0.405
Financial strain	0	8.3 ± 15.4 (0-33.3)	0.170

TP: Total pancreatectomy; QoL: Quality of life; DVPPSP: Duodenum and ventral pancreas preserving subtotal pancreatectomy.

results regarding pancreatic endocrine function after DVPPSP are summarized in Table 4. Of the 10 patients who had DVPPSP, seven patients had no diabetes before surgery. There were no patients with new-onset diabetes. Of the 3 patients who had diabetes before surgery in the DVPPSP group, only one patient (1/3, 33%) had the diabetes worsen after surgery. This patient changed treatment with oral medication to treatment with insulin. The quality of life (QoL) according the EORTC QLQ-C30 is summarized in Table 5.

A total 16 patients were qualified to complete questions about the EORTC QLQ-C30 at the time of this study. Each group included 8 patients. Due to the recurrence of IPMNs, two patients in the DVPPSP group and 5 patients in the TP group mentioned above were excluded from the inquiry. As presented in Table 5, patients in the DVPPSP group could have a higher quality of life status after surgery compared with the TP group. A high scale score in the global health, physical, working ability, emotional, cognitive and social functioning represented a high quality of life status. Regarding the symptoms and financial impact of the disease, the low scale score indicated no important problems. There were significant differences existing in social (P = 0.042) and global health (P = 0.047) on functional scale. There was a significant difference existing in appetite loss (P = 0.049) on the symptom scale.

DISCUSSION

Although TP could be performed safely as reported by different authors in the literature, patients would suffer from the pain of replacement therapy throughout their lives[15-17]. When faced with benign or low-grade malignant disease with long life expectancy such as pNETs, IPMNs and SPTs, the need for a proper treatment has to be balanced with the risk of long term impairment of exocrine/endocrine function of the gland. Herein, we described the outcomes after 10 DVPPSPs performed for low-grade malignant disease compared with 13 TPs performed for the same spectrum of disease, showing that DVPPSP is safe and effective for preserving the exocrine/endocrine function of the gland. This procedure could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas.

It is a difficult question when we find the low-grade malignant neoplasm originating from the distal pancreas across the pancreatic neck. TP might bring negative margins but be accompanied by a loss of exocrine/endocrine function. When choosing distal pancreatectomy, positive margins might be inevitable. We used to choose primary or complete TP for this type of neoplasm in our department 8 years ago. After the first DVPPSP was performed successfully, this procedure

became our first choice when negative margins could be achieved at the pancreatic head. Intraoperative EUS and frozen sectioning are indispensable to decide where to perform limited pancreatic resection and to achieve negative margins. It is worth noting that only patients with benign or low-grade pancreatic neoplasms are selected as candidates for DVPPSP. The patients with such neoplasms could obtain long disease-free life expectancy if negative margins are achieved[18-20]. If invasive features or any doubts about the low-grade character of the stump were found in the frozen section, DVPPSP was immediately converted to TP with formal lymphadenectomy.

Moreover, diseases such as IPMN might predispose the entire gland, especially for the type of main duct, so detailed examination (such as endoscopic ultrasonography) could be needed before DVPPSP to detect the condition of the main duct[21]. DVVPSP is suitable for disease with a normal Wirsung duct in the ventral pancreas. If the entire duct is involved, the ventral pancreas should not be preserved. In this case, duodenum preserving total/subtotal pancreatic resection might be the best choice[22].

Due to the biological features of low-grade malignant neoplasms, normal interstitial space exists between the neoplasm and the peri-pancreatic tissue. This space is the key prerequisite to perform DVPPSP successfully. According to Yoshihiro's research[14] based on autopsy specimens, the dorsal pancreas makes up the pancreatic neck, body, and tail, as well as the anterior segment of the head, whereas the ventral pancreas comprises the posterior segment of the head and uncinate process. Moreover, Thayer *et al*[8] described the embryonic fusion plane of the two segments as a clear fibrous septum. These anatomic features make the duodenum and ventral pancreas preserving subtotal pancreatectomy (DVPPSP) a theoretically feasible procedure. As this plane was difficult to identify by some researchers[23] and by us in practice, we performed the pancreatic transection along the anterior side of the CBD and exposed the entire distal CBD (except 1-2 cm from the major papilla) to achieve negative margins. The confluence of the CBD with the ventral duct and ventral pancreas was carefully preserved.

A case of complete dorsal pancreatectomy was presented previously[8], with the first part of the duodenum resected due to ischemic damage. The gastroduodenal artery (GDA) was ligated in that procedure, while both the gastroduodenal artery and the posterior pancreaticoduodenal arterial arcade were preserved in our series. Additionally, the Kocher manoeuvre was suggested to increase the risk of duodenal ischemia in another previous case report[23]. However, we performed a complete Kocher manoeuvre to make the potential haemorrhage easier to control and to make the surgical procedure smoother during resection of the cephalic side of the pancreas. The duodenum was not found to be cyanotic in our present study with 10 DVPPSP cases. Careful dissection and reservation of the GDA and the posterior pancreaticoduodenal arterial arcade appeared to be essential for preservation of the duodenum in DVPPSP. The successful preservation of the duodenum prevents reconstruction in the upper digestive tract.

Our results (Table 2) also demonstrated that, compared with the TP group, DVPPSP had comparable short-term postoperative morbidities. Postoperative pancreatic fistula (POPF) is the most common complication after pancreatectomy[24,25]. However, clinical POPF (Grade B or C) was not detected in the 10 patients who underwent DVPPSP according to the revised definition by the International Study Group on Pancreatic Fistula (ISGPF)[26]. DVPPSP appears to be a safe and feasible procedure for selected low-grade pancreatic neoplasms based on our results. As shown in Table 2, 33.3% IPMN in the DVPPSP group and 45.5% IPMN in the TP group were shown to have recurrences (P = 1.000). The rate of postoperative recurrence of IPMN appeared slightly high compared with previous reports (0%-30%)[27-29]. The malignant component was suggested to be associated with tumor recurrence[29,30]. The high rate of malignancy (high-grade dysplasia or invasive carcinoma, 13/17, 76.5%; Table 1) of IPMN in our study might play a role in this recurrence, which was reported by others[29,31,32] to range from 35.7% to 68%. Furthermore, the small number of patients prevented us from making strong conclusions.

Given the long life expectancy after surgery for these diseases, the postoperative long-term pancreatic function and QoL become a major concern. Compared with the TP group, we found that DVPPSP did preserve better long-term pancreatic function with less supplementary therapy and resulted in better QoL after surgery in the follow-up of our study. No new-onset diabetes occurred in the DVPPSP group in the follow-up. Only one out of three patients had diabetes worsen, as shown in Table 4. This reservation could be attributed to less loss of the pancreatic parenchyma in DVPPSP.

In recent years, several investigators have advocated that postoperative QoL is as significant a clinical outcome as morbidity and mortality. The QoL of DVPPSP compared with TP in this study appeared to be better no matter replacement therapy or survey of quality of life. The high dependence of pancreatic enzyme replacement and insulin mainly negatively impacted the social and global quality of life in TP patients. The diabetes after TP could cause a negative impact on QoL[33]. More patients in the TP group felt a loss of appetite compared with patients in the DVPPSP group, perhaps because of manual changes of the digestive tract. The enterogastric reflux was considered an

unavoidable consequence after reconstruction in the upper digestive tract[34]. The great advantage of DVPPSP is preservation of the natural digestive channel. Other reports have demonstrated better QoL in duodenum preserving pancreatic surgery[35-37].

Our study is limited by the small number of cases because of the infrequent indications for DVPPSP. Some questions need to be researched further. A major limitation of the present study was its retrospective nature, which prevented us from making strong conclusions. Quality of life metrics were administered only once and were not compared with preoperative baseline measurements.

In conclusion, DVPPSP is a feasible and safe procedure that could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas, with a better metabolic function and QoL after surgery.

COMMENTS

Background

Parenchyma-sparing procedures are increasingly performed for low-grade pancreatic neoplasms, such as intraductal papillary mucinous neoplasms (IPMNs), neuroendocrine tumors, and pseudo-papillary tumors. These procedures preserve the pancreatic parenchyma and decrease the risk of long-term endocrine and exocrine dysfunction. The conventional parenchyma-sparing procedures, such as enucleation and central pancreatectomy, are unable to substitute for total pancreatectomy (TP) for lesions involving the head, body and tail of the pancreas. Herein, we report our experience of duodenum and ventral pancreas preserving subtotal pancreatectomy (DVPPSP), which could be an alternative procedure to TP for low-grade pancreatic neoplasms.

Research frontiers

Although TP could be performed safely as reported by different authors in studies, patients would suffer from the pain of replacement therapy throughout their lives. These results showed that DVPPSP is safe and effective for preserving the exocrine/endocrine function of the gland, with a better metabolic function and QoL after surgery. DVPPSP could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas.

Innovations and breakthroughs

This study described the indications, operative technique and outcomes of the novel surgical procedure DVPPSP. Furthermore, comparisons between DVPPSP with TP were presented in the present study. The findings indicate that DVPPSP is a feasible and safe procedure and could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas, with a better metabolic function and QoL after surgery.

Applications

DVPPSP is a feasible and safe procedure. It could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas, with a better metabolic function and QoL after surgery.

Terminology

The DVPPSP is a parenchyma-sparing procedure and could be an alternative to TP for low-grade neoplasms arising from the body and tail region but across the neck region of the pancreas.

Peer-review

The present study explored a new surgical technique so called "duodenum and ventral pancreas preserving subtotal pancreatectomy" as a choice of treatment for low-grade pancreatic neoplasms, such as IPMN, neuroendocrine tumors and pseudo-papillary tumors. The results showed DVPPSP was superior to total pancreatectomy by shorter operative time, better postoperative pancreatic function and better quality of life. But there was no detail pathological examination information of surgical specimens and follow-up data about tumor recurrence in the study. AS mentioned in the result of this article, why the rate of postoperative recurrence of IPMN was so high, 2 out of 6 for DVPPSP and 5 out of 11.

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ORIGINAL ARTICLE

Retrospective Study

Validated preoperative computed tomography risk estimation for postoperative hepatocellular carcinoma recurrence

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Institutional review board statement:: The study was reviewed and approved by the Affiliated Tumor Hospital of Guangxi Medical University Institutional Review Board.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymized clinical data that were obtained after each patient agreed by written consent to undergo computed tomography and curative resection.

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

Data sharing statement: No additional data are available.

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Abstract

AIM

To develop and validate a risk estimation of tumor recurrence following curative resection of operable hepatocellular carcinoma (HCC).

METHODS

Data for 128 patients with operable HCC (according to Barcelona Clinic Liver Cancer imaging criteria) who underwent preoperative computed tomography (CT) evaluation at our hospital from May 1, 2013 through May 30, 2014 were included in this study. Follow-up data were obtained from hospital medical records. Follow-up data through May 30, 2016 were used to retrospectively analyze preoperative multiphasic CT

findings, surgical histopathology results, and serum α-fetoprotein and thymidine kinase-1 levels. The χ^2 test, independent *t*-test, and Mann-Whitney *U* test were used to analyze data. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

During the follow-up period, 38 of 128 patients (29.7%) had a postoperative HCC recurrence. Microvascular invasion (MVI) was associated with HCC recurrence (χ^2 = 13.253, *P* < 0.001). Despite postoperative antiviral therapy and chemotherapy, 22 of 44 patients with MVI experienced recurrence after surgical resection. The presence of MVI was 57.9% sensitive, 75.6% specific and 70.3% accurate in predicting postoperative recurrence. Of 84 tumors without MVI, univariate analysis confirmed that tumor margins, tumor margin grade, and tumor capsule detection on multiphasic CT were associated with HCC recurrence (*P* < 0.05). Univariate analyses showed no difference between groups with respect to hepatic capsular invasion, Ki-67 proliferation marker value, Edmondson-Steiner grade, largest tumor diameter, necrosis, arterial phase enhanced ratio, portovenous phase enhanced ratio, peritumoral enhancement, or serum α-fetoprotein level.

CONCLUSION

Non-smooth tumor margins, incomplete tumor capsules and missing tumor capsules correlated with postoperative HCC recurrence. HCC recurrence following curative resection may be predicted using CT.

Key words: Hepatocellular carcinoma; Microvascular invasion; Computed tomography; Recurrence; Tumor margin; Tumor capsule

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Core tip: We discuss risk estimation for recurrence following curative resection of operable hepatocellular carcinoma that meets Barcelona Clinic Liver Cancer imaging criteria. Preoperative multiphasic computed tomography findings, including non-smooth tumor margins, incomplete tumor capsule and missing tumor capsule, can predict hepatocellular carcinoma recurrence following curative resection. Treatment should include wide margins during curative resection, followed by antiviral therapy and chemotherapy.

Zhang W, Lai SL, Chen J, Xie D, Wu FX, Jin GQ, Su DK. Validated preoperative computed tomography risk estimation for postoperative hepatocellular carcinoma recurrence. *World J Gastroenterol* 2017; 23(35): 6467-6473 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6467.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6467

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third most common cause of cancer-related death worldwide[1-3]. Surgical resection and liver transplantation are potentially curative treatment options for patients with HCC[4]. However, the 5-year HCC recurrence rate may be as high as 70%[5]. Recent studies have shown that tumor size and number[6], microvascular invasion (MVI)[5,7-9] and α-fetoprotein (AFP) levels[10,11] are associated with recurrence following surgical resection. Although many potential risk factors for HCC postoperative recurrence have been described, a reliable preoperative method to estimate this risk has not been established.

Kanai *et al*[12] first described nodular HCC sub-classification based on microscopic, clinical and prognostic features; these being single nodular type, single nodular type with extra-nodular growth, and contiguous multinodular type. Studies have shown that non-smooth tumor margins are associated with increased MVI[13-16]. Although computed tomography (CT) examination is part of the standard of care for HCC evaluation, no study has demonstrated the validity of preoperative CT assessment to predict postoperative recurrence of solitary HCC.

Thymidine kinase 1 (TK1), a DNA synthesis enzyme, is an important serum proliferation marker that correlates with clinical stage and is an independent prognostic factor for recurrence-free survival in HCC[17-20]. However, to our knowledge, there are no reports about serum TK1 levels and postoperative HCC recurrence.

In this study, we retrospectively assessed whether preoperative CT findings could predict postoperative HCC recurrence. We specifically evaluated largest tumor diameter, tumor margins, tumor capsule, necrosis, peritumoral enhancement, tumor enhanced ratio and hepatic capsular invasion. Furthermore, we assessed whether MVI, serum AFP, and serum TK1 levels were correlated with postoperative recurrence of solitary HCC.

MATERIALS AND METHODS

Patients

This retrospective study included 128 patients (105 males and 23 females), aged 24 to 79 years (mean, 47.7 years), with operable HCC (according to the Barcelona Clinic Liver Cancer imaging criteria) who underwent preoperative CT evaluation at our hospital from May 1, 2013 through May 30, 2014. Follow-up data were obtained from hospital medical records. HCC recurrence was defined as a pathologic or radiologic diagnosis of recurrent HCC during the follow-up period that ended on May 30, 2016. All patients

in this study: (1) had no cancer-related treatment or liver biopsy prior to CT imaging; (2) underwent partial hepatectomy and had HCC diagnosis confirmed by histopathology; (3) had no macrovascular invasion or metastasis on CT imaging; and (4) had livers with a Child-Pugh classification of A or B. This study was approved by our hospital institutional review board and all patients provided written informed consent.

CT imaging protocol

Hepatic CT images were obtained using a 64-MDCT scanner (SOMATOM Sensation 64; Siemens, Forchheim, Germany) with Z-axis modulation, a spiral pitch of 1, a 5 mm section thickness, a 2 mm reconstruction gap, a field of view of 311 mm, 120 kVp, 230 mA, and a standard reconstruction algorithm. Nonionic contrast medium (300 mg I/mL iopromide) was administered at an injection rate of 3 mL/s for a total dose of 100 mL. For the hepatic arterial and portovenous phases, scanning was begun approximately 25 and 60 s after contrast media injection, respectively. Equilibrium phase images were acquired approximately 180 s after contrast media injection. The scanning range was the whole-liver zone while patients held their breath. Coronary and sagittal images were reconstructed with 5 mm section thickness.

Image evaluation

Tumor margins on the liver map (*i.e.* transverse, coronary and sagittal planes) were defined as three subtypes (smooth margin, non-smooth margin focal extranodular and non-smooth margin multinodular), based on previous reports[12-16,21,22]. The tumor margins were categorized into one of the following three grades: Grade 0: smooth margin; Grade 1: focal extranodular type; and Grade 2: multinodular type. The tumor capsule was defined as a linear and enhanced structure surrounding the tumor detected by equilibrium phase CT imaging. Tumor capsules were categorized into the following three groups: Grade 0: complete tumor encapsulation; Grade 1: incomplete tumor encapsulation; and Grade 2: no tumor encapsulation. Negative or positive peritumoral enhancement was determined by multi-phase CT imaging. Positive peritumoral enhancement was defined in comparison to liver parenchyma as a hyperdense area proximal to the tumor border during arterial phase imaging that changed to an isodense area during equilibrium phase imaging. The tumor enhanced ratio was calculated as ($CT_{enhanced}$-$CT_{unenhanced}$)/$CT_{unenhanced}$.

All CT findings were reviewed by two radiologists experienced in liver CT evaluation: Lai SL (22 years of experience) and Xie D (21 years of experience). In the case of disagreements over CT findings, a third radiologist helped resolve discrepancies to achieve consensus.

Serum samples and histopathologic analysis

The serum TK1 assay was performed using a commercial kit, based on an enhanced chemiluminescence dot blot assay. Serum AFP detection was carried out using enzyme immunoassay. Ki67 expression, hepatic capsular invasion and MVI were confirmed by immunohistochemistry. The Edmondson-Steiner (E-S) grade was determined using the WHO Liver Cancer Study Group guidelines[23].

Statical analysis

Statistical analysis was performed using SPSS version 16.0 statistical software (SPSS, Chicago, IL, United States). An independent *t*-test was used to compare recurrence and non-recurrence groups with respect to largest tumor diameter, arterial phase enhanced ratio, portovenous phase enhanced ratio, serum AFP, serum TK1 and Ki67. Receiver operating characteristic curve analyses were used to determine the optimal cut-off value and diagnostic performance for $P < 0.05$. The chi-square test was used to compare tumor margins, necrosis, peritumoral enhancement, MVI and hepatic capsular invasion between groups. The tumor margin grade, E-S grade and tumor capsule were analyzed using the Mann-Whitney *U* test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Quantitative and qualitative findings are summarized in Table 1. MVI was higher in the recurrence group (22 of 38 patients) compared to the non-recurrence group (22 of 90 patients) ($\chi^2 = 13.253$, $P < 0.001$). Despite antiviral therapy and chemotherapy during follow-up, 22 of 44 patients with MVI experienced recurrence after surgical resection. The presence of MVI was 57.9% sensitive, 75.6% specific and 70.3% accurate in predicting postoperative recurrence.

Of 84 recurrences without MVI, univariate analyses showed no difference between groups with respect to hepatic capsular invasion ($\chi^2 = 1.691$, $P = 0.193$), Ki67 value ($P = 0.686$), E-S grade ($Z = -0.460$, $P = 0.646$), largest tumor diameter ($P = 0.366$), necrosis ($\chi^2 = 0.412$, $P = 0.521$), arterial phase enhanced ratio ($P = 0.725$), portovenous phase enhanced ratio ($P = 0.685$), peritumoral enhancement ($P = 0.240$) or serum AFP level ($P = 0.882$). In 27 of 84 recurrences without MVI, serum TK1 did not differ between groups ($P = 0.377$). Univariate analyses showed that non-smooth tumor margins ($\chi^2 = 5.042$, $P = 0.025$), tumor margin grade ($Z = -2.355$, $P = 0.019$) and absence of tumor capsule on CT ($Z = -2.279$, $P = 0.023$) were

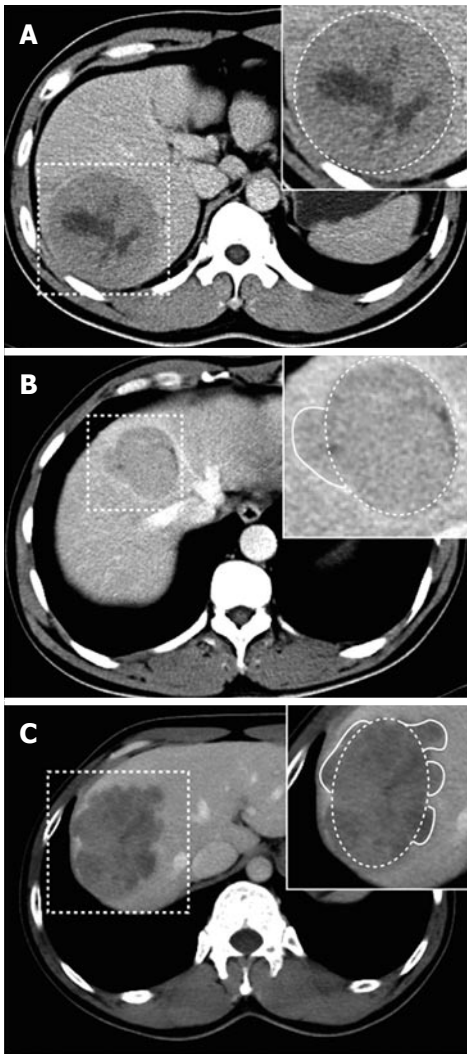


Figure 1 Tumor margins on the liver computed tomography map. A: Portovenous phase CT showing smooth margin at liver segment VI in a 36-year-old male patient without postoperative recurrence (dashed box); B: Portovenous phase CT showing focal extranodular type at liver segments V and VIII in a 38-year-old female patient with postoperative recurrence (dashed box); C: Portovenous phase CT showing multinodular type at liver segments V and VI in a 35-year-old male patient with postoperative recurrence (dashed box). CT: Computed tomography.

correlated with tumor recurrence (Figure 1A-C and Figure 2A-C).

DISCUSSION

Surgical resection of HCC suffers from high rates of tumor recurrence[5]. A reliable and validated prognostic method to estimate individual HCC recurrence risk would help guide future treatment strategies. Factors commonly associated with high risk for postoperative HCC recurrence include tumor vascular invasion and poorly differentiated tumor grade[24]. In this study, however, E-S grade was not associated with tumor recurrence. We did confirm, however, that MVI may be a powerful independent prognostic factor for postoperative recurrence and metastasis[5,7,14,25-27].

Table 1 Quantitative and qualitative factors associated with recurrence of hepatocellular carcinoma following curative resection

Risk factor		Follow-up recurrence		*P* value
		Yes	No	
MVI	Positive	22	22	0.000[b]
	Negative	16	68	
MVI-negative cases				
Hepatic capsular invasion	Positive	4	29	0.193
	Negative	12	39	
Ki67		30.3 ± 20.4	28.0 ± 21.0	0.686
E-S grade[1]	I	1	6	0.646
	II	8	36	
	III	7	26	
Largest tumor diameter, cm		5.5 ± 3.0	4.8 ± 2.6	0.366
Arterial phase enhanced ratio		0.62 ± 0.34	0.66 ± 0.40	0.725
Portovenous phase enhanced ratio		0.77 ± 0.23	0.80 ± 0.33	0.685
Tumor capsule	Complete	3	35	0.023[a]
	Incomplete	7	19	
	Negative	6	14	
Necrosis	Positive	8	28	0.521
	Negative	8	40	
Tumor margins	Smooth	9	56	0.019[a]
	Focal extranodular	5	11	
	Multinodular	2	1	
Peritumoral enhancement	Positive	2	3	0.24
	Negative	14	65	
AFP, ng/mL		336.8 ± 480.4	357.6 ± 505.6	0.882
TK1, U/L		5.7 ± 7.8	2.6 ± 2.5	0.377

[1]E-S classification rubric as in ref. 23. [b]$P < 0.01$ and [a]$P < 0.05$, statistically significant; Statistics are presented as mean ± SD. AFP: α-fetoprotein; E-S: Edmondson-Steiner; HCC: Hepatocellular carcinoma; MVI: Microvascular invasion; TK1: Thymidine kinase-1.

Moreover, in this retrospective study, we further validated MVI as an independent prognostic factor correlated with HCC recurrence. A 50% HCC recurrence rate was found following surgical resection for tumors with MVI, despite antiviral therapy and chemotherapy. Thus, the possibility of recurrence for HCC with MVI is substantial. However, MVI can rarely be confirmed preoperatively and, consequently, it is difficult to choose an appropriate procedure (*e.g.*, wide resection margins) to prevent HCC recurrence[28]. Thus, it is important to identify preoperative imaging markers that could predict HCC recurrence.

It has been reported that conventional CT could be used to reconstruct global HCC gene expression patterns[29]. When Zhou *et al*[30] investigated the correlation between CT-based radiomics signature and early HCC recurrence, they identified internal arteries and necrosis as independent risk factors. Their results, which were similar to those of previous studies[5,29], led to their hypothesis that internal arteries may be correlated with MVI. The finding that necrosis is a marker of early HCC recurrence[30], however, is inconsistent with our study findings. We suspect that different follow-up times (1 year in their study *vs* 2 years in ours), was responsible for this difference.

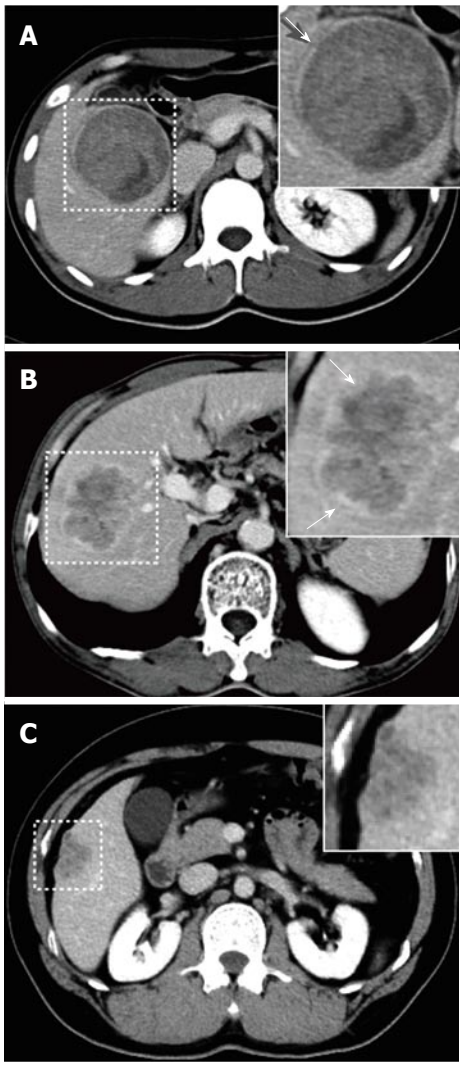


Figure 2 Tumor capsules on the liver computed tomography map. A: Equilibrium phase CT showing complete tumor capsule (red arrow) involvement at liver segment V in a 33-year-old male patient without postoperative recurrence (dashed box); B: Equilibrium phase CT showing incomplete tumor capsule (red arrow) at liver segments V and VI in a 57-year-old female patient with postoperative recurrence (dashed box); C: Equilibrium phase CT showing missing tumor capsule at liver segment VI in a 41-year-old male patient with postoperative recurrence (dashed box). CT: Computed tomography.

In this study, we further investigated whether preoperative CT findings correlate with HCC recurrence after surgical resection. Our finding that non-smooth tumor margins on CT could predict tumor recurrence has not been evaluated in previous studies. However, a previous study did show that non-smooth tumor margins on CT were predictive of MVI, and that MVI is an independent predictor of poor outcomes following surgical resection[13]. In our stratified analysis of tumors without MVI, tumor margins were significantly associated with postoperative recurrence during the follow-up period. This finding may be related to HCC multi-centricity being an important independent prognostic factor[31], and tumor aggressiveness should be considered[32]. We believe that non-smooth tumor margins can be considered an independent predictor of HCC recurrence.

Adachi *et al*[33] reported that vessels of the fibrous liver capsule were often invaded by tumor cells and that complete liver capsule fibrosis was a predictor of portal venous invasion. However, complete liver capsule fibrosis has been reported as a favorable prognostic marker because it can prevent HCC invasion into adjacent liver parenchyma[13]. Our results also showed that CT evidence of complete tumor capsules were associated with a low risk of postoperative recurrence. Peritumoral enhancement on magnetic resonance imaging (MRI) has been associated with a high risk of MVI[34]. In our study, peritumoral enhancement on CT was not related to HCC recurrence. The discrepancy in findings may be due to differences in imaging modalities used in various studies. Lu *et al*[35] reported that, when HCC was larger than 3 cm in diameter, the tumor tended to be more aggressive and was associated with a poor prognosis[36]. However, large tumor diameter was not a significant risk factor for postoperative recurrence in our study. At present, it is clear that HCC nodule size is not the only prognostic factor for recurrence[32]. Additionally, the relationship between postoperative HCC recurrence and AFP level remains controversial[5,10,11].

This study has two limitations. One potential limitation is the use of CT instead of MRI. Although CT is commonly used for HCC evaluation, MRI may provide additional information. A second limitation is the relatively small number of postoperative recurrences, except among those with preoperative evidence of MVI on CT. However, we further analyzed all cases and found similar results for the MVI-positive and MVI-negative groups. Thus, the study sample may reflect true population postoperative recurrences.

In conclusion, evaluation of patients with HCC should include preoperative CT findings of non-smooth tumor margins, incomplete tumor capsules and missing tumor capsules, which indicate possible postoperative recurrence. The treatment plan should include wide resection margins during curative resection, followed by antiviral therapy and chemotherapy.

COMMENTS

Background

Surgical resection of hepatocellular carcinoma (HCC) suffers from high rates of tumor recurrence. Although many potential risk factors for HCC postoperative recurrence have been described, a reliable preoperative method to estimate this risk has not been established. It has been reported that conventional computed tomography (CT) could be used to reconstruct global HCC gene expression patterns. Therefore, preoperative CT risk estimation for postoperative HCC recurrence should be clarified.

Research frontiers

A reliable and validated prognostic method to estimate individual HCC recurrence risk would help guide future treatment strategies. The results of this study contribute to clarifying the correlation of CT signature with postoperative HCC recurrence.

Innovations and breakthroughs

In the study, preoperative CT provides evidence of non-smooth tumor margins, incomplete tumor capsules and missing tumor capsules, which had been clarified as independent risk factors of postoperative HCC recurrence.

Applications

This study suggests that preoperative CT is useful for predicting postoperative HCC recurrence. If the preoperative CT findings of a patient are non-smooth tumor margins, incomplete tumor capsules and missing tumor capsules, the treatment plan should include wide resection margins during curative resection, followed by antiviral therapy and chemotherapy.

Peer-review

The manuscript from Zhang W *et al* reported the correlation of preoperative CT findings with postoperative HCC recurrence. HCC recurrence following curative resection may be predicted using CT. The entire sets of data are nicely presented, and highly supportive of the conclusion.

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ORIGINAL ARTICLE

Observational Study

Diagnostic delay in inflammatory bowel disease increases the risk of intestinal surgery

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Informed consent statement: The requirement for obtaining informed patient consent was waived because the present study has been based on the retrospective analysis of existing medical records.

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Data sharing statement: No additional data are available.

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Abstract

AIM

To investigate the factors affecting diagnostic delay and outcomes of diagnostic delay in inflammatory bowel disease (IBD)

METHODS

We retrospectively studied 165 patients with Crohn's disease (CD) and 130 patients with ulcerative colitis (UC) who were diagnosed and had follow up durations > 6 mo at Korea University Ansan Hospital from January 2000 to December 2015. A diagnostic delay was

defined as the time interval between the first symptom onset and IBD diagnosis in which the 76th to 100th percentiles of patients were diagnosed.

RESULTS

The median diagnostic time interval was 6.2 and 2.4 mo in the patients with CD and UC, respectively. Among the initial symptoms, perianal discomfort before diagnosis (OR = 10.2, 95%CI: 1.93-54.3, *P* = 0.006) was associated with diagnostic delays in patients with CD; however, no clinical factor was associated with diagnostic delays in patients with UC. Diagnostic delays, stricturing type, and penetrating type were associated with increased intestinal surgery risks in CD (OR = 2.54, 95%CI: 1.06-6.09; OR = 4.44, 95%CI: 1.67-11.8; OR = 3.79, 95%CI: 1.14-12.6, respectively). In UC, a diagnostic delay was the only factor associated increased intestinal surgery risks (OR = 6.81, 95%CI: 1.12-41.4).

CONCLUSION

A diagnostic delay was associated with poor outcomes, such as increased intestinal surgery risks in patients with CD and UC.

Key words: Diagnostic delay; Intestinal surgery; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Core tip: As the manifestations of inflammatory bowel disease (IBD) were nonspecific, the diagnosis is often established following considerable delay. There have been few reports about diagnostic delay associated with poor outcomes in Asian IBD patients. We aimed to investigate the factor affecting diagnostic delay and its effect in Korean IBD patients. In present study, a diagnostic delay was significantly associated with poor outcomes, such as increased IBD related intestinal surgery risks in patients with Crohn's disease and ulcerative colitis. Therefore, it is important for the improvement of clinical outcomes in IBD patients to early diagnose and manage adequately.

Lee DW, Koo JS, Choe JW, Suh SJ, Kim SY, Hyun JJ, Jung SW, Jung YG, Yim HJ, Lee SW. Diagnostic delay in inflammatory bowel disease increases the risk of intestinal surgery. *World J Gastroenterol* 2017; 23(35): 6474-6481 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6474.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6474

INTRODUCTION

Inflammatory bowel disease (IBD) represented by Crohn's disease (CD) and ulcerative colitis (UC) is characterized by chronic inflammation in all or part of digestive tract without definite cause[1]. Persisting and relapsing inflammation causes not only functional dysfunction in the gut, but also structural destruction of bowel such as stenosis, fistula formation and perforation[2]. Recent studies have reported that an early intensive control of inflammation with immunosuppressive agents or biological agents improved the prognosis of newly diagnosed patients with IBD, which emphasized the importance of early treatment following early diagnosis[3-5].

IBD has a waxing and waning course with asymptomatic remission period and with episodes of disease where patients present with symptoms, such as hematochezia, fever, and abdominal pain[6]. Because symptoms were not specific and not constant over time and the findings of diagnostic studies overlapped with those of other disease, such as tuberculosis and connective tissue disease, the diagnosis is often established following a considerable delay[7-9]. Indeed, in the Swiss IBD cohort study, the median duration of diagnostic delay period in CD patients was 9 mo, and about 25% of them had a duration of > 24 mo from symptom to diagnosis[9]. The median diagnostic delay period in patients with UC was 4 mo, which was shorter than that in patients with CD. Further, in the French CD cohort with 364 patients, about 40% of patients had a duration of > 12 mo from symptom to diagnosis[10]. A diagnostic delay was associated with poor clinical outcomes in patients with CD, such as an increased risk of bowel stenosis, fistula and abscess formation, and intestinal surgery[11,12]. However, no study has reported the association between the clinical outcomes and diagnostic delays in patients with UC.

Unlike in Western countries, there are few studies on diagnostic delays associated with IBD in Asia. Considering the significant differences in the epidemiological and clinical features of IBD according to ethnicities and environmental factors, diagnostic delays and the associated factors may differ according to countries[13,14]. Therefore, we aimed to investigate the clinical factors and outcomes associated with diagnostic delays in Korean patients with CD and UC.

MATERIALS AND METHODS

Subjects

This study included 177 patients with CD and 143 patients with UC who were definitely diagnosed at Korea University Ansan Hospital from January 1, 2000 to December 31, 2015. The CD and UC diagnoses were confirmed based on the clinical, endoscopic, radiological and histological findings. The patients younger than 18 years at the time of diagnosis, those who had a follow-up duration of < 6 mo, and those who had incomplete medical records were excluded from this study. In the present study, 25 patients with IBD were excluded because they were under 18 years old (CD 3), had insufficient medical records (CD 3, UC 7), or had a

follow up duration time of < 6 mo (CD 5, UC 6). The remaining 165 patients with CD and 130 patients with UC were analyzed in the present study

This study was approved by the Institutional Review Board of Korea University Ansan Hospital (AS16206) and conducted in accordance with the Declaration of Helsinki. The need for informed consent was waived in view of the retrospective observational design of the study.

Methods

The clinical and demographic data such as age at diagnosis, sex, smoking status, the first IBD related symptom, family history of IBD and prescribed medications were collected from each patient's medical records.

The diagnostic time interval was the duration from the first symptom to the diagnosis of IBD. This diagnostic time interval included patient-dependent delay (time interval from first symptoms to the physician visit) and physician-dependent delay (time interval from first physician visit to IBD diagnosis). Because there are no established criteria to define diagnostic delays, it was defined based on the time interval in which the 76th to 100th percentile of the patients were diagnosed. Follow-up duration was defined as the time from the date of first diagnosis of IBD to the date of the last follow up.

Disease extent and behavior at the initial diagnosis in CD and UC were classified in accordance with the Montreal classification[15]. Disease severity was evaluated using the C-reactive protein level at diagnosis. In UC, the Mayo score was additionally used for severity assessment. Data on the prescribed medications for IBD, including 5-aminosalicylic acid, systemic corticosteroids, immunomodulator and anti-tumor necrosis factor alpha (anti-TNFα) agents were also obtained from medical records.

To assess the clinical outcomes associated with a diagnostic delay, the following outcomes were measured: the number and type of intra-abdominal surgery, number and type of any surgery including perianal surgical procedures, number and date of hospitalization, and time duration from diagnosis to immunomodulator or anti-TNFα administration. Frequent admission was defined as two or more hospitalizations during the follow-up period.

Statistical analysis

The values for the continuous variables are expressed as mean ± (SD) or medians ± (SE). Categorical or discrete variables are presented as percentages. The groups were compared using the Student *t*-test or χ^2 test. Logistic regression was used to calculate the odds ratios (ORs) with 95% (CIs) for evaluating the risk factors for a long diagnostic delay. The covariates used in our multivariate analyses included variables with a significant result on the univariate analysis in addition to variables associated with diagnostic delays in previous studies and clinical experience. The Kaplan-Meier method was used to evaluate the association between diagnostic delays and clinical outcomes, such as IBD-related surgery and admission. The difference between the groups according to diagnostic time interval was analyzed using the log-rank test. The Cox proportional hazard regression analysis was performed to evaluate the association of long diagnostic delays with the clinical outcomes; the result was expressed as hazards ratio with 95%CI. The statistical methods of this study were reviewed by biostatistician from Korea University Ansan Hospital. And all statistical analyses were performed using the Statistical Package for the Social Science statistical software (version 18; SPSS-IBM, Chicago, IL, United States) or R version 3.02 (R Foundation for Statistical Computing, Vienna, Austria). All $P < 0.05$ was considered statistically significant.

RESULTS

The mean age at diagnosis was 28.2 ± 13.8 years in patients with CD and 38.8 ± 15.6 years in patients with UC. Male sex was predominant in both patients with CD and UC. Abdominal pain (51.5%) was the most prevalent symptom among the chief complaints in patients with CD and hematochezia (66.9%) in patients with UC. Regarding the disease location at diagnosis, the ileocolic area (L3, 51.5%) was the most common in CD and proctitis (40.3%) was the most prevalent in UC. The median diagnostic time interval was 6.2 mo in the patients with CD and 2.4 mo in the patients with UC. In the present study, the diagnostic time interval of ⩾ 21.4 mo and 6.2 mo were defined as long diagnostic delays in CD and UC, respectively, based on the highest quartile cut off limit.

Tables 1 and 2 showed the baseline characteristics of the patients with CD and UC stratified by presence of a long diagnostic delay. Although age, sex, smoking status, IBD family history, and disease location were not different between the diagnostic delayed group and non-delayed group, the disease behavior type was significantly different; the stenosis type was especially more prevalent in the CD delayed group ($P = 0.021$). However, there was no significant baseline characteristic difference, except for the follow-up duration in UC.

The factors associated with a diagnostic delay in the patients with CD and UC are summarized in Tables 3 and 4. In the patients with CD, perianal discomfort was the only clinical factor associated with diagnostic delays (OR 10.23, 95%CI: 1.93-54.37). However, there was no clinical factor associated with significant diagnostic delays in the patients with UC.

Medication history during the follow-up period in the patients with IBD is shown in Supplementary Table 1. There was no difference in the types and frequencies of prescribed medications between the diagnostic delay and non-diagnostic delay groups in

Table 1 Baseline characteristics of Crohn's disease patients *n* (%)

	Total (*n* = 165)	Delayed (*n* = 41)	Non-delayed (*n* = 124)	*P* value
Age, yr (± SD)	28.2 (± 1.1)	29.2 (± 12.7)	27.9 (± 14.2)	0.269
Age ⩾ 40	27 (16.4)	5 (12.2)	22 (17.7)	0.405
Male,	126 (76.4)	33 (80.5)	93 (75.0)	0.473
Family history,	6 (3.6)	0 (0)	6 (4.8)	0.249
Symptom to diagnosis[1], d	185 (45.0-642.5)	1108 (810-2331)	79.5 (32.0-253.5)	0.000
Symptom to visit[1], d	57.0 (14.0-255.8)	739 (98.8-996)	33.5 (10.8-109.8)	0.000
Visit to diagnosis[1], d	20.0 (4.0-139.0)	150 (5.8-1188)	14.0 (4.0-85.0)	0.002
Smoking,	41 (24.8)	12 (29.3)	29 (23.4)	0.450
Chief complain				0.241
Diarrhea	29 (17.6)	4 (9.8)	25 (20.2)	
GI bleeding	20 (12.1)	4 (9.8)	16 (12.9)	
Perianal discomfort	25 (15.2)	10 (24.4)	15 (12.1)	
Abdominal pain	85 (51.5)	22 (53.7)	63 (50.8)	
Others	6 (3.6)	1 (2.4)	5 (4.0)	
Location,				0.688
L1	39 (23.6)	12 (29.3)	27 (21.8)	
L2	18 (10.9)	3 (7.3)	15 (12.1)	
L3	85 (51.5)	20 (48.8)	65 (52.4)	
L4	23 (13.9)	6 (14.6)	17 (13.7)	
Behavior,				0.021
B1	116 (70.3)	23 (56.1)	93 (75.0)	
B2	32 (19.4)	14 (34.1)	18 (14.5)	
B3	17 (10.3)	4 (9.8)	13 (10.5)	
Perianal disease	31 (18.8)	5 (12.2)	26 (21.0)	0.213
CRP at diagnosis, (± SD)	4.17 (± 7.76)	3.73 (± 4.35)	4.33 (± 8.68)	0.677

[1]The duration was expressed as median (interquartile range); CRP: C-reactive protein.

Table 2 Baseline characteristics of ulcerative colitis patients *n* (%)

	Total (*n* = 130)	Delayed (*n* = 32)	Non-delayed (*n* = 98)	*P* value
Age at diagnosis (± SD)	38.9 ± 15.5	36.9 ± 13.2	39.6 ± 16.1	0.395
Male,	71 (54.6)	17 (53.1)	54 (55.1)	0.845
Family history	7 (5.4)	2 (6.3)	5 (5.1)	0.803
Symptom to diagnosis[1], d	73.0 (35.0-186.5)	635 (360.5-1219)	50.5 (31.0-90.0)	0.000
Symptom to visit[1], d	57.5 (27.0-147.8)	409.5 (197.3-1183)	37.0 (15.8-70.0)	0.000
Visit to diagnosis[1], d	7.00 (3.0-17.0)	10.0 (3.0-28.5)	6.00 (3.0-16.3)	0.215
Smoking	26 (20.2)	6 (18.8)	20 (20.6)	0.819
Chief complaints				0.55
Hematochezia	87 (66.9)	20 (62.5)	67 (68.4)	
Diarrhea	31 (23.8)	10 (31.3)	21 (21.4)	
Abdominal pain	9 (6.9)	2 (6.3)	7 (7.1)	
Others	3 (2.3)	0 (0)	3 (3.1)	
Location				0.594
Proctitis	52 (40.3)	12 (38.7)	40 (40.8)	
Left sided	37 (28.7)	11 (35.5)	26 (26.5)	
Pancolitis	40 (31.0)	8 (25.8)	32 (32.7)	
Mayo score at diagnosis	5.60 ± 1.98	5.38 ± 1.95	5.67 ± 1.99	0.460
Severity[2]				0.748
Remission (0-2)	2 (1.5)	1 (3.1)	1 (1.0)	
Mild (3-5)	53 (40.8)	14 (43.8)	39 (39.8)	
Moderate (6-10)	74 (56.9)	17 (53.1)	57 (58.2)	
Severe (11-12)	1 (0.8)	0 (0)	1 (1.0)	
CRP at diagnosis, (± SD)	1.82 ± 3.88	1.85 ± 3.96	1.82 ± 3.88	0.970

[1]The duration was expressed as median (interquartile range); [2]The severity was classified according to the Mayo score.

patients with CD and UC. However, the median time interval from diagnosis to anti-TNFα administration was significantly shorter in the non-diagnostic delay group of the patients with CD than in the diagnostic delay group. In the patients with UC, the time interval from diagnosis to systemic steroid, immunomodulator and anti-TNFα administration was not different between groups.

After diagnosis, 28 (17.0%) patients with CD underwent intestinal surgeries due to CD related problems, such as uncontrolled internal fistula, stenosis, or abdominal abscess and 43 (26.1%) underwent any surgical treatments including perianal surgery due to perianal abscess or fistula. Among the patients with UC, 6 (4.6%) underwent intestinal surgeries due to uncontrolled inflammation and UC-related colon cancer. Figure 1 show that a long diagnostic delay was associated with an increased risk of intestinal surgery in the patients with CD and UC, which was analyzed using Kaplan-Meier method. Tables 5 and 6 show the clinical risk factors associated with intestinal or any surgery in the patients with IBD. In patients with CD, a long diagnostic delay (OR = 2.54, 95%CI: 1.06-6.09) was a significant risk factor for intestinal surgery, but not for any surgery. Further, stricturing and penetrating types were significantly associated with intestinal and any surgery. However, the diagnostic delay was the only clinical risk factor associated with intestinal surgery in UC (OR = 6.81, 95%CI: 1.12-41.4).

Kaplan Meier analysis showed that the diagnostic delay group of the patients with CD had an increased risk of admission and frequent admission as shown in Figure 2. However, after adjusting for covariates, such as age and sex, a long diagnostic delay was not associated with admission and frequent admission in the patients with CD (OR = 1.60, 95%CI: 0.93-2.75 and OR 2.05; 95%CI: 0.84-5.01, respectively), as shown in Supplementary Table 2. In the patients with UC, pancolitis was the only risk factor of admission (OR = 3.94; 95%CI: 1.67-9.30), as shown in supplementary Table 3. A diagnostic delay was not associated with admission and frequent admission in patients with UC (OR = 1.04; 95%CI: 0.50-2.17 and OR = 2.05; 95%CI: 0.68-6.24, respectively).

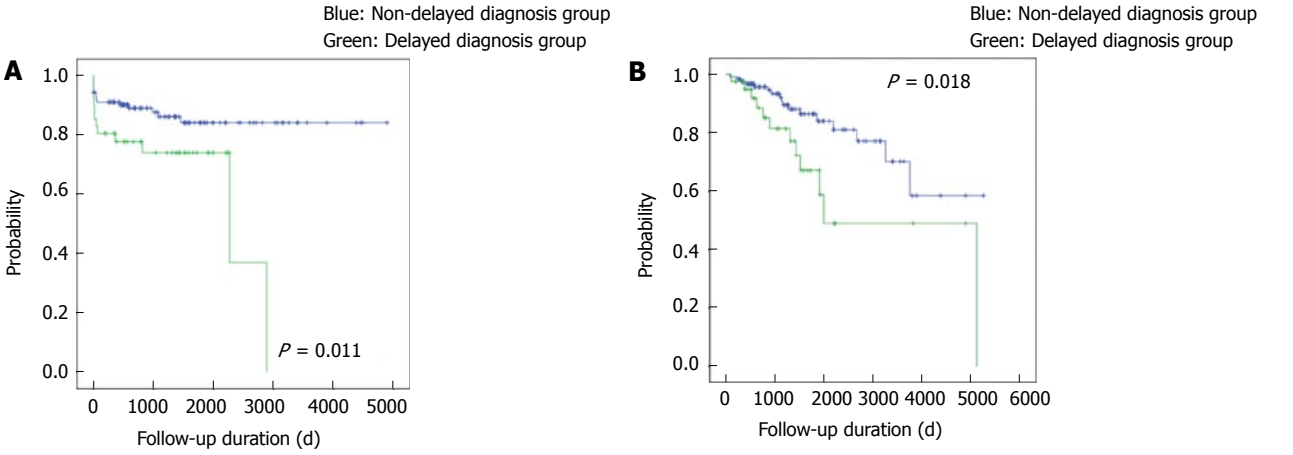


Figure 1 Surgery-free survival according to the presence of a long diagnostic delay in the patients with inflammatory bowel disease. A: Intestinal surgery in the patients with CD; B: Intestinal surgery in the patients with UC. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Factors associated with a long diagnostic delay in Crohn's disease

Variable	*n*	OR (95%CI)	*P* value
Age	-	1.00 (0.96-1.03)	0.787
Male	126	0.72 (0.23-2.26)	0.575
Smoking	41	2.26 (0.85-5.98)	0.101
Chief complaint			
Diarrhea	29	Ref.	-
GI bleeding	20	1.94 (0.37-10.1)	0.432
Perianal discomfort	25	10.23 (1.93-54.37)	0.006
Abdominal pain	85	2.20 (0.64-7.62)	0.213
Others	6	1.13 (0.09-14.38)	0.925
Location			
L1	39	Ref.	-
L2	18	0.71 (0.14-3.67)	0.684
L3	85	0.77 (0.28-2.11)	0.613
L4	23	0.66 (0.18-2.43)	0.533
Behavior			
B1	116	Ref.	
B2	32	2.33 (0.90-6.04)	0.081
B3	17	0.49 (0.12-1.97)	0.312
Perianal disease	31	0.28 (0.07-1.17)	0.080
CRP at diagnosis	-	0.98 (0.91-1.05)	0.572

GI: Gastrointestinal; OR: Odds ratio; CRP: C-reactive protein; CI: Confidence interval.

Table 4 Factors associated with a long diagnostic delay in ulcerative colitis

Variable	*n*	OR (95%CI)	*P* value
Age	-	0.99 (0.96-1.02)	0.484
Male	71	0.78 (0.30-2.04)	0.607
Smoking	26	1.26 (0.33-4.87)	0.733
IBD family history	7	1.40 (0.24-8.07)	0.709
Chief complaint			
Hematochezia	87	Ref.	-
Diarrhea	31	1.34 (0.49-3.65)	0.566
Abdominal pain	9	1.03 (0.18-5.92)	0.975
Others	3	Not assessed	-
Location			
Proctitis	39	Ref.	-
Left sided	18	1.45 (0.48-4.39)	0.513
Left sided	85	0.90 (0.28-2.92)	0.860
Severity[1]			
Mild	53	Ref.	
Moderate to severe	75	0.78 (0.29-2.13)	0.634

[1]The severity was classified according to the Mayo score. IBD: Inflammatory bowel disease; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

This study demonstrated a considerable diagnostic delay in patients with IBD, especially in patients with CD. Further, a long diagnostic delay was significantly associated with a higher risk of IBD-related intestinal surgery in both the patients with CD and UC.

In the present study, the diagnostic period for CD was longer than that for UC; this finding is consistent with previous studies[9,12]. In western IBD registries, such as the Swiss IBD cohort and the Romanian national registry, about 25% of patients with CD have a considerable diagnostic delay period of about ⩾ 1.5 years. The diagnostic period of UC ranged from 1 mo to 4 mo. The significant gap in the diagnostic period between CD and UC might be because of initial symptoms[16]. Abdominal pain is the most prevalent initial chief complaint in patients with CD; however, it is also a common symptom of other digestive disorders, such as irritable bowel syndrome[17]. Furthermore, when the pain is not severe, it is easily regarded as trivial, and the diagnosis may be missed because the tests needed for the diagnosis of IBD are not performed. In contrast, hematochezia, which is a major symptom of UC, is not only a rare symptom but also causes great fear to the public[18]. This may cause patients to visit the hospital relatively quickly, leading to early diagnosis.

Among the initial chief complaints, perianal discomfort was significant clinical factor associated with long

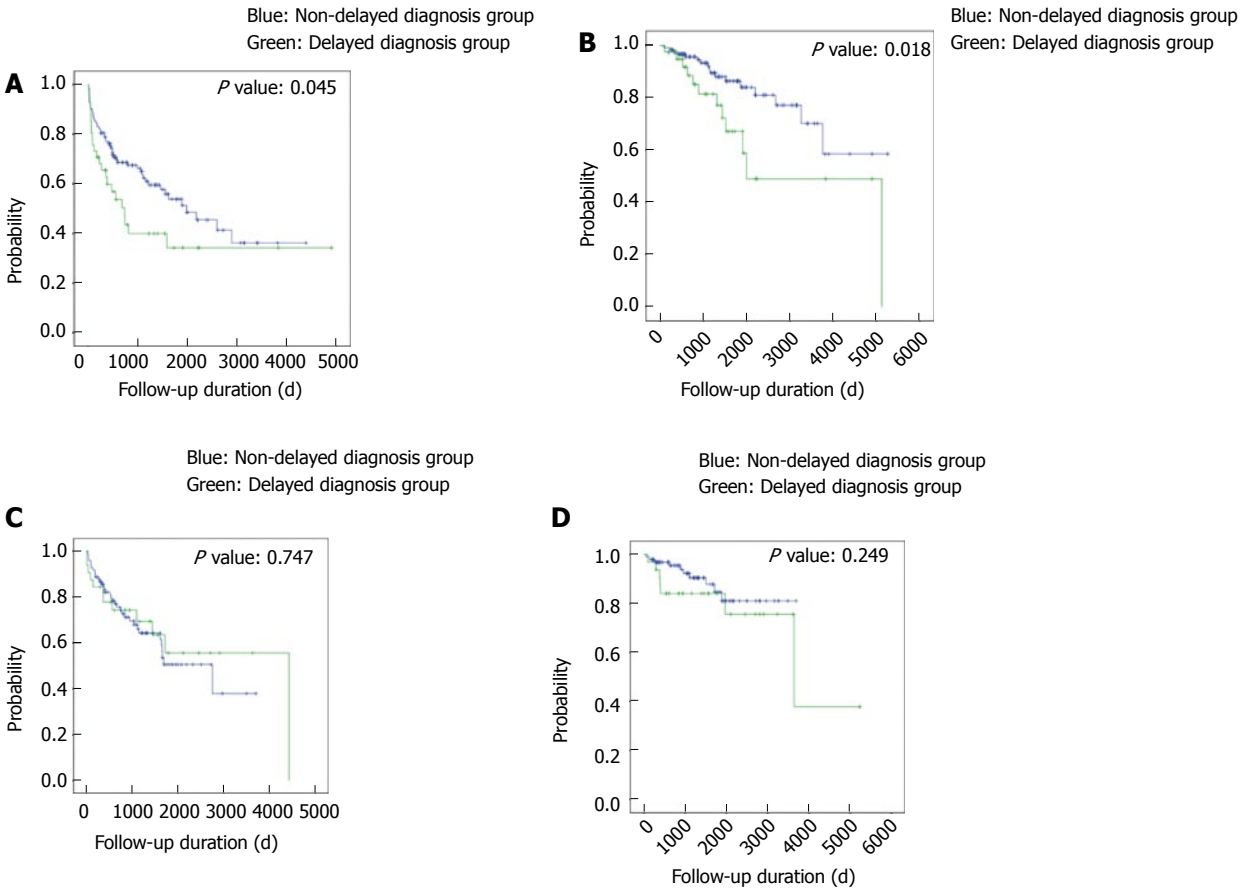


Figure 2 Admission-free survival according to the presence of a long diagnostic delay in the patients with inflammatory bowel disease. A: Admission in the patients with CD; B: Frequent admission in the patients with CD; C: Admission in the patients with UC; D: Frequent admission in the patients with UC. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

diagnostic delay in our study. The patients with perianal discomfort were significantly younger than those with other symptoms and tended to have longer duration from symptom to diagnosis (Supplementary Table 4). Interestingly, the time from the visit to the diagnosis (physician-dependent delay) was significantly longer in the patients with perianal discomfort, although there was no difference in the time from symptom to the hospital visit (patient-dependent delay) in this study. This is related to the tendency of CD patients with perianal discomfort to visit the colorectal/anus surgery clinic or general doctor's clinic for the first time in South Korea. However, they might tend to overlook and miss the diagnosis of CD because anal disorders, such as hemorrhoids, are relatively common and IBD is rare in the East than in the West[19]. Therefore, strengthening IBD education for general doctors and general surgeons is considered to be a good way to reduce the delay of diagnosis of IBD disease, especially CD patients with perianal disease.

In our analyses, a long diagnostic delay was an independent risk factor of CD-related intestinal surgery in addition to stricturing and penetrating behavior types at diagnosis, which was similar to those of other studies[11,12,20]. Further, a long diagnostic delay in patients with UC was an independent risk factor of intestinal surgery. Considering that, there was no difference in treatments between the diagnostic delay group and the non-diagnostic delay group after the diagnosis in the present study, greater irreversible damage to the intestines might occur and accumulated, as the exposure period to the disease before the diagnosis is extended. Such damages may reduce the responsiveness to medical treatments and increase the risks of intestinal surgeries. To our knowledge, this is the first study to demonstrate the association between long diagnostic delays and poor clinical outcomes, such as an increased risk of intestinal surgery in patients with UC. This result is in part owing to the relatively long diagnostic delay compared with those of other UC diagnostic delay studies and to ethnic differences[9,12]. In present study, the duration from symptom onset to first hospital visit is significantly different between the delayed and non-delayed groups. This difference is caused by various factors such as patient's perception, attitude toward the disease and sensitivity to symptoms, and these factors are thought to influence patient's prognosis. However, a large multicenter study is needed to reveal the exact association between the diagnostic delay and

Table 5 Risk factors associated with Crohn's disease related surgery

Variables	Intestinal surgery			Any surgery		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age	0.99	0.96-1.03	0.743	1.00	0.97-1.03	0.973
Male	0.41	0.14-1.22	0.108	0.94	0.36-2.45	0.902
Smoking	1.90	0.74-4.87	0.182	1.10	0.51-2.38	0.812
Location						
L1	1 (Ref.)	-	-	1 (Ref.)	-	-
L2	1.19	0.32-4.50	0.793	1.74	0.57-5.31	0.332
L3	0.53	0.17-1.71	0.291	0.82	0.32-2.09	0.676
L4	1.07	0.29-3.97	0.924	0.91	0.28-2.94	0.878
Behavior						
B1	1 (Ref.)	-	-	1 (Ref.)	-	-
B2	4.44	1.67-11.8	0.003	2.93	1.30-6.60	0.009
B3	3.79	1.14-12.6	0.030	3.67	1.40-9.60	0.008
Perianal disease	0.97	0.20-4.81	0.968	1.84	0.69-4.87	0.222
CRP at diagnosis	1.02	0.96-1.08	0.520	1.01	0.96-1.06	0.792
Delayed diagnosis	2.54	1.06-6.09	0.036	1.76	0.87-3.57	0.119

CD: Crohn's disease; CRP: C-reactive protein; CI: Confidence interval; OR: Odds ratio.

prognosis in patients with UC.

A diagnostic delay was not directly associated with an increased risk of IBD-related admission in our analyses. However, stricturing behavior and penetrating type at diagnosis were significantly associated with admission and frequent admission in patients with CD. Among the patients with UC, those with pancolitis at diagnosis had an increased risk of admission. Considering that the disease behavior of CD tends to change from an inflammatory type to either stricturing or penetrating type and the disease extent progresses proximally from the rectum in UC, an early diagnosis might be associated with a better prognosis, such as reduced hospitalization[18,21-23].

Our study has a few limitations. First, as a limitation of retrospective study, important clinical information, such as the onset of IBD-related symptoms and first hospital visit date, may be inaccurate owing to a recall bias. Second, this study was conducted at a single center, and its results might not fully reflect the overall patients with IBD in South Korea. Therefore, a large multicenter prospective study is needed for to a better understanding of the association between the diagnostic delay and prognosis of IBD.

Our study demonstrated a considerable diagnostic delay in patients with IBD. In addition, a long diagnostic delay was significantly associated with an increased risk of IBD-related intestinal surgeries in patients with CD and UC. Based on these results, efforts should be made to reduce diagnostic delays, such as increasing awareness for physicians and the public, to improve the prognosis of patients with IBD.

Table 6 Risk factors associated with ulcerative colitis related surgery

Variables	Intestinal surgery		
	OR	95%CI	*P* value
Age	1.00	0.93-1.07	0.986
Male	1.18	0.17-8.37	0.868
Smoking	0.82	0.05-13.8	0.890
IBD family history	5.39	0.44-66.5	0.189
Location			
Proctitis	1 (Ref.)	-	-
Left sided	0.85	0.10-7.24	0.878
Pancolitis	1.05	0.12-9.46	0.969
Severity[1]			
Mild	1 (Ref.)	-	-
Moderate to severe	2.67	0.24-30.2	0.427
Delayed diagnosis	6.81	1.12-41.4	0.037

[1]The severity was classified according to the Mayo score. UC: Ulcerative colitis; IBD: Inflammatory bowel disease; CI: Confidence interval; OR: Odds ratio.

COMMENTS

Background

The diagnosis of inflammatory bowel diseases (IBD) is often established following considerable delay due to nonspecific and inconsistent symptoms. In previous western studies, the delayed diagnosis was associated with poor outcome in patients with Crohn's disease (CD).

Research frontiers

There are few studies on diagnostic delays associated with IBD in Asia. Because there were significant differences in the epidemiological and clinical features of IBD according to ethnicities and environmental factors, the authors aimed to investigate the clinical factors and outcomes associated with diagnostic delays in Korean patients with IBD.

Innovations and breakthroughs

In present study, a long diagnostic delay was defined based on the time interval in which the 76th to 100th percentile of the patients was diagnosed due to no established criteria. This study demonstrated a considerable diagnostic delay in patients with Asian patients with IBD. Further, a long diagnostic delay was significantly associated with poor outcome such as a higher risk of IBD-related intestinal surgery in both the patients with CD and ulcerative colitis.

Applications

Efforts should be made to reduce diagnostic delays, such as increasing awareness and strengthening educations about IBD for physicians and the public, to improve the prognosis of patients with IBD.

Peer-review

The manuscript has convincing data and is publishable.

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ORIGINAL ARTICLE

Prospective Study

Fecal marker levels as predictors of need for endoscopic balloon dilation in Crohn's disease patients with anastomotic strictures

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Abstract

AIM

To evaluate the accuracy and best cut-off value of fecal calprotectin (FC) and fecal lactoferrin (FL) to predict disease recurrence in asymptomatic patients presenting with anastomotic strictures.

METHODS

This was a longitudinal single tertiary center study based on prospectively collected data (recorded in a clinical database created for this purpose) performed between March 2010 and November 2014. Crohn's disease (CD) patients with anastomotic stricture who submitted to postoperative endoscopic evaluation were included. Stools were collected on the day before bowel cleaning for FC and FL. Endoscopic balloon dilation (EBD) was performed if the patient presented an anastomotic stricture not traversed by the colonoscope, regardless of patients' symptoms. Successful dilation was defined as passage of the colonoscope through the dilated stricture into the neotermimal ileum.

Author contributions: Lopes S and Magro F were responsible for study design; Lopes S and Andrade P were responsible for data collection; Afonso J performed all laboratorial procedures; Rodrigues-Pinto E and Andrade P were responsible for statistical analysis; Lopes S and Andrade P wrote the manuscript; Magro F and Macedo G revised and approved the final manuscript; All the named authors, contributing directly to the work described, have agreed on the respective roles of each author, read the manuscript and approved submission.

Institutional review board statement: The study was approved by the Ethics Committee of Centro Hospitalar São João, Porto, Portugal.

Informed consent statement: All patients gave informed written consent to participate in the study.

Conflict-of-interest statement: The authors of this manuscript have no conflict of interest to declare.

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Postoperative recurrence was defined as a modified Rutgeerts score of ⩾ i2b.

RESULTS

In a total of 178 patients who underwent colonoscopy, 58 presented an anastomotic stricture, 86% were asymptomatic, and 48 (54% male; median age of 46.5 years) were successfully dilated. Immediate success rate was 92% and no complications were recorded. FC and FL levels correlated significantly with endoscopic recurrence ($P < 0.001$) with an optimal cut-off value of 90.85 μg/g (sensitivity of 95.5%, specificity of 69.2%, positive predictive value (PPV) of 72.4%, negative predictive value (NPV) of 94.7% and accuracy of 81%] for FC and of 5.6 μg/g (sensitivity of 77.3%, specificity of 69.2%, PPV of 68%, NPV of 78.4% and accuracy of 72.9%) for FL.

CONCLUSION

Fecal markers are good predictors of CD endoscopic recurrence in patients with asymptomatic anastomotic stricture. FC and FL may guide the need for EBD in this context.

Key words: Crohn's disease; Anastomotic strictures; Endoscopic balloon dilation; Fecal markers; Postoperative recurrence

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Core tip: This longitudinal study evaluated the accuracy of fecal calprotectin (FC) and fecal lactoferrin (FL) to predict disease recurrence in postoperative Crohn's disease asymptomatic patients with an anastomotic stricture. FC and FL levels accurately predicted endoscopic recurrence in the presence of anastomotic stricture and thus may guide the need for endoscopic balloon dilation (EBD) in this context. A normal value of fecal markers can reassure clinicians and be safely used to avoid balloon dilation if we only aim to diagnose recurrence. A high value of fecal markers has a high likelihood of recurrence so EBD should be performed in order to provide adequate endoscopic therapy and adjust or optimize medical therapy.

Lopes S, Andrade P, Rodrigues-Pinto E, Afonso J, Macedo G, Magro F. Fecal marker levels as predictors of need for endoscopic balloon dilation in Crohn´s disease patients with anastomotic strictures. *World J Gastroenterol* 2017; 23(35): 6482-6490 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6482.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6482

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder with progression to penetrating or stricturing phenotype as part of the natural history. More than half of CD patients experience these complications during disease lifetime and will need surgery[1,2]. It is well known that disease recurrence proximal or at the anastomosis is almost universal, with progression to luminal narrowing and stricturing behavior[3]. It is known that despite the permanent reduction of luminal caliber and disease progression, the majority of patients remain asymptomatic, with normal biomarkers. As the primary therapeutic goal of CD has shifted from clinical remission to achieving mucosal healing[4,5], it may be important to access the mucosa proximal to strictures to evaluate disease recurrence and escalate therapy if needed. Over the last decade there is increasing evidence for endoscopic balloon dilation (EBD) as a safe and minimally invasive effective method for the treatment of stricturing disease[6-14]. Median technical success has been reported as 90%, with major complication rate around 3%-10%[6,9,10]. Symptomatic recurrence is common, with a reported frequency ranging from 13% to 100%[12]. However, it has been shown that repeated dilations do not reduce the efficacy of the procedure and may prevent surgery in compliant patients[8-12].

Fecal markers, namely fecal calprotectin (FC) and fecal lactoferrin (FL), have proved to be useful and accurate non-invasive tools in evaluating disease activity in CD. Recent works have also demonstrated their validity in diagnosing recurrence in the postoperative setting, suggesting that normal values of fecal biomarkers can obviate the need for endoscopic evaluation[15-18]. In patients with elevated levels of fecal biomarkers, endoscopy should be performed in order to confirm recurrence and escalate therapy if indicated[19]. To our knowledge there are no studies evaluating the performance of FC and FL in patients with asymptomatic anastomotic CD strictures and limited data is available on the long-term effect of medical therapy escalation after balloon dilation of anastomotic strictures.

The aims of this study were, therefore, to evaluate the accuracy of FC and FL in the diagnosis of recurrent disease in the neoterminal ileum in asymptomatic/mild disease patients that had undergone bowel resection for CD and present with stricture of the anastomosis, to evaluate the best cut-off value of FC and FL to diagnose recurrence, and to evaluate the immediate technical success and safety rate of EBD.

MATERIALS AND METHODS

A longitudinal single tertiary center study based on prospectively collected data (recorded in a clinical database created for this purpose), was performed between March 2010 and November 2014. All patients gave informed written consent to participate in the study that was approved by the Ethics Committee of our Institution. From a cohort of consecutive CD

patients who submitted to postoperative endoscopic evaluation after ileocolectomy, we selected the group of patients with anastomotic stricture. All patients were followed at our inflammatory bowel disease (IBD) outpatient clinic and were referred for endoscopic evaluation at our institution.

Inclusion criteria were definitive diagnosis of CD established by standard clinical, radiographic, endoscopic and histological criteria[20], previous ileocolectomy, and existence of an anastomotic stricture. Exclusion criteria were: age less than 18-years-old; strictures length greater than 6 cm; fistulae or deep ulceration of the strictured segment; technical impossibility of passing the catheter/balloon through the strictures; and active disease in the colon or upper digestive tract.

Clinical disease activity was assessed on the day of endoscopic examination, according to the clinical criteria of the Harvey-Bradshaw index (HBI)[21,22]. Clinically inactive disease was defined as HBI < 5. All procedures were performed under propofol sedation, with CO_2 insufflation, by a single senior endoscopist (SL) and on an outpatient basis. Mechanical intestinal bowel preparation was done the day before colonoscopy with polyethylene glycol bowel preparation solution (Klean Prep®; Helsinn Birex Pharmaceuticals, Dublin, Ireland). Stool samples were collected the day before beginning bowel preparation (preferably from the first stool in the morning) and then kept in the refrigerator until being brought to the hospital. For FC, within a maximum 7 d after collection, stools were extracted in accordance with the manufacturer's instructions, using the Fecal Sample Preparation Kit (Roche Diagnostics, Mannheim, Germany) and analyzed using immunoassay (EliA Calprotectin®; Thermo Fisher Scientific, Freiburg, Germany). For FL evaluation, stools were stored at -80 ℃ upon arrival at the laboratory, and samples were thawed and analyzed using a commercially available quantitative enzyme-linked immunoassay test (IBD-Scan®; Tech-Lab, Blacksburg, VA, United States). The techniques for measurement of fecal markers followed the manufacturer´s guidelines.

Postoperative disease activity of the neoterminal ileum was evaluated according to the modified Rutgeerts score[23] (i0: no lesions in the distal ileum; i1: < 5 aphthous lesions in the distal ileum; i2a: lesions confined to the ileocolonic anastomosis, including anastomotic strictures; i2b: > 5 aphthous ulcers or larger lesions, with normal mucosa in between, in the neoterminal ileum, with or without anastomotic lesions; i3: diffuse aphthous ileitis with diffusely inflamed mucosa; i4: large ulcers with diffuse mucosal inflammation or nodules or strictures in the neoterminal ileum). Postoperative recurrence was defined as a modified Rutgeerts score of ⩾ i2b.

EBD was performed if the patient presented an anastomotic stricture not traversed by the colonoscope, regardless of the patient's symptoms. All dilations were performed with the same type of colonoscope (Olympus® CF type H180AL; Tokyo, Japan) under fluoroscopic control to allow the endoscopist to characterize the strictures, exclude peristricture fistulae, evaluate the optimal diameter of the balloon to use and to prompt identify any complication during the procedure. Dilations were performed with a guidewire (Boston Scientific® Jagwire 0.035 in; Marlborough, MA, United States) placed through the strictures [after contrast instillation through a catheter (Olympus® Ball Tip/6Fr) over which a through-the-scope balloon (Cook Medical®, Bloomington, IN, United States) was placed]. The balloon was inflated using contrast agent and the pressure maintained for 2 min, to a maximum diameter of 18 mm.

The procedure could be repeated at the discretion of the endoscopist. Successful dilation was defined as passage of the colonoscope through the dilated stricture into the neotermimal ileum. Only patients with a successful dilation were analyzed, as progression to the neoterminal ileum was mandatory to evaluate disease recurrence. Major complications were defined as major bleeding requiring surgery, blood transfusion or hospital admission and perforation. Minor, self-limited bleeding was not registered as a complication.

Statistical analysis

SPSS 20.0 for Windows (SPSS, Chicago, IL, United States) was used for statistical analysis. Categorical variables were described as absolute frequencies (*n*) and relative frequencies (%); continuous variables were described as mean ± SD (parametric distributions) or as median and percentiles (non-parametric distributions). The normality of the continuous variables was tested using the Kolmogorov-Smirnov test and the respective histogram. Student's *t*-test was used to compare quantitative variables with a normal distribution, and the Mann-Whitney *U* test was used to compare the quantitative variables without a normal distribution. Any groups with more than two quantitative variables were compared using the Kruskal-Wallis test. A Pearson χ^2 test was used to compare categorical variables. Kaplan-Meier analysis with log rank statistics was used to estimate event-free interval. A logistic regression was performed to assess predictors of disease recurrence and need for dilation. Statistical significance was set at $P < 0.05$.

RESULTS

Patient characteristics

One hundred and seventy-eight consecutive CD patients (51.7% male; median age 46.4 years) who previously submitted to right ileocolectomy were evaluated by colonoscopy. At the time of endoscopic evaluation, 31 (17.4%) patients complained of subocclusive symptoms, and 66.3% of patients were being treated with immunomodulators and 38.2% with

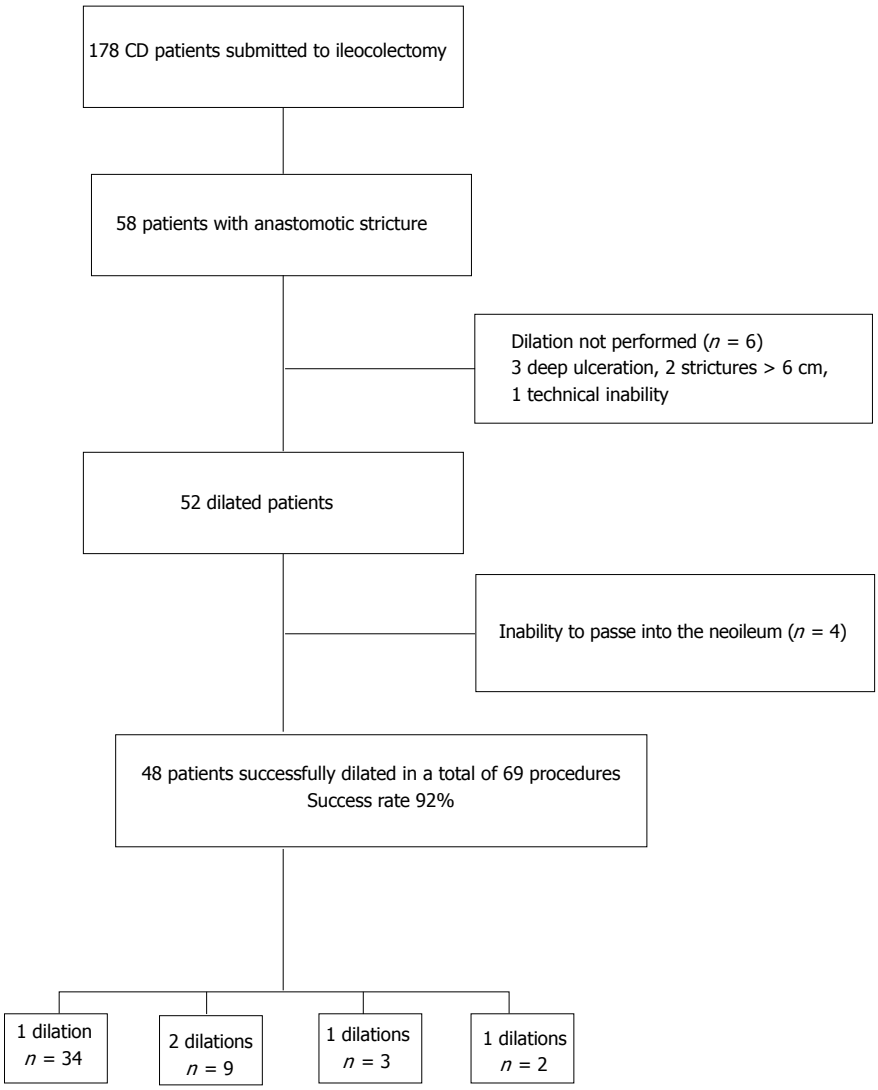


Figure 1 Study flowchart. CD: Crohn's disease.

biologics.

Of the 178 evaluated patients, 58 (32.6%) presented with an anastomotic stricture. The majority were asymptomatic, with only 8 (13.8%) patients presenting with subocclusive symptoms. All patients were in clinical remission (HBI < 5 in 83.3%) or with mild clinical disease (HBI 5-7 in 16.7%). Among the 58 patients presenting with anastomotic stricture, 52 were dilated (6 were excluded due to deep ulceration, stricture size > 6 cm or technical inability). Of the total 52 dilated patients, 4 were excluded as it was not possible to evaluate the neoterminal ileum (Figure 1). Baseline characteristics of the 48 successfully dilated patients are summarized in Table 1. The majority were men (54%) with a median age of 46.5 years. At the time of dilation, 42% of patients were being treated with biologics and 54% with immunomodulators.

EBD

Overall, technical success rate was 92% (48/52) and no major complications were recorded. The 18-mm balloon diameter was used in 69.2% of the patients and the 15-mm diameter in 25.0%. Redilation was required in 14 patients (29.2%) during a median follow-up time of 34 mo (27.5-52.5 mo). During the follow-up period, only 2 patients needed surgery, both cases due to long strictures that did not allow EBD (Table 2).

Fecal markers and endoscopic recurrence

Of the 48 successfully dilated patients, 22 presented with endoscopic recurrence defined as modified Rutgeerts score of $\geqslant$ i2b. Of these, 16 patients presented with severe disease (i3 = 3 and i4 = 13). Recurrence was diagnosed only after dilation of the anastomotic stricture and intubation of the neoterminal ileum. Comparing FC and FL levels in patients with endoscopic recurrence and in patients with endoscopic remission we found a significantly higher level in patients with endoscopic recurrence [FC: 257.0 μg/g, interquartile range (IQR): 161.0-565.0 μg/g) *vs* 53.9 μg/g, IQR: 23.9-146.0 μg/g; $P < 0.001$ and FL: 9.1

Table 1 Patient characteristics, *n* = 48

Characterization	*n* (%)
Women,	22 (45.8)
Median time between diagnosis and surgery, mo (IQR)	36.0 (14.0-120.0)
Median time between surgery and endoscopic evaluation, mo (IQR)	114.5 (60.8-199.0)
Median age at endoscopic evaluation, yr (IQR)	46.5 (39.3-53.4)
Montreal classification	
Age at diagnosis	
A1, ⩽ 16 yr	6 (12.5)
A2, 17-40 yr	33 (68.8)
A3, > 40 yr	9 (18.8)
Location	
L1: ileal	30 (62.5)
L3: ileocolonic	17 (35.4)
L4: upper gastrointestinal tract	1 (2.1)
Behavior	
B1: non-stricturing, non-penetrating	1 (2.1)
B2: stricturing	27 (56.2)
B3: penetrating	20 (41.7)
Perianal disease	14 (29.2)
Smoking	
Never	26 (54.2)
Current	12 (25.0)
Past	10 (20.8)
Concomitant treatment	
Corticosteroids	9 (18.8)
Immunomodulators (Azathioprine/6MP/ Methotrexate)	26 (54.2)
Biologics (Infliximab, adalimumab)	20 (41.7)
5-ASA	17 (35.4)
Median fecal calprotectin, μg/g (IQR)	134.0 (35.3-321.0)
Median, fecal lactoferrin, μg/g (IQR)	6.2 (2.0-22.4)
Modified Rutgeerts score	
i0, i1, i2a	26 (54.2)
i2b, i3, i4	22 (45.8)
Subocclusive symptoms	8 (16.7)
Harvey-Bradshaw index	
Remission (HBI < 5)	40 (83.3)
Mild disease (HBI 5-7)	8 (16.7)
Need of redilation	14 (29.2)
Surgery after dilation	2 (1.4)
Median follow up, mo (IQR)	34.0 (27.5-52.5)

HBI: Harvey-Bradshaw index; IQR: Interquartile range.

μg/g, IQR: 5.5-27.8 μg/g *vs* 3.9 μg/g, IQR: 1.5-21.9 μg/g; *P* = 0.042] (Figure 2). No other clinical variable or biomarker reached statistical difference between the two groups (Table 3).

The best calculated cut-off value for FC to predict recurrent disease was 90.85 μg/g, with a sensitivity of 95.5%, a specificity of 69.2%, a positive predictive value (PPV) of 72.4%, a negative predictive value (NPV) of 94.7% and an accuracy of 81%. The area under the ROC curve for diagnosing endoscopic recurrence was 0.786 (95%CI: 0.646-0.926, *P* < 0.05) for FC. Concerning FL, the best calculated cut-off was 5.6 μg/g, with sensitivity of 77.3%, specificity of 69.2%, PPV of 68%, NPV of 78.4% and accuracy of 72.9%. The area under the ROC curve for diagnosing endoscopic recurrence was 0.672 (95%CI: 0.511-0.834, *P* = 0.042) (Figure 3).

Table 2 Characterization of the dilation procedure

Characterization	*n* (%)
Patients with stricture	58 (32.6)
Stricture type	
Anastomotic	58 (100)
Dilated patients	52 (29.2)
Causes for non-dilation	
Length of stenosis	2 (33.3)
Ulceration of mucosa	3 (50.0)
Technical inability	1 (16.7)
Successful dilated patients	
Balloon size, *n* = 52	48 (92.3)
15 mm	13 (25.0)
16.5 mm	3 (5.8)
18 mm	36 (69.2)
Complications	0 (0.0)

DISCUSSION

One of the major drawbacks of CD surgical therapy is the high recurrence rate of the disease at the anastomotic site or in the neoterminal ileum, with the development of stricture and bowel obstruction[8]. As the primary therapeutic goal of CD has shifted from clinical remission to achieving mucosal healing[4,5], it may be important to access the mucosa proximal to strictures to evaluate disease recurrence and escalate therapy if needed. The recently published POCER trial[16] demonstrated that initial postoperative treatment according to clinical risk stratification and early endoscopic evaluation and step-up therapy if there was recurrent disease was superior to standard medical therapy.

In this study, endoscopy was the gold standard method to define recurrent disease. With respect to non-invasive methods, both serum biomarkers and clinical activity indexes have demonstrated a poor correlation with disease activity[24-26]. The use of fecal markers in the postoperative period has been studied in small groups of patients with variable results. Recently, however, some studies evaluated the value of fecal biomarker measurement after surgery for CD. In 2015, Wright *et al*[15] demonstrated that FC was sensitive enough to diagnosis CD recurrence in 135 patients submitted to bowel resection with a high enough NPV to reassure clinicians that few patients with recurrence would be missed. More recently, Lopes *et al*[27] showed that both FC and FL strongly correlated with endoscopic findings in the evaluation of CD after surgery and accurately predicted endoscopic recurrence in 99 CD patients who submitted to ileocolonic resection.

The results of both of these studies suggests that fecal biomarkers may be incorporated in the postoperative management algorithm, both to diagnose recurrence and to assess response to therapy. In our study, we evaluated if in asymptomatic CD patients with anastomotic strictures not traversed

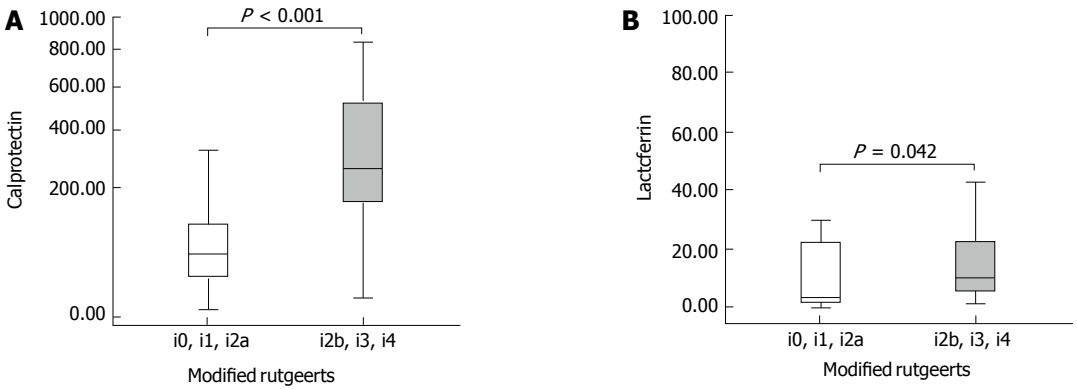


Figure 2 Box plots of fecal markers according to endoscopic activity defined by the modified Rutgeerts score, in asymptomatic patients presenting with anastomotic strictures. A: Fecal calprotectin; B: Fecal lactoferrin.

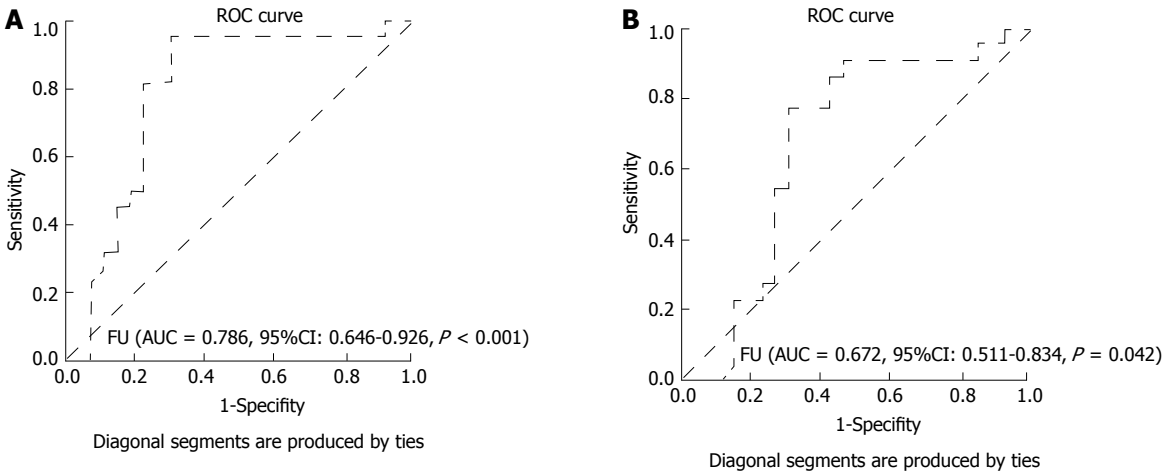


Figure 3 Receiver operating characteristic curves for fecal markers for discriminating between endoscopic recurrence and remission in asymptomatic patients presenting with anastomotic strictures. A: Fecal calprotectin; B: Fecal lactoferrin.

by the colonoscope, fecal markers perform as well as a predictor of disease recurrence, defining groups of patients that will need more invasive methods. In this series of 48 patients with strictures, only 18% complained of subocclusive symptoms. Similarly to what has been published[28-30], we also did not find any correlation between patient symptoms, serum biomarkers and HBI and endoscopic or radiographic findings, supporting the belief that using only symptoms or C-reactive protein levels to inform treatment decisions may increase the risk of disease progression and complications.

It is controversial whether or not asymptomatic strictures should be endoscopically treated. Despite being a safe and minimally invasive technique, there is a 3%-10%[6,9,10] described risk of major complications, especially in centers with limited procedural volume per year. Our data confirmed the safety and efficacy of EBD in the context of CD anastomotic strictures, with a technical success rate of 92%. We had no serious complications, which may be explained by several factors: careful patient selection; use of fluoroscopic image to evaluate, in real time, stricture characteristics and the therapeutic procedure indicated; maximum diameter of the balloon used (18 mm); use of carbon dioxide as type of insufflation; and experience of the endoscopist performing the technique with a uniform technical approach. The dilation of the anastomosis allowed the diagnosis of recurrence in 22 patients, that otherwise would have been missed if we only relied on symptoms or biochemical markers.

We used the modified Rutgeerts score to diagnose endoscopic recurrence, although it is not yet validated. Despite being used for several decades in clinical trials and clinical practice, the Rutgeerts score is also an invalidated score. In this study, we chose to use the modified Rutgeerts score, as demonstrated in a previous work[27] as having a better correlation between the modified score and fecal markers to diagnose recurrent disease. Recurrence was defined by a modified Rutgeerts score of i2b, not considering the presence of only anastomotic stricture as a criterion, as many other factors may be implicated in stricture development[1,2]. In that paper[27] the calculated best cut-off level for FC for predicting recurrence was 100 μg/g, with a sensitivity of 74% and a NPV of 91%.

Table 3 Comparison between patients with and without endoscopic recurrence

	Endoscopic recurrence, *n* = 22	No endoscopic recurrence, *n* = 26	*P* value
Sex, M:F	11:11	15:11	0.404
Median age, yr (IQR)	47.0 (35.5-53.3)	46.5 (39.8-54.8)	0.472
Median hemoglobin, g/dL (IQR)	13.8 (12.5-15.1)	13.9 (13.1-15.0)	0.715
Median albumin, g/L (IQR)	42.4 (36.1-45.9)	42.1 (39.8-44.6)	0.886
Median C-reactive protein, mg/L (IQR)	5.6 (1.6-8.2)	2.4 (0.9-9.9)	0.457
Median fecal calprotectin, μg/g (IQR)	257.0 (161.0-565.0)	53.9 (23.9-146.0)	< 0.001
Median fecal lactoferrin, μg/g (IQR)	9.1 (5.5-27.8)	3.9 (1.5-21.9)	0.042
Smoking, yes/no/past	3/13/6	9/13/4	0.196
Subocclusive symptoms, yes/no	2/20	6/20	0.183
HBI, remission/mild	17/5	24/2	0.145
Concomitant treatment			
Anti-TNF- α agents, yes/no	12/10	14/12	0.596
Immunomodulators, yes/no	9/13	11/15	0.578
Steroids, yes/no	4/18	5/21	0.611

HBI: Harvey-Bradshaw index; IQR: Interquartile range.

This is in accordance with other studies[15] that defined in the post-operative setting a higher cut-off than that of 50 μg/g used to diagnose inflammatory bowel disease[31].

In the present study, both FC and FL were also significantly higher in patients with endoscopic recurrence, with an area under the receiver operating characteristic curve for detection of endoscopic recurrence of 0.786 for FC and of 0.672 for FL. The best cut-off value of FC as predictor of recurrence was 90.85 μg/g, with sensitivity of 95.5%, NPV of 94.7% and accuracy of 81%. If we adopted the commonly used cut-off value of 7.25 μg/g for lactoferrin[32-34], we would have missed patients with recurrence (false negative results). The cut-off value of 5.6 μg/g had sensitivity of 77.3%, NPV of 78.4% and accuracy of 72.9%. Our findings support the potential value of these two noninvasive markers in the monitoring of patients submitted to bowel resection and presenting with an anastomotic stricture.

Indeed, a FC and/or FL concentration lower than 90.85 μg/g and 5.6 μg/g respectively, have high accuracy to exclude disease recurrence, with no need to further therapeutic intervention. This may be of particular interest in centers with low expertise in EBD and/or with a lower procedural volume per year, in order to avoid complications and/or be used as an indication for referring patients to tertiary centers. If these results are reproduced and validated by others, in a large number of patients, this conservative strategy could be adopted, reserving balloon dilation for symptomatic patients and those with high levels of fecal markers, in order to facilitate step-up therapy.

To our knowledge this is the first report on the performance of FC and FL in the context of anastomotic strictures in CD. Our results suggest that low values of fecal markers can predict, with a great amount of certainty, disease remission. This may avoid application of endoscopic dilation in asymptomatic patients in centers with less endoscopic expertise, reassuring physicians that the use of fecal markers serves as a good indicator to monitor disease recurrence. The serial monitoring of FC and FL can help to make decisions in indeterminate results and a persistently elevated value may serve as another useful indicator when considering therapy intensification.

In conclusion, postoperative FC and FL levels accurately predicted endoscopic recurrence in the presence of anastomotic stricture. Considering that a significant number of patients remain asymptomatic, with normal serum biomarkers, despite the permanent reduction of luminal caliber, a normal value of fecal markers can reassure clinicians and be safely used to avoid balloon dilation if we only aim to diagnose recurrence. In asymptomatic patients with a high FC and/or FL level, there is a great chance of having disease activity proximal to the stricture, so EBD should be performed in order to provide adequate endoscopic therapy and adjust or optimize medical therapy.

COMMENTS

Background

Recurrent disease in the neoterminal ileum and anastomotic strictures are frequent complications of Crohn´s disease (CD). Despite the permanent reduction of luminal caliber and disease progression, the majority of patients remain asymptomatic. Fecal calprotectin (FC) and lactoferrin have been suggested as surrogate non-invasive markers for diagnosing postoperative disease recurrence. There are no studies evaluating the performance of fecal markers as predictors of disease recurrence in asymptomatic patients with an anastomotic stricture.

Research frontiers

The results demonstrated that FC and lactoferrin are good predictors of CD endoscopic recurrence in patients with asymptomatic anastomotic stricture and may guide the need for endoscopic balloon dilation in this context.

Innovations and breakthroughs

A normal value of fecal markers can reassure clinicians and be safely used to avoid balloon dilation if we only aim to diagnose recurrence. A high value of fecal markers has a high likelihood of recurrence, so endoscopic balloon dilation should be performed in order to provide adequate endoscopic therapy and adjust or optimize medical therapy.

Applications

Fecal markers may avoid the need of endoscopic balloon dilation in asymptomatic patients with anastomotic stricture if we only aim to diagnose disease recurrence.

Terminology

Calprotectin is a protein complex, constituting up to 60% of neutrophil cytosol

protein that is released upon neutrophil activation. Lactoferrin, an iron-binding protein, is the main component of secondary granules that degranulate during inflammatory process. Both these proteins are remarkably stable and resistant to degradation, easily detected and have been proved to reflect endoscopic disease activity in CD, predicting endoscopic inflammation and being a surrogate marker of mucosal healing.

Peer-review

The authors have performed a very interesting and important study. They concluded that postoperative FC and FL levels accurately predicted endoscopic recurrence in the presence of anastomotic stricture.

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SYSTEMATIC REVIEWS

Gastrosplenic fistula occurring in lymphoma patients: Systematic review with a new case of extranodal NK/T-cell lymphoma

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Author contributions: Kang DH and Huh J performed the literature selection, data extraction, and initial drafting of the manuscript; Lee JH and Jeong YK conceptualized and designed the review and performed critical revision/editing of the manuscript; Cha HJ provided pathology data and performed critical revision/editing of the manuscript; all authors reviewed and approved the final manuscript as submitted.

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Abstract

AIM

To provide the overall spectrum of gastrosplenic fistula (GSF) occurring in lymphomas through a systematic review including a patient at our hospital.

METHODS

A comprehensive literature search was performed in the MEDLINE database to identify studies of GSF occurring in lymphomas. A computerized search of our institutional database was also performed. In all cases, we analyzed the clinicopathologic/radiologic features, treatment and outcome of GSF occurring in lymphomas.

RESULTS

A literature search identified 25 relevant studies with 26 patients. Our institutional data search added 1 patient. Systematic review of the total 27 cases revealed that GSF occurred mainly in diffuse, large B-cell lymphoma (*n* = 23), but also in diffuse, histiocytic lymphoma (*n* = 1), Hodgkin's lymphoma (*n* = 2), and NK/T-cell lymphoma (*n* = 1, our patient). The common clinical presentations are constitutional symptoms (*n* = 20) and abdominal pain (*n* = 17), although acute gastrointestinal bleeding (*n* = 6) and infection symptoms due to splenic abscess (*n* = 3) are also noted. In all patients, computed tomography scanning was very helpful for diagnosing GSF and for evaluating the lymphoma extent. GSF could occur either post-chemotherapy (*n* = 10) or spontaneously (*n* = 17). Surgical resection has been

the most common treatment. Once patients have recovered from the acute illness status after undergoing surgery, their long-term outcome has been favorable.

CONCLUSION
This systematic review provides an overview of GSF occurring in lymphomas, and will be helpful in making physicians aware of this rare disease entity.

Key words: Gastrosplenic fistula; Lymphoma; NK/T-cell lymphoma; Systematic review

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Core tip: Gastrosplenic fistula (GSF) is a very rare complication occurring in lymphomas. Due to its rarity, GSF had not been well-investigated. Therefore, we intend to provide an overall spectrum of GSF occurring in lymphomas through a systematic review. GSF occurred mainly in diffuse, large B-cell lymphoma, but also in various kinds of lymphoma. Common clinical presentations are constitutional symptoms and abdominal pain. Occasionally, acute gastrointestinal bleeding and splenic abscess have occurred. Computed tomography was helpful for diagnosing GSF and for evaluating the lymphoma extent. GSF could occur either post-chemotherapy or spontaneously. Surgical resection has been the most common treatment.

Kang DH, Huh J, Lee JH, Jeong YK, Cha HJ. Gastrosplenic fistula occurring in lymphoma patients: Systematic review with a new case of extranodal NK/T-cell lymphoma. *World J Gastroenterol* 2017; 23(35): 6491-6499 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6491.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6491

INTRODUCTION

Gastrosplenic fistula (GSF) is a rare and potentially fatal complication of various diseases, including lymphoma, gastric adenocarcinoma, Crohn's disease, splenic abscess, and trauma[1]. Lymphoma is the most common cause of GSF. Of these diseases, the majority have occurred in patients with diffuse, large, B-cell lymphoma (DLBCL), and there has been no patient with NK/T-cell lymphoma. In our hospital, a tertiary cancer center, we recently encountered a patient with GSF developed in NK/T-cell lymphoma.

A multidisciplinary team was organized in our hospital for management of this patient. Our team performed an extensive literature search, however, and determined that the characteristics of GSF occurring in lymphoma patients have not been thoroughly investigated due to its extreme rarity. In contrast, the number of case reports regarding these patients has increased over the last 10 years[1-24]. Despite the increasing number of these patients, there has been no systematic review to summarize the variable presentations of GSF occurring in lymphomas. Therefore, we performed the current, systematic review with the addition of our single patient in order to provide a perspective regarding this rare disease entity.

MATERIALS AND METHODS

Systematic literature search

A computerized search of the MEDLINE database was conducted to find relevant studies published prior to February 10, 2017. Studies were eligible for inclusion if they described the clinicopathologic features, imaging findings, treatment and outcome of the cases with regard to GSF occurring in lymphoma. The following search terms were used: "GSF" and "lymphoma". To expand the search, the bibliographies of articles that remained after the selection process were screened for other potentially suitable articles. We did not limit the language of the articles.

Institutional data search and case presentation

Our institutional review board approved the search of the electronic medical records for this study. Informed consent was waived from our institutional review board. We performed a systematic computerized search of our institutional database from January 2000 to January 2017 using the diagnostic codes of "GSF", "splenic fistula", and "lymphoma". Using these search terms, we identified only one case which we recently encountered, as described below. In our institutional database, the patient's record was anonymized and provided to us. We present here the clinical course, pathologic findings, and imaging features of this case. We also included this case in the systematic review of GSF of lymphoma.

Analysis of clinicopathologic and radiologic features

For patients with GSF occurring in lymphoma identified in the literature and at our institution, we analyzed their clinicopathologic features, imaging findings, treatment and outcome.

RESULTS

Literature selection

Our study selection process is shown in Figure 1. The literature search in the MEDLINE database generated 24, initial candidate articles. After reviewing the titles and the abstracts, all 23 articles were included. One study was subsequently excluded due to the lack of a full-text manuscript. Therefore, we reviewed the full text of all 23 articles, including 2 written in Spanish and 1 written in Korean. Search of the bibliographies of these articles found two, additional, eligible studies[8,14]. Therefore, a total of 25 articles were included in our

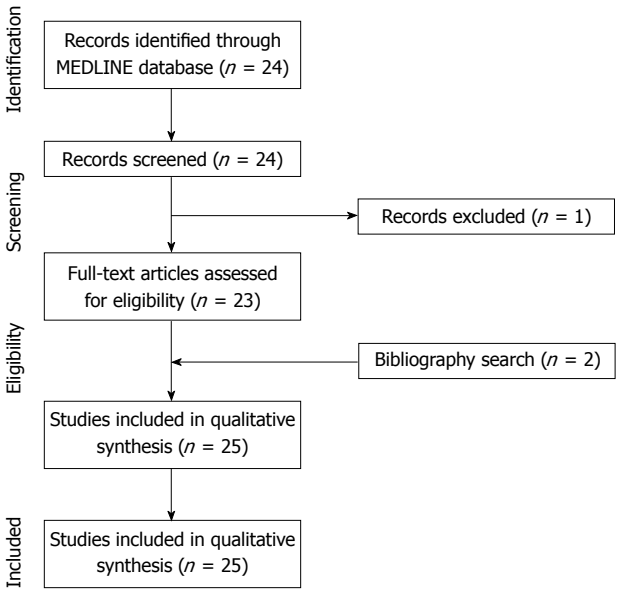


Figure 1 Flow diagram for the selection of studies.



Figure 2 On a coronal computed tomography image taken 2 mo after autologous stem-cell transplantation, the spleen was enlarged, measuring 17 cm in the longest dimension, and indicative of recurred lymphoma. The enlarged spleen abutted to the gastric fundus.

systematic review. Of these articles, 24 described one case, while 1 described two cases[3]. Therefore, a total of 27 cases were retrieved from the systematic literature search.

Presentation of our case

A 50-year-old man was admitted to our hospital for a biopsy-proven NK/T-cell lymphoma of the right anterior nasal cavity. On contrast-enhanced computed tomography (CT), there was an ill-defined, homogeneous, enhancing, soft-tissue mass in the right anterior nasal cavity. There was no significantly enlarged lymph node in his neck, chest or abdomen-pelvis. However, hepatosplenomegaly was noted with numerous, ill-defined, low, attenuating nodules in the liver. His spleen measured 15.5 cm in the longest dimension. Hypermetabolic enlargement of the liver and spleen was revealed on positron emission tomography scanning. These findings were indicative of lymphoma involvement in the liver and spleen.



Figure 3 On an axial computed tomography image taken after chemotherapy, a huge fistula was shown between the gastric lumen and the spleen. The spleen was totally infarcted.

The patient was subsequently started on the combination of a SMILE chemotherapy regime consisting of methotrexate, leucovorin, ifosfamide, etoposide, and L-asparaginase followed by autologous stem-cell transplantation (ASCT). After completing the ASCT, complete remission was confirmed by a bone marrow biopsy and CT scans with resolution of the nasal cavity mass, hepatosplenomegaly, and focal liver lesions.

Two months later, follow-up CT showed a huge splenomegaly measuring 17.5-cm in its longest diameter, which was indicative of lymphoma recurrence (Figure 2). Chemotherapy was started for the lymphoma recurrence. Tumor lysis syndrome occurred, and the patient underwent dialysis treatment.

He then soon experienced left, upper quadrant, abdominal pain and nausea/vomiting. Follow-up abdominal CT showed a diffusely enlarged spleen with nearly total splenic infarction. A huge fistula track was also seen between the gastric lumen and the infarcted spleen (Figure 3).

He underwent emergency surgery for gastric wedge resection and splenectomy. During the surgery, a large GSF was observed and there was adhesion between the infarcted spleen and the stomach. These surgical findings suggested that the extensive lymphoma lysis due to chemotherapy resulted in disruption of the splenic capsule, adhesion to the adjacent gastric fundus, and eventually led to the large GSF. In the gross and microscopic specimen, there was extensive hemorrhagic infarction in the spleen with a large GSF. The atypical lymphoma cells, which were positive for CD3 on immunohistochemistry stain and positive for Epstein-Barr virus (EBV) on EBV RNA stain, were found in the stomach wall near the GSF as well as in the whole spleen (Figure 4). The histopathological diagnosis was NK/T-cell lymphoma and these findings suggest that lymphoma cells may infiltrate from the spleen to the stomach wall through the adhesion/perforation site.

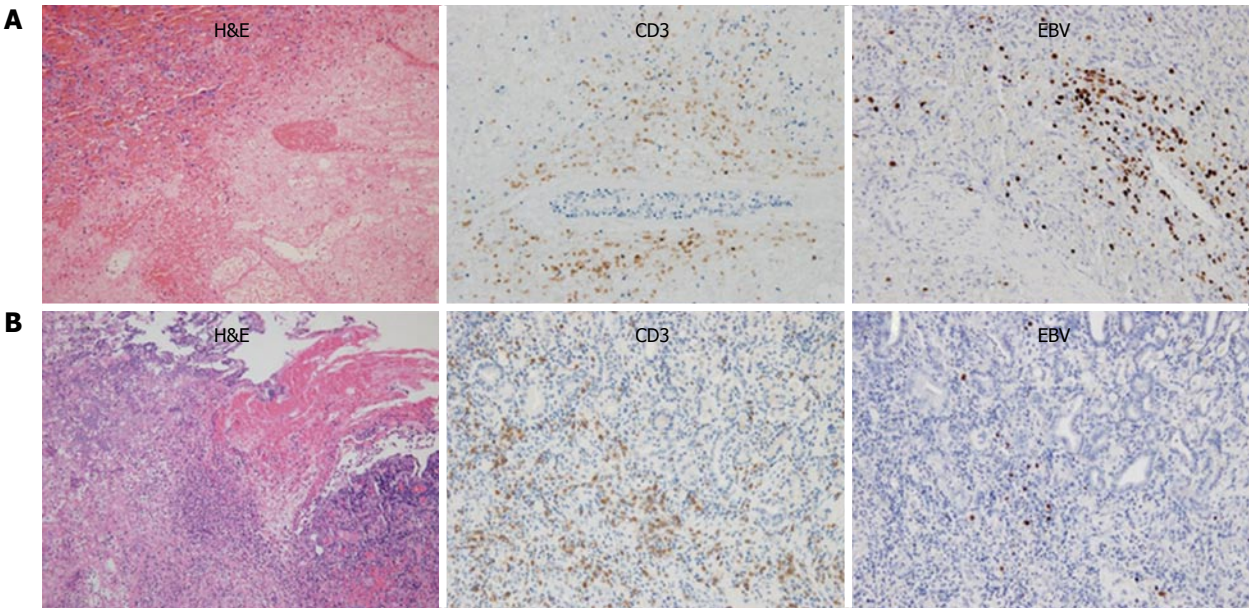


Figure 4 Microscopic specimen of the spleen and stomach. A: Atypical lymphoma cells were found in the spleen on hematoxylin-eosin stain (left). These cells showed positivity for CD3 on immunohistochemistry stain (middle) and EBV on EBV RNA stain (right). There was also extensive coagulative necrosis indicative of splenic infarction; B: Lymphoma cells were found in the stomach wall near the gastrosplenic fistula on hematoxylin-eosin stain (left), and which also showed positivity for CD3 (middle) and EBV (right). NK/T-cell lymphoma was diagnosed. These findings suggested that lymphoma cells may have infiltrated from the spleen to the stomach wall through the perforation site. EBV: Epstein-Barr virus.

Systematic review

The characteristics of the 27 included cases (26 literature cases and 1 presented case) of GSF occurring in lymphomas are summarized in Table 1. Although there was a substantial diversity in the clinicopathologic and radiologic features, the systematic review allowed us to summarize the characteristic features of GSF occurring in lymphomas.

Clinicopathologic features

The mean age of these patients was 50.6 ± 16.8 years with a range of 16-76 years. The male to female ratio was 4.4 (22 males, 5 females). Regarding the presenting symptoms or signs, constitutional symptoms such as weight loss, fever and fatigue (*n* = 20, 74.1%), were followed by abdominal/flank pain (*n* = 17, 63.0%), acute gastrointestinal bleeding such as hematemesis (*n* = 6, 22.2%), and infection due to splenic abscess (*n* = 3, 11.1%).

Among the different histological types, GSF occurred most commonly in DLBCL (*n* = 23, 85.2%). The other types of lymphoma associated with GSF include diffuse histiocytic lymphoma (*n* = 1, 3.7%), Hodgkin's lymphoma (*n* = 2, 7.4%), and extranodal NK/T-cell lymphoma (*n* = 1, 3.7%). Of these, GSF occurred due to splenic lymphoma involvement (*n* = 8, 29.6%, including the present case), gastric lymphoma involvement (*n* = 6, 22.2%), extensive lymphoma including both gastric and splenic involvement (*n* = 1, 3.7%), and not specified (*n* = 12, 44.4%). GSF occurred either post-chemotherapy (*n* = 12, 44.4%) or spontaneously (*n* = 17, 63.0%).

Radiologic features

In all 27 cases, the GSF was depicted or suspected on CT scans, as there was a defect in the gastric wall and splenic capsule where the stomach and spleen were closely attached. Of these, 10 cases described the size of the GSF which ranged from 0.25 cm to 6 cm (mean size: 2.87 cm). In 21 cases, endoscopy was performed to confirm the diagnosis of GSF and to evaluate the bleeding or other concomitant disease, such as a gastric ulcer.

The CT scan was excellent for identifying the extent of lymphoma involvement. The splenic involvement was most commonly noted as a moderate to massive splenomegaly (*n* = 13). Five of seven cases specifically noted the gastric lymphoma as a diffuse/segmental, gastric wall thickening or mass. There were five cases with extensive lymphoma masses in the left upper quadrant, including diaphragm involvement.

The CT scan was also very helpful for identifying any associated GSF complications. Indeed, a splenic abscess was diagnosed on CT in three cases. Acute bleeding was seen as leakage of contrast agent in the gastric lumen. Even when there was no leakage of contrast agent seen on CT, the presence of a hematoma in the stomach and/or spleen could suggest a recent hemorrhagic event.

Treatment and outcome

Regarding the treatment, surgical resection was performed in 23 patients (88.9%). Two patients underwent chemotherapy without surgery, and one patient refused any treatment.

Table 1 Summary of the 27 cases of gastrosplenic fistula occurring in lymphomas

Ref.	Diagnosis	Sex/age	Size of lymphoma	Disease status	Presentation	Diagnostic modality	Intervention/therapy	Outcome
Bubenik *et al*[1] (1983)	Diffuse histiocytic lymphoma	Male/58	Not available	Post-CTx	Nonspecific LUQ discomfort	CT abdomen followed by endoscopy of upper GI tract	Splenectomy, gastric greater curvature resection, distal pancreatectomy	Uneventful post-operative period; no further details
Hiltunen *et al*[2] (1991)	Gastric DLBCL	Male/36	Not available	Post-CTx	Hematemesis, splenomegaly	CT abdomen followed by endoscopy	Laparotomy without details	Followed-up over 3 yr
Blanchi *et al*[3] (1995) case 1	Splenic DLBCL	Male/62	Not available	Initial presentation	Left abdominal pain and fever	Endoscopy of upper GI tract followed by CT abdomen	Resection of spleen, tail of pancreas, and involved stomach	At 6 mo after the operation, the patient was in complete remission after CTx
Blanchi *et al*[3] (1995) case 2	Splenic DLBCL	Male/45	Not available	Initial presentation	Epigastric pain and weight loss	Endoscopy of upper GI tract followed by CT abdomen	No further details.	No further details
Carolin *et al*[4] (1997)	Gastric DLBCL	Male/46	Not available	Initial presentation	Epigastric pain, fatigue, weight loss and splenomegaly	Endoscopy of upper GI tract followed by CT abdomen	Laparotomy, but no further details.	No further details
Bird *et al*[5] (2002)	Splenic DLBCL	Male/36	Not available	Initial presentation	Hematemesis, melena, fatigue, weight loss and splenomegaly	Endoscopy of upper GI tract followed by CT abdomen	Splenic artery embolization, near total gastrectomy and splenectomy	Disease-free after three cycles of CTx; no further details
Choi *et al*[6] (2002)	Splenic DLBCL	Male/24	Not available	Initial presentation	LUQ pain and constitutionals symptoms (splenic mass)	CT abdomen followed by endoscopy of upper GI tract/ biopsy	CTx followed by splenectomy, gastric wedge resection, and distal pancreatectomy	Not available
Yang *et al*[7] (2002)	Gastric and splenic DLBCL	Male/21	Not available	Initial presentation	LUQ pain, fatigue, weight loss, fever, and splenomegaly	CT abdomen followed by endoscopy of upper GI tract	Splenectomy, gastric wedge resection, and distal pancreatectomy	After surgery, the patient underwent CTx
Puppala *et al*[8] (2005)	DLBCL	Female/66	Not available	Initial presentation	LUQ pain	CT abdomen oral contrast	CTx	Died after 2 mo of Ctx
Kerem *et al*[9] (2006)	DLBCL	Male/57	10 cm × 7 cm × 2 cm in the stomach and 8 cm × 5 cm × 4 cm in the spleen	Initial presentation	Abdominal pain, epigastric tenderness and splenomegaly	CT abdomen followed by PETCT and endoscopy of upper GI tract	Splenectomy, proximal gastrectomy, esophagojejunostomy, proximal pyroloplasty followed by CTx	Uneventful post-op period; underwent chemotherapy.
Al-Ashgar *et al*[10] (2007)	Hodgkin's lymphoma-(nodular sclerosis)-IIIS	Female/16	Not available	Initial presentation	LUQ pain, constitutional symptoms and splenomegaly	Endoscopy of upper GI tract, barium swallow, CT abdomen	Laparoscopic surgical repair followed by seven cycles CTx	Alive and in remission after 1 yr
Aribaş *et al*[11] (2008)	DLBCL	Male/25	Not available	Post-CTx	Abdominal pain, weight loss, fever, chill and splenomegaly	CT cystography followed by USG	Gastric wedge resection, fistulectomy and splenectomy	Discharged after a month and died 2 mo later due to progression of lymphoma and infection due to pancreatic and gastric fistulas
Palmowski *et al*[12] (2008)	DLBCL	Male/56	15 cm of spleen	After three cycles of CTx	Fever and signs of acute infection (splenic mass)	CT abdomen	Splenectomy with partial gastric resection	Finished six cycles of CTx
Seib *et al*[13] (2009)	Hodgkin's lymphoma	Male /49	3.6-cm splenic mass	Relapsed post-CTx	LUQ pain and constitutional symptoms (splenic mass)	CT abdomen	Partial gastrectomy and fistulectomy	Died after 5 mo

Moran *et al*[14] (2009)	DLBCL	Male/35	5.4 cm × 5.3 cm of gastrosplenic mass	Initial presentation	LUQ pain and constitutional symptoms	CT abdomen followed by endoscopy of upper GI tract	Abscess drainage; splenectomy, total gastrectomy, Roux-en-Y esophagojejunostomy followed by CTx	Received CTx after surgery; no further details available
Maillo *et al*[15] (2009)	Splenic DLBCL	Female/76	Not available	Initial presentation	Massive hematemesis, fever and fatigue (splenic abscess)	CT abdomen followed by endoscopy of upper GI tract	splenectomy, partial gastrectomy, diaphragmatic primary repair, drainage chest tube and a feeding tube jejunostomy	At 2 mo later the patient developed a pulmonary infection and died because of multi-organic failure
García *et al*[16] (2009)	Gastric DLBCL	Male/76	Not available	Initial presentation	Epigastric pain, weight loss and splenomegaly	CT abdomen followed by endoscopy of upper GI tract	Total gastrectomy, splenectomy and distal pancreatectomy	Remained asymptomatic at the 36-mo follow-up, no further details
Khan *et al*[17] (2010)	Gastric DLBCL	Female/43	18.9 cm × 10 cm × 8.6 cm of splenic mass	Initial presentation	Upper abdominal pain and constitutional symptoms (splenic mass)	Endoscopy of upper GI tract followed by CT	CTx	Complete remission after two cycles of CTx; no further details
Rothermel *et al*[18] (2010)	Splenic DLBCL	Male/74	Not available	Initial presentation	Fever, chill and weight loss	Endoscopy of upper GI tract followed by CT	Splenectomy, stapled gastric-sleeve resection	After surgery, the patient underwent CTx; good prognosis for long-term survival
Dellaportas *et al*[19] (2011)	Splenic DLBCL	Male/68	Not available	Initial presentation	Hematemesis (splenic mass)	Endoscopy of upper GI tract followed by CT abdomen	Surgical *en bloc* resection followed by chemotherapy	Post-CTx on follow up No details available
Jain *et al*[20] (2011)	DLBCL	Male/55	Not available	Post-CTx	Progressive weakness, fatigue, melena and splenomegaly	CT abdomen followed by endoscopy of upper GI tract	Splenectomy and partial gastrectomy	Received CTx after surgery; no further details available
Ding *et al*[21] (2012)	DLBCL	Male/62	7 cm of splenic segment	Initial presentation	LUQ pain with constitutional symptoms and splenomegaly	CT abdomen followed by endoscopy of upper GI tract	Splenectomy, gastric wedge resection, and distal pancreatectomy followed by CTx and RT	Well at follow up; no further details available
Favre Rizzo *et al*[22] (2013)	Gastric DLBCL	Male/55	Not available	Initial presentation	Hematemesis, epigastric pain, weight loss and splenomegaly	CT abdomen	Partial gastrectomy, splenectomy and distal pancreatectomy	After surgery; no further details available
Senapati *et al*[29] (2014)	DLBCL	Male/57	Splenomegaly of 15 cm	Post-CTx	No symptom but splenomegaly	PET/CT followed by endoscopy of upper GI tract	Refused any surgical intervention	Lost to follow-up
Gentilli *et al*[23] (2016)	Gastric DLBCL	Female/66	7.5 cm × 3 cm of splenic mass	Post-CTx	Weakness, fatigue, weight loss and splenomegaly	Endoscopy of upper GI tract followed by CT	Gastric wedge resection, splenectomy	Discharged after surgery; no further details
Sousa *et al*[24] (2016)	Gastric DLBCL	Male/52	Not available	Post-CTx	Hematemesis	Endoscopy of upper GI tract	Total gastrectomy, splenectomy, distal pancreatectomy	Patient was lost to follow-up after discharge
Present case	NK/T cell lymphoma	Male/50	11 cm × 5 cm × 13 cm of spleen	Post-CTx	LUQ pain, nausea, vomiting and splenomegaly	CT abdomen	Gastric wedge resection and splenectomy	At 3 mo later, gastric perforation occurred and the patient expired due to sepsis

CT: Computed tomography; CTx: Chemotherapy; DLBCL: Diffuse large B-cell lymphoma; GI: Gastrointestinal; LUQ: Left upper quadrant; PET: Positron emission tomography; RT: Radiation therapy; USG: Ultrasonography.

Among the 27 cases, death was reported in 5 patients due to a gastric perforation (n = 1), progression of lymphoma and infection (n = 1), pulmonary infection with multi-organic failure (n = 1), and no further details (n = 2). The time to death ranged from 2-5 mo after the initial surgery or diagnosis. In 18 cases, the patients were alive at that time or were discharged. Patient outcomes were not reported for three patients, and two patients were lost to follow-up.

DISCUSSION

GSF is a very rare complication of lymphoma, as only 27 cases have been reported including the present case. It is very difficult to identify the spectrum of presentation,

clinicopathologic/radiologic features, and outcome in this kind of extremely rare disease entity. Systematic review of pre-existing cases might be the best way to evaluate the overview of this rare disease[25]. From that perspective, our systematic review may provide a systematic summary and overview of GSF occurring in lymphomas.

As our systematic review includes all of the available articles in PubMed without the limitation of language, it is the largest reported series of GSF cases occurring in lymphomas. Although there was a diversity in the clinicopathologic features, imaging features and outcomes, we can summarize that GSF occurs mainly in DLBCL (85.2%) and it can occur either post-chemotherapy (37%) or spontaneously (63%). GSF can also cause fatal complications such as acute bleeding and splenic abscess. Once these fatal complications in the acute illness stage are controlled, the long-term outcome is good.

Regarding the pathologic type, DLBCL is the most common type associated with GSF. The present case of GSF occurred in extranodal NK/T-cell lymphoma of the nasal type and, to our knowledge, is the first reported case. In general, NK/T-cell lymphoma occurs in the nasal cavity. If NK/T-cell lymphoma involves an area outside the nasal cavity, the gastrointestinal tracts and skin are common and preferential extranodal organs of lymphoma involvement[26]. Rare sites of involvement include the spleen, prostate, pancreas and adrenal glands[27]. From this perspective, our case demonstrated a very rare complication of GSF which occurred in a rarely involved site of NK/T-cell lymphoma of the nasal type.

In general, NK/T-cell lymphoma is characterized by its aggressive behavior and with an angiocentric/angiodestructive growth pattern. In our case, we at first postulated that the GSF might occur due to these aggressive behaviors. Thereafter, a multidisciplinary discussion at our medical center indicated that extensive tumor lysis from chemotherapy might also be an important causative factor of GSF. The spleen parenchyma and capsule were largely infiltrated by NK/T-cell lymphoma cells, and thus caused splenomegaly. When the enlarged spleen was attached to the adjacent gastric fundus, tumor cells might infiltrate into the gastric wall. When chemotherapy caused tumor lysis and necrosis, the defect in the spleen capsule and gastric wall occurred, eventually leading to the GSF.

When we systematically reviewed the literature reports, we discovered that there has been a commonly proposed theory of GSF development, *i.e.*, necrosis of lymphoma tissue involving the spleen or stomach may cause GSF. According to this theory, invasion of the gastric wall and splenic capsule is required. If the lymphoma predominantly involves the spleen, the tumor cells should also infiltrate the gastric wall, and *vice versa*. The rapid necrosis of infiltrated lymphoma cells in the gastric wall and splenic capsule may result in the formation of GSF which may occur either spontaneously or post-chemotherapy[6,28]. Most of the GSFs described in the published literature are small. However, if a larger part of the stomach is infiltrated, it is possible for a large opening in the stomach wall to arise, as was reported in our case.

As the initial presentations of GSF occurring in lymphomas are somewhat non-specific, such as constitutional symptoms and abdominal pain, the diagnosis of GSF may be difficult and delayed. CT scanning can have an important role in the diagnosis of GSF, can identify GSF-associated complications such as abscess or bleeding, and can evaluate the disease extent and surgical planning. If the diagnosis and treatment of GSF are delayed, the clinical outcome might not be favorable, as GSF in lymphomas recover spontaneously. Therefore, radiologists should be aware of this rare disease entity and should notify physicians when GSF is suspected in patients with lymphoma involving the spleen or stomach.

There are various therapeutic options for managing GSF. Surgery is regarded as the most important and curative treatment in the vast majority of literature reports. The surgical method can be determined based on the tumor extent and the surgeon's preference. The most common surgical method in the literature reports is partial gastrectomy (mostly laparoscopic) with/without splenectomy. However, in some cases with a large tumor extent, near-total gastrectomy and splenectomy has also been performed. When patients were too ill to have surgery or refused it, the outcome was not good. Once patients had surgery and overcame the acute illness status, the long-term outcome was generally good. Considering all of these cases, we propose that aggressive surgery at the early stage of disease might be the best way to save patients with GSF occurring in lymphomas.

In conclusion, this systematic review provides an overview and spectrum of GSF occurring in lymphomas and covering the clinicopathologic features, radiologic features, treatment and outcome. We also included a rare case of GSF occurring in extranodal NK/T-cell lymphoma. This information will be helpful for physicians so that they can become aware of this rare disease entity.

COMMENTS

Background

Gastrosplenic fistula (GSF) is a very rare complication occurring in lymphomas. Due to its rarity, GSF have not been well-investigated. Therefore, the intent of our study was to provide the overall spectrum of GSF occurring in lymphomas through a systematic review including a patient at our hospital who had extranodal NK/T-cell lymphoma.

Research frontiers

A systematic review of 27, published studies was conducted. The clinicopathologic/radiologic features, treatment and outcome of GSF occurring

in lymphomas were analyzed.

Innovations and breakthroughs

This systematic review includes all of the available articles in PubMed without the limitation of language and it is the largest reported series of GSF cases occurring in lymphomas. It provides a systematic summary and overview of GSF occurring in lymphomas in order to understand the spectrum of presentation, clinicopathologic/radiologic features and outcome in this type of extremely rare disease entity. The authors also added our present case of GSF occurring in extranodal NK/T-cell lymphoma of the nasal type and, to our knowledge, this is the first reported case of this type.

Applications

When lymphoma patients present symptoms or signs of constitutional symptoms, such as weight loss, fever and fatigue, abdominal/flank pain, acute gastrointestinal bleeding, such as hematemesis or any infection signs with or without history of chemotherapy, computed tomography scanning was very helpful for diagnosing GSF and evaluating the lymphoma extent. GSF could occur either post-chemotherapy or spontaneously. Surgical resection has been the most common treatment. Once patients have recovered from their acute illness status after undergoing surgery, their long-term outcome has been good. The authors should be aware of GSF, which is a rare disease entity in patients with lymphoma, especially involving the spleen or stomach.

Terminology

GSF is a communication between the stomach lumen and spleen parenchyma.

Peer-review

This manuscript systematically reviewed a very rare complication of GSF occurring in lymphomas. The review analyzed the current literature regarding the clinicopathologic/radiologic features, treatment and outcome of GSF. It therefore provides useful recording of the disease.

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META-ANALYSIS

Proton pump inhibitors therapy and risk of *Clostridium difficile* infection: Systematic review and meta-analysis

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Abstract

AIM

To perform a systematic review and meta-analysis on proton pump inhibitors (PPIs) therapy and the risk of *Clostridium difficile* infection (CDI).

METHODS

We conducted a systematic search of MEDLINE/PubMed and seven other databases through January 1990 to March 2017 for published studies that evaluated the association between PPIs and CDI. Adult case-control and cohort studies providing information on the association between PPI therapy and the development of CDI were included. Pooled odds ratios (ORs) estimates with 95% confidence intervals (CIs) were calculated using the random effect. Heterogeneity was assessed by I^2 test and Cochran's Q statistic.

Potential publication bias was evaluated *via* funnel plot, and quality of studies by the Newcastle-Otawa Quality Assessment Scale (NOS).

RESULTS

Fifty-six studies (40 case-control and 16 cohort) involving 356683 patients met the inclusion criteria and were analyzed. Both the overall pooled estimates and subgroup analyses showed increased risk for CDI despite substantial statistical heterogeneity among studies. Meta-analysis of all studies combined showed a significant association between PPI users and the risk of CDI (pooled OR = 1.99, CI: 1.73-2.30, $P < 0.001$) as compared with non-users. The association remained significant in subgroup analyses: by design-case-control (OR = 2.00, CI: 1.68-2.38, $P < 0.0001$), and cohort (OR = 1.98, CI: 1.51-2.59, $P < 0.0001$); adjusted (OR = 1.95, CI: 1.67-2.27, $P < 0.0001$) and unadjusted (OR = 2.02, CI: 1.41-2.91, $P < 0.0001$); unicenter (OR = 2.18, CI: 1.72-2.75, $P < 0.0001$) and multicenter (OR = 1.82, CI: 1.51-2.19, $P < 0.0001$); age ≥ 65 years (OR = 1.93, CI: 1.40-2.68, $P < 0.0001$) and < 65 years (OR = 2.06, CI: 1.11-3.81, $P < 0.01$). No significant differences were found in subgroup analyses (test for heterogeneity): $P = 0.93$ for case-control *vs* cohort, $P = 0.85$ for adjusted *vs* unadjusted, $P = 0.24$ for unicenter *vs* multicenter, $P = 0.86$ for age ≥ 65 years and < 65 years. There was significant heterogeneity across studies ($I^2 = 85.4\%$, $P < 0.001$) as well as evidence of publication bias (funnel plot asymmetry test, $P = 0.002$).

CONCLUSION

This meta-analysis provides further evidence that PPI use is associated with an increased risk for development of CDI. Further high-quality, prospective studies are needed to assess whether this association is causal.

Key words: Proton pump inhibitors; *Clostridium difficile* infection; Risk; Systematic review; Meta-analysis

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Core tip: A possible association between the use of proton pump inhibitors (PPIs) and the risk of *Clostridium difficile* infection (CDI) have been suggested by several studies. This meta-analysis, including the largest number of studies published to date found the risk of CDI almost two-times higher in PPIs users than in nonusers. Because all the studies analyzed were observational, the causality could not be confirmed. Nevertheless, clinicians should be aware of such potential association and prescribe the PPIs only where they are clearly indicated.

Trifan A, Stanciu C, Girleanu I, Stoica OC, Singeap AM, Maxim R, Chiriac SA, Ciobica A, Boiculese L. Proton pump inhibitors therapy and risk of *Clostridium difficile* infection: Systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(35): 6500-6515 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6500.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6500

INTRODUCTION

Over the past two decades *Clostridium difficile* (*C. difficile*) infection (CDI) has registered an increasing trend worldwide both in incidence and severity[1-5], with healthcare costs varying between 1.2 and 4.7 billion dollars each year in the United States alone[6-9]. In addition to the broad-spectrum antimicrobial therapy which has been the most prominent causative factor for CDI[10,11], other potential risk factors have been identified such as: advanced age, hospitalization [particularly in intensive care units (ICU)], immunosuppression, renal insufficiency, hypoalbuminemia, lengthy hospital stay, the use of nasogastric tubes, invasive gastrointestinal procedures, chemotherapy, the presence of comorbidities, environment-related factors, and the emergence of a hypervirulent strain of the bacterium known as North American pulso-type 1 in some areas[12-21]. However, there might be some other risk factors for the CDI epidemic in the recent years despite tighter control on the use of antibiotics and stricter control policies on hospital-related infections[17]. A possible association between the use of proton pump inhibitors (PPIs) and the development of CDI has been suggested and numerous studies have examined it, reporting conflicting results[22-43].

Since their release in the late 1980s, PPIs have become some of the most widely prescribed agents both in outpatient and inpatient settings throughout the world[44-53], with sales totalling billions dollars worldwide[54,55]. These drugs have proven effective in the treatment of ulcer disease (including bleeding peptic ulcer), gastroesophageal reflux disease, *Helicobacter pylori* (in combination with antibiotics), Zollinger-Ellison syndrome, in the prophylaxis of upper gastrointestinal complications with nonsteroidal anti-inflammatory drugs (NSAIDs) therapy, stress ulcer prophylaxis in ICU patients, and functional dyspepsia[50,53,56-60]. The widespread use of PPIs during the last 25 years in clinical practice is the result not only of their high efficacy but also of their excellent safety profile, proving to be one of the safest class of medication used in gastroenterology[57,61-64].

Nevertheless, like in the case of other drugs, PPIs are not as safe as it has been thought and more recently, concerns have been raised about their potential association with pneumonia[65-67], bone fractures[68-70], interstitial nephritis and acute kidney injury[71]. More recently, reports of other potential PPIs adverse events such as risk for chronic kidney disease[72,73], dementia[74], spontaneous bacterial peritonitis[75,76], acute myocardial infarction[77,78], micronutrient

deficiency (magnesium, calcium, iron)[79,80] were published, although the quality of evidence for these is consistently low to very low[81].

An association between PPIs use and CDI is, at least theoretically, rational. Thus, intestinal homeostasis is maintained by host defense mechanisms in which gastric acid plays an important role as a barrier to ingested bacteria and bacterial overgrowth[82]. PPIs therapy profoundly inhibits gastric acid production leading to the proliferation of spores and their ability to convert to a vegetative form of *C. difficile* [83]. Moreover, PPIs impair leukocyte function by inhibiting phagocytosis and acidification of phagolysosome[84].

Several systematic reviews and meta-analyses have reported conflicting results regarding the association between PPIs use and increased risk of CDI. Thus, no less than six meta-analyses[85-90] found a significant association between PPIs therapy and increased risk of CDI. These findings were also supported by several studies[19,22-26,39,91-114] which reported a risk for CDI two or three times higher in PPIs users than in nonusers. Moreover, the United States Food and Drug Administration (FDA) informed the public about a possible correlation between PPIs use and CDI[115]. Still, other studies and meta-analyses have failed to associate PPIs use with the development of CDI[11,27,34,38,40-43,116-123]. It should be mentioned that PPIs continue to be among the most used drugs despite the above mentioned concerns about long-term side effects. Furthermore, beside a marked overuse of PPIs, over half of prescriptions are for non-indicated reasons[29]. One study reported that 60.7% of patients with CDI used PPIs, of whom only 47.1% had an evidence-based indication[30].

The aim of this systematic review and meta-analysis is to summarize data on the association between PPIs use and the risk of CDI as presented in the published studies.

MATERIALS AND METHODS

Information sources

A systematic literature search was independently conducted by four study investigators (Girleanu I, Stoica OC, Singeap AM and Chiriac SA) using a variety of databases including MEDLINE/PubMed, Web of Science (ISI Web of Knowledge), Scopus, EMBASE, Science Direct, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Excerpta Medica Database, and Cochrane Library, from January 1990 (the first PPI received FDA approval in 1989) to March 2017. The database searches were performed using the following medical subject heading (MeSH) terms: "proton pump inhibitors", "acid suppressive therapy", "omeprazole", "pantoprazole", "lansoprazole", "rabeprazole", "esomeprazole", combined with "*C. difficile* infection", "*C. difficile*-associated diarrhea", "pseudomembranous colitis". Reference lists of all retrieved papers were hand-searched to identify any additional studies that may have been missed in the computed-assisted literature search. The investigation was limited to studies performed in adult human beings, written and published in English, French, and German, in any geographic region.

Inclusion and exclusion criteria

Selection of the studies. Inclusion and exclusion criteria were established a priori by two authors (Trifan A and Stanciu C). First, duplicate citations were identified and removed, then three of us (Ciobica A, Maxim R and Singeap AM) independently reviewed the titles and abstracts of the studies and excluded those which did not answer the search question. Adult case-control and cohort studies providing information on the association between PPI therapy and the development of CDI were included. Studies conducted on pediatric patients, systematic reviews and meta-analyses, consensus documents, studies using PPIs simultaneously with H2 receptor antagonists (H2RA) or reporting exclusively on H2RA, case reports, editorials, protocols, and studies presented only as abstracts were excluded. There was no restriction related to the type of PPI regimen or diagnostic methods of CDI. Any disagreements about study inclusion were resolved in consensus with a third author (Stanciu C or Trifan A) after the full-text of the potential study had been reviewed; all eligible studies were assessed in full. They were subsequently included in this meta-analysis only if reported odds ratios (ORs) or risk ratios (RRs) for (adjusted or unadjusted) case-control and cohort studies, respectively, or data for their calculation were available.

Data extraction

Extracted data were cross-checked independently by four authors (Girleanu I, Stoica OC, Chiriac SA and Ciobica A) from each included study using a standardized data extracting sheet which included the last name of first author, journal and year of publication, country where the study was carried out, study design, sample size, age (mean or median) and gender distribution of patients, duration of the PPI treatment, effect estimates ORs or RRs, and 95% confidence intervals (CIs) of PPI exposure with and without adjustment for confounding variables. Any disagreement between reviewers was resolved in consensus with a third reviewer (Stanciu C or Trifan A).

Study quality assessment

Assessment of study quality was made independently by two authors (Boiculese L and Girleanu I) using the Newcastle-Ottawa Quality Assessment Scale (NOS; ranging 0-9)[124] as recommended by the Cochrane Handbook for Systematic Reviews of Interventions[125]. The NOS comprises three domains: selection, comparability, and outcome for cohort studies or exposure for case-control studies. A maximum of

four stars were awarded for selection, two stars for comparability, and three stars for exposure/outcome. Studies with cumulative score ⩾ 7 were considered high quality, 6 stars to be of moderate quality, and less than 6 stars to be of low quality. When disagreement, after discussion with the third author (Trifan A or Stanciu C) a consensus was reached. The final analysis included 56 high and moderate quality studies.

As none of the studies was randomized, and all were observational (case-control and cohort), the methods used in our systematic review and meta-analysis followed the MOOSE (Meta-Analysis of Observational Studies in Epidemiology) criteria[126].

Statistical analysis

Meta-analyses were performed both for all studies together and separately for case-control and cohort studies using DerSimonian and Laird[127] random effects model due to expected heterogeneity between studies. Our primary analysis focused on the association between PPIs therapy and the risk for developing CDI and because all of PPIs have similar efficacy we have not performed meta-analyses stratified by type of PPIs. The results are reported as pooled ORs with 95%CIs for primary and subgroup analyses.

Heterogeneity between studies was assessed by I^2 statistic and Cochran's *Q*-statistic. The level of heterogeneity was considered as high when I^2 > 75% or P < 0.10 for the *Q* statistic[128]. I^2 values between 61%-75%, 30%-60%, and < 30% were considered to represent substantial, moderate and low level of heterogeneity, respectively[129]. Seven potential confounders were considered: study design, effect estimate (adjusted *vs* unadjusted), setting (community *vs* inpatient), number of centers (single center *vs* multicenter), age, study quality, and geographical region.

Publication bias was assessed quantitatively using Egger's regression asymmetry test[130] and a P < 0.1 was considered statistically significant for asymmetry, and qualitatively by visual inspection of funnel plots of the logarithmic OR *vs* their standard errors[131]. Asymmetrical funnel plots were regarded to indicate high risk of publication bias.

Number needed to harm (NNH) estimates the number of patients needed to be treated with PPI for one additional person to have a CDI, and was calculated using the pooled OR (95%CI) from the meta-analysis and Patient Expected Event Rate (1.67%)[120].

All statistical tests were two tailed, and results associated with P < 0.05 (except for heterogeneity and publication bias) were considered significant. All analyses were performed using R version 3.2.3 software for the metaphor package 1.9-8, which provides a comprehensive collection of validated functions[132]. The statistical analyses of this study were performed by an expert in biostatistics from "Grigore T. Popa" University of Medicine and Pharmacy, Department of Medical Informatics and Biostatistics.

RESULTS

Search results

The initial online databases search identified 944 studies and 12 more were found from the reference lists of the articles retrieved. After reviewing all titles and abstracts, 216 studies were selected for full-text review, from which 56 studies were found to fulfill the inclusion criteria and were included in meta-analysis. Five of the 56 studies were published after the last meta-analysis (Figure 1).

Characteristics of included studies

The characteristics of the included studies are shown in Table 1. Of the included 56 studies, 40 (71.4%) were case-control, and 16 (28.6%) cohort studies, addressed to hospital-acquired (n = 43), community-acquired (n = 6), and both hospital and community-acquired CDI (n = 7). Most of the studies (n = 31) were single-center. The size of the study population ranged from 40 to 101796. In total, 356683 subjects were included, most of them from North-American and European studies (n = 46).

Quality assessment

The median value of NOS quality assessment was 7, with a mean 6.67 ± 0.74, range 6-8. In studies reporting gender, the proportion of men ranged from 47% to 67%, and from those that reported the age, the average age ranged between 18 and 82.2 years. Thirty-eight studies identified confounding factors (sex, age, antibiotic use, comorbidities) used for adjustment of the association between PPI therapy and risk of CDI. The majority of the studies were retrospective (85.7%) and only 8 were prospective (14.3%). None of the studies was randomized.

Meta-analysis

Meta-analysis of all studies combined. The results of pooled analysis for all 56 studies showed a significant association between PPI therapy and the risk of CDI as compared with non-PPI users (OR = 1.99, CI: 1.73-2.30, P < 0.001) (Figure 2). There was significant heterogeneity of effects across studies (I^2 = 85.41%; P < 0.001).

Subgroup analyses of case-control and cohort studies also showed a significant higher risk of CDI with PPI use (Table 2). There was no significant difference of effects between cohort and case-control studies (P = 0.931). The pooled OR for the cohort studies was 1.98 similar to OR for case-control that was 2.0.

The association remained also significant after limiting meta-analysis to studies with both adjusted (OR = 1.95, CI: 1.67-2.27, P < 0.001) and unadjusted

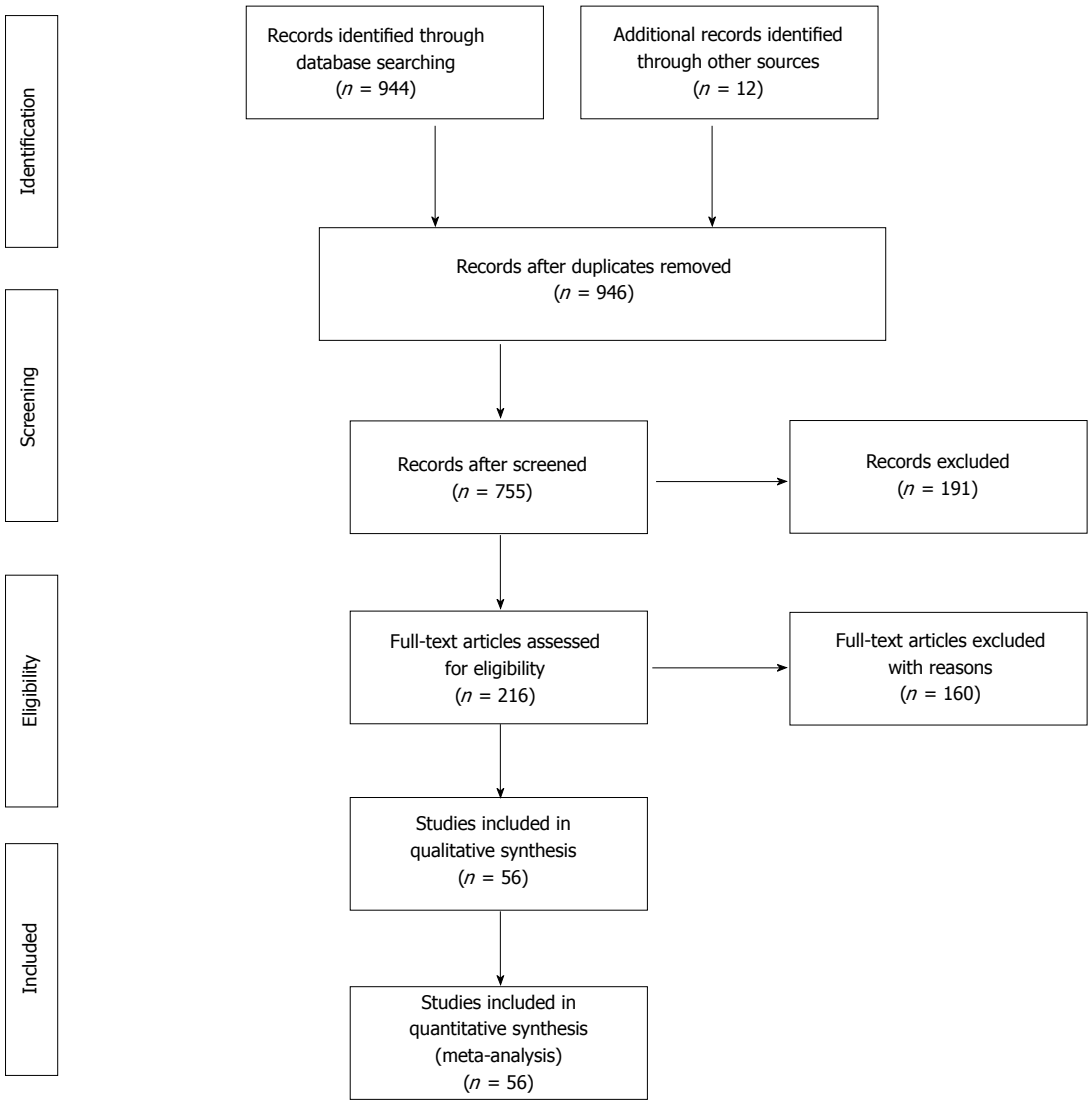


Figure 1 Study selection process.

data (OR = 2.02, CI: 1.41-2.91, $P < 0.001$). There was also no significant difference of effects between adjusted and unadjusted studies ($P = 0.856$).

PPIs use was found to be associated with an increased risk of CDI in both single-center studies (OR = 2.18, 95%CI: 1.72-2.75) and multicenter studies (OR = 1.82, 95%CI: 1.51-2.19).

There was no significant difference between inpatients and outpatients regarding CDI risk ($P = 0.868$). For both inpatients and outpatients the PPIs use almost doubled the risk of CDI (OR = 1.95, OR = 2.10, respectively).

When grouped by region, a direct association was found in the European group (OR = 1.78, 95%CI: 1.35-2.34), the North American group (OR = 2.00, 95%CI: 1.67-2.40), while the highest risk of CDI after PPI treatment was demonstrated in the Asian group (OR = 2.31, 95%CI: 1.96-2.72).

The subgroup of high-quality studies (NOS $\geqslant$ 7) showed a direct association (OR = 1.88, 95%CI: 1.55-2.28) between PPIs and risk of CDI, and this association was also significant in the medium-quality group (OR = 2.11, 95%CI: 1.69-2.62), with no difference between the two groups ($P = 0.441$).

There was no statistical difference regarding the risk for CDI for elderly ($\geqslant$ 65 years) compared with younger group (< 65 years) ($P = 0.860$).

Publication bias

We have drawn the funnel plot for 3 levels of confidence interval (90%, 95% and 99% corresponding to shades white, gray and dark gray) (Figure 3). The Egger's test of asymmetry proved no significance ($Z = 0.3699$, $P = 0.711$).

Number needed to harm

Based on reported incidence of CDI (at 14 d after hospital admission) of 1.67% in patients who have not used PPI, we estimate a NNH of 63 (95%CI: 48-78), if these patients will receive PPIs.

Table 1 Characteristics of studies included in the meta-analysis

Author, yr	Region	Study design	Centers	Setting	Sample size, *n*	Mean age, yr	Identified confounders	OR (95%CI)
Akhtar *et al* Shaheen[91], 2007	America	Case-control	Unicenter	Inpatient	1290		Adjusted for age, sex, comorbidities, antibiotics, chemotherapy	2.1 (1.6-2.7)
Al-Tureihi *et al*[19], 2005	America	Case-control	Unicenter	Inpatient	53		Adjusted for age, antibiotics	3.1 (1.0-9.7)
Aseeri *et al*[23], 2008	America	Case-control	Unicenter	Inpatient	188		Adjusted for admission date, sex, age group, antibiotic use, patient location, and room type	4.4 (2.3-8.2)
Bajaj *et al*[133], 2010	America	Case-control	Multicenter	Mixt	162		Adjusted for antibiotics, PPI	37.6 (6.2-227.6)
Barletta *et al*[92], 2014	Asia	Case-control	Unicenter	Inpatient	408		Adjusted for PPI exposure, antibiotics, immunosuppression	2.1 (1.2-3.8)
Baxter *et al*[93], 2008	America	Case-control	Multicenter	Inpatient	4493		Adjusted for antibiotics, age, hospital stay, other infections	1.2 (1.0-1.4)
Beaulieu *et al*[27], 2007		Cohort	Unicenter	Inpatient	827		Adjusted for age, sex, length of stay, comorbidities, APACHE score, NGT feeding, tracheal tube placement, antibiotics	1.3 (0.9-2.0)
Branch *et al*[94], 2007	America	Case-control	Unicenter	Inpatient	787	66.02	No	13.0 (7.5-22.7)
Buendgens *et al*[95], 2014	Europe	Case-control	Multicenter	Inpatient	3286		Adjusted for age, sex, antibiotics, comorbidities, other treatment	3.1 (1.1-8.7)
Campbell *et al*[38], 2013	America	Case-control	Unicenter	Inpatient	96		Adjusted for antibiotics, hospitalization	2.2 (0.6-8.0)
Cunningham *et al*[96], 2003	Europe	Case-control	Unicenter	Inpatient	320		Adjusted for antibiotics and chemotherapy	2.5 (1.5-4.1)
Dalton *et al*[22], 2009	America	Cohort	Multicenter	Inpatient	14719	74.7	Adjusted for number of medication groups, antibiotic days, age, length of stay, medical service, PPI days	1.9 (1.4-2.7)
Debast *et al*[116], 2009	Europe	Case-control	Unicenter	Inpatient	154		Adjusted for age, hospital stay, comorbidities, antibiotics	1.1 (0.5-2.4)
Dial *et al*[26], 2004 (case-control)	America	Case-control	Multicenter	Inpatient	188		Adjusted for age, antibiotics	2.6 (1.3-5.0)
Dial *et al*[26], 2004 (cohort)	America	Cohort	Multicenter	Inpatient	1187		Adjusted for age, antibiotics	2.1 (1.2-3.5)
Dial *et al*[98], 2005	Europe	Case-control	Multicenter	Outpatient	13563		Adjusted for age, sex, antibiotics	2.9 (2.4-3.5)
Dial *et al*[97], 2006	Europe	Case-control	Multicenter	Outpatient	3484		Adjusted for PPI and antibiotics	3.5 (2.3-5.3)
Dial *et al*[134], 2008	America	Case-control	Multicenter	Outpatient	9196	79.8	Adjusted for age, sex, antibiotics, comorbidities, physician visits, hospital admissions, length of stay	1.6 (1.3-1.9)
Dubberke *et al*[99], 2007	America	Cohort	Unicenter	Inpatient	36086		Adjusted for age, admissions, antibiotics, albumin level, leukemia/lymphoma, mechanical ventilation, antimotility agents	1.6 (1.3-2.1)
Elseviers *et al*[100], 2015	Europe	Case-control	Multicenter	Inpatient	743	71.9	Adjusted for age, co-morbidity, endoscopic procedures	1.9 (1.1-3.4)
Faleck *et al*[42], 2016	America	Cohort	Unicenter	Inpatient	11230	66	Adjusted for age, sex, antibiotics, comorbidities, length of stay	0.6 (0.4-0.8)
Garzotto *et al*[43], 2015	Europe	Case-control	Multicenter	Inpatient	225		No	0.4 (0.2-0.8)
Hebbard *et al*[135], 2017	Asia	Case-control	Unicenter	Inpatient	200	59.7	Adjusted for age, chemotherapy, abdominal surgery, antibiotics	2.4 (1.0-5.7)
Hensgens *et al*[117], 2011	Europe	Case-control	Unicenter	Inpatient	169		Adjusted for age, co-morbidity, antibiotics, ICU stay	1.1 (0.5-2.5)
Howell *et al*[136], 2010	America	Cohort	Unicenter	Inpatient	101796	65.4	Adjusted for age, comorbidities, antibiotics	1.7 (1.3-2.1)
Ingle *et al*[40], 2011	Asia	Cohort	Unicenter	Mixt	99	47	Adjusted for immunosuppression	1.8 (0.4-7.4)
Ingle *et al*[118], 2013	Asia	Case-control	Unicenter	Community	150	45.3	no	2.3 (0.6-9.2)
Jayatilaka *et al*[101], 2007	America	Case-control	Unicenter	Inpatient	366		Adjusted for PPI	2.7 (1.6-4.8)

Kazakova *et al*[102], 2006	America	Case-control	Unicenter	Mixt	195		Adjusted for antibiotics, PPI, length of stay, psychosis, depression	5.0 (1.3-19.3)
Khan *et al*[39], 2012	Asia	Cohort	Unicenter	Inpatient	123		Adjusted for surgery, PPI, antibiotics, hospitalization, Underlying debilitating conditions	3.2 (1.2-8.5)
Khanafer *et al*[119], 2013	Europe	Cohort	Unicenter	Inpatient	40			2.5 (0.6-9.6)
Kuntz *et al*[2], 2011	America	Case-control	Unicenter	Mixt	3344		no	2.3 (1.5-3.3)
Kurti *et al*[3], 2015	Europe	Case-control	Multicenter	Inpatient	979	72.4	Adjusted for antibiotics, PPI, length of stay,	1.6 (1.1-2.2)
Kutty *et al*[41], 2010	America	Case-control	Multicenter	Outpatient	144	62	No	1.7 (0.7-4.0)
Lewis *et al*[103], 2016	America	Cohort	Unicenter	Inpatient	41663		No	6.4 (3.6-11.5)
Lin *et al*[137], 2013	Asia	Case-control	Multicenter	Inpatient	86	59	Age, sex, unit, antibiotics, length of stay	10.1 (1.2-87.4)
Linney *et al*[24], 2010	America	Case-control	Unicenter	Inpatient	284		Age, sex, discharge date and hospital unit, antibiotics, diabetes mellitus, IBD, cancer, enteral feeding, length of stay	2.4 (1.4-4.3)
Loo *et al*[120], 2005	America	Case-control	Multicenter	Inpatient	474		no	1.0 (0.7-1.4)
Loo *et al*[138], 2011	America	Cohort	Multicenter	Inpatient	4143	67.4	Adjusted for age, PPI, antibiotics, chemotherapy	2.6 (1.7-4.0)
Lowe *et al*[121], 2006	America	Case-control	Multicenter	Inpatient	13692	78.7	Adjusted for antibiotics, other medications, and comorbidities	0.9 (0.7-1.0)
McFarland *et al*[122], 2007	America	Case-control	Multicenter	Mixt	368		No	0.8 (0.5-1.4)
Mizui *et al*[104], 2013	Asia	Case-control	Multicenter	Inpatient	2716	71.7	No	3.2 (1.4-7.3)
Modena *et al*[105], 2005	America	Case-control	Unicenter	Inpatient	250		Adjusted for macrolides, ICU, length of stay, infections	3.3 (1.6-6.8)
Mori *et al*[123], 2015	Asia	Case-control	Unicenter	Outpatient	78	58.2	No	0.4 (0.1-2.0)
Muto *et al*[106], 2005	America	Case-control	Multicenter	Inpatient	406		Adjusted for PPI, antibiotics, diabetes mellitus, organ transplantation	2.4 (1.3-4.4)
Pakyz *et al*[107], 2014	America	Case-control	Multicenter	Inpatient	14164		No	1.4 (1.3-1.5)
Peled *et al*[108], 2007	America	Cohort	Unicenter	Inpatient	217		Adjusted for PPI, low albumin level,	3.7 (1.5-9.3)
Pepin *et al*[11], 2005	America	Cohort	Unicenter	Inpatient	5619		Adjusted for age, length of stay, antibiotics	1.0 (0.7-1.2)
Ro *et al*[139], 2016	Asia	Cohort	Unicenter	Inpatient	1005	64.8	Adjusted for age, antibiotics, comorbidities	3.3 (1.5-7.2)
Roughead *et al*[109], 2016	Asia	Cohort	Multicenter	Mixt	54957		Adjusted for antibiotics, PPI, length of stay,	2.4 (1.9-3.1)
Shah *et al*[34], 2000	Europe	Case-control	Unicenter	Inpatient	252		No	0.8 (0.4-1.5)
Southern *et al*[110], 2010	Europe	Cohort	Multicenter	Inpatient	3904	65.5	No	2.3 (1.1-4.5)
Vesteinsdottir *et al*[111], 2012	Europe	Case-control	Multicenter	Mixt	333		No	1.6 (1.0-2.6)
Yang *et al*[112], 2011	Asia	Case-control	Multicenter	Inpatient	1420	67.12	No	1.9 (1.3-2.7)
Yearsley *et al*[25], 2006	Europe	Case-control	Unicenter	Inpatient	308	79.1	Adjusted for PPI, antibiotics, female sex	1.9 (1.1-3.2)
Yip *et al*[140], 2001	America	Case-control	Unicenter	Inpatient	54		No	3.0 (0.8-11.1)

CI: Confidence interval; IBD: Inflammatory bowel disease; ICU: Intensive care unit; PPI: Proton pump inhibitor; NGT: Naso-gastric tube; OR: Odds ratio.

DISCUSSION

This systematic review and meta-analysis which includes 56 studies and 356683 subjects[2,3,11,19,22-27,34,38-43,91-112,116-123,133-140] found a significant association between PPI therapy and the risk for CDI development. Both the overall pooled estimates (OR = 1.99, CI: 1.73-2.30, $P < 0.001$) and subgroup analyses showed a significant increased risk for CDI in patients on PPI therapy compared to nonusers, despite substantial statistical heterogeneity among studies and evidence of publication bias. Thus, in line with previous meta-analyses, our results add further evidence to PPIs use as a risk factor for development of CDI [85-89].

Since 2001, when Yip *et al*[140] first suggested a possible association between PPIs use and the risk of CDI, other studies, systematic reviews, and meta-analyses have reported such an association. It should be mentioned that a decade earlier (1993), Walker *et al*[141] suggested that the H2RAs therapy was a potential risk factor for CDI. In an earlier systematic review which included 11 studies with 126999 patients, Leonard *et al*[113] reported a significant association between PPI therapy and CDI (OR = 2.05, 95%CI: 1.47-2.85) although there was significant heterogeneity among the studies ($\chi^2 = 50.9$, $P <$

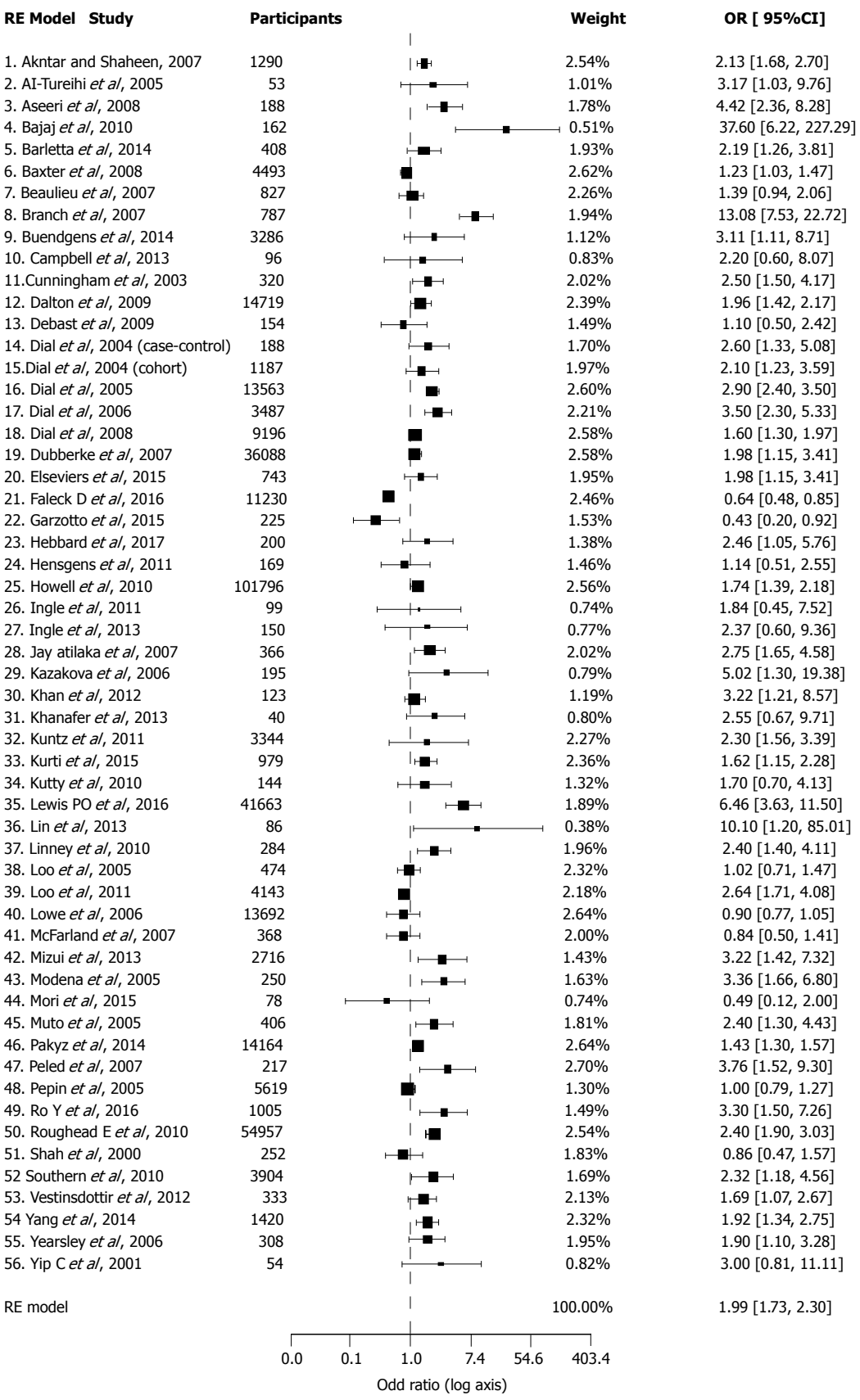


Figure 2 Forest plot of the meta-analysis.

0.0001). During the last years, six meta-analyses have been published on this topic, and all reported a positive association between PPIs use and the risk for CDI. Thus, Janarthanan *et al*[88] in a meta-analysis including

Table 2 Subgroup analysis

Subgroup analysis	No. of studies (n = 56)	ORs	95%CI	Heterogeneity, I^2, %	Heterogeneity between groups, P value
Study design					
Case-control	40	2	1.68-2.38	85.54	0.931
Cohort	16	1.98	1.51-2.59	85.99	
Study type					
Adjusted	38	1.95	1.67-2.27	85.02	0.856
Unadjusted	18	2.02	1.41-2.91	85.58	
Centers					
Unicentric	31	2.18	1.72-2.75	83.99	0.241
Multicentric	25	1.82	1.51-2.19	86.97	
Type					
Inpatient	43	1.95	1.67-2.29	84.99	0.868
Outpatient	6	2.1	1.36-3.24	84.84	
Mixt	7	2.19	1.39-3.45	76.77	
Region					
Europe	14	1.78	1.35-2.34	74.33	0.231
America	31	2	1.67-2.40	88.58	
Asia	11	2.31	1.96-2.72	89.18	
Age					
Age < 65 yr	6	2.06	1.11-3.81	35.39	0.86
Age ⩾ 65 yr	13	1.93	1.40-2.68	92.11	
NOS					
NOS ⩾ 7	26	1.88	1.55-2.28	87.65	0.441
NOS < 7	30	2.11	1.69-2.62	81.98	

CI: Confidence interval; NOS: Newcastle-Ottawa Quality Assessment Scale; ORs: Odds ratio.

23 observational studies with nearly 300000 patients found a 65% increase in the incidence of CDI among PPIs users with an estimated risk of 1.69 and 95%CI from 1.395 to 1.974. In another meta-analysis (30 studies, 202965 patients), Desphande *et al*[85] reported that PPI therapy was associated with a 2-fold increase in risk for CDI, but their study is limited by unadjusted risk estimates. Recently, the same team[90] performed a meta-analysis examining the relationship between PPI therapy and the risk for recurrent CDI, and found a positive association with the pooled risk ratio of 1.58 (95%CI: 1.13-2.21). A third meta-analysis by Kwok *et al*[87] including 42 studies (313000 participants) also found a statistically significant association between PPIs use and the risk for CDI compared with nonusers (OR = 2.51; 95%CI: 1.47-2.85; P = 0.05). Tleyjeh *et al*[86] in a systematic review and meta-analysis including 51 observational studies (37 case-control and 14 cohort) examining healthcare and community-associated CDI, found a very low quality evidence for an association between PPI therapy and CDI not supporting a cause-effect relationship. Authors reported a pooled OR of 1.65 (95%CI: 1.47-1.85) with evidence of publication bias and significant statistical heterogeneity among the studies (I^2 = 89.9%). More recently, Arriola *et al*[89] suggest, in a meta-analysis including only inpatients, that PPIs use significantly increases the risk of hospital-acquired CDI (OR = 1.81). Bavishi *et al*[114] in a systematic review regarding the use of PPI and increased susceptibility to enteric infection found 27 studies evaluating an association between PPI therapy and the risk of CDI, 17 of which reported a significant association. Based on an analysis of 28 studies, US FDA issued a warning on the risk of CDI with PPIs use[115], and similar warnings are found in CDI treatment guidelines[142].

Several studies reported that PPIs use is also a risk factor for community-acquired CDI. Dial *et al*[26], in a study including over 1000 cases of community-acquired CDI, found that patients who had received PPIs within the previous 90 d had a nearly 3-fold increased risk for CDI. A similar result was reported by Kutty *et al*[41] who found a 2-3-fold increased for community-acquired CDI in patients treated with PPIs within the previous 6 mo. Marwick *et al*[143] in a study including patients aged 65 years or older identified all cases of community-acquired CDI and found that patients prescribed PPIs within the previous 6 mo had a 1.7-fold increased risk for CDI compared to matched controls. A study assessing the epidemiology of community-acquired CDI found rates of PPI use of nearly 30% among patients with this infection compared to less than 3% in the general population[144]. These results indicate a similar degree of association between PPIs use and CDI risk, be it community-acquired CDI or hospital-acquired CDI[145].

Nevertheless, the association between PPIs use and the risk for CDI remains to a certain extent controversial despite the results reported above, as several studies failed to find such an association[11,27,34,94,122]. Beaulieu *et al*[27] found that the use of gastric acid-suppression therapy does not predispose to development of CDI, while McFarland *et al*[122] reported no relation between CDI and the use of PPIs. Branch *et al*[94] found that

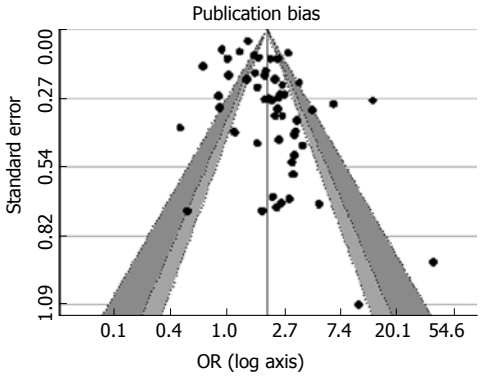


Figure 3 Funnel plot with 95% confidence limits.

PPI use did not increase the incidence of CDI in hospitalized patients.

The mechanism by which PPI therapy contributes to an increased risk of CDI is unclear. It has been proposed that a vegetative form of *C. difficile* survives in conditions of gastric pH greater than 4[114]-the threshold for enteric infections acquisition, including *C. difficile*. Howell *et al*[136] reported that the risk of nosocomial CDI rose with increasing levels of acid suppression. Hegarty *et al*[146] reported that PPI therapy decreased the expression of genes holding an important role in colonocyte integrity, thus favoring the development of CDI. Other studies show that long-term use of PPIs decreases microbial diversity, a condition found in patients with CDI[147].

As we have already mentioned, our subgroup analyses also showed an increased risk for CDI. There were no significant differences of effects between cohort and case-control studies, adjusted and unadjusted data, single-center and multicenter studies, hospitalized-and community-acquired CDI or among geographic regions. Advanced age is a well-known risk factor for CDI. To our surprise, we found no increased risk of CDI in elderly patients (≥ 65 years) using PPIs compared with youngers (OR = 1.93 *vs* OR = 2.06, P = 0.860). A possible explanation is that many of such patients may have atrophic gastritis with low gastric acid output[148] and PPIs use cannot further lower gastric acid secretion, without any additional risk of CDI[32].

As data regarding the association between PPI therapy and risk of CDI are supported only by observational studies, a final estimation of the real risk is not possible. It should be mentioned that randomized placebo-controlled clinical trials evaluating the association of PPIs use and the risk for CDI are ethically unfeasible and therefore, such studies could not be performed in the future. Thus, a weak association between PPI therapy and CDI does not confirm causality and could be the result of bias and uncontrolled confounding (*e.g.*, comorbidities, comedication use, *etc.*) which were lacking in most studies.

Our meta-analysis has some strengths such as the largest number of studies published to date, adjusted effect estimates concerning the association between PPI use and the risk of CDI, and subgroup analyses based on age, region, type, design and quality of the study. However, it also has several limitations: the included studies were observational, influenced by confounding variables despite statistical adjustment, the significant heterogeneity among most of them and lack of information regarding the dose and duration of PPI use as well as patient compliance to PPI therapy.

Although the above presented data from several meta-analyses and many studies demonstrated an association between PPI therapy and the risk for development of CDI, PPIs continue to be overused even in patients who are at high risk of CDI, because they are still considered "safe" drugs by most physicians. There is evidence that over half of PPI users who developed CDI had no valid indications for such therapy[25]. While in many countries PPIs are now totally available as over-the-counter medication, clinicians should inform their patients about the risk of CDI when PPIs are used on the long-term and without valid indication.

In spite of the aforementioned limitations of our and several other meta-analyses, clinicians should be aware of the risk of CDI when prescribing long-term PPI therapy, particularly in patients at high risk (*e.g.*, hospitalized patients on antibiotics). It should be underlined that PPIs remain, on the whole, a safe group of drugs[149], providing enormous benefits when prescribed for well-established indications. Unfortunately, many prescriptions fall outside accepted indications[90].

In conclusion, this systematic review and meta-analysis provides further evidence that PPI use significantly increases the risk for developing CDI, despite the substantial heterogeneity and publication bias present among studies. Due to the fact that all the studies included in our analysis are observational and cannot confirm causality, further large, high quality, prospective studies are needed to assess the association between PPI use and the risk of CDI.

COMMENTS

Background

Proton pump inhibitors (PPIs) are among the most widely prescribed agents by gastroenterologists because of their high efficacy and excellent safety profile. However, more recently, concerns have been raised about association between PPI therapy and several potentially serious adverse events including *Clostridium difficile* (*C. difficile*) infection (CDI). This systematic review and meta-analysis explored the existing evidence regarding the association of PPI therapy and CDI.

Research frontiers

Many observational studies and meta-analyses have reported conflicting results regarding the association between PPI therapy and the risk for CDI.

Innovations and breakthroughs

This systematic review and meta-analysis, including the largest number

of studies published to date, provides further evidence that PPI therapy is associated with an increased risk for development of CDI. Because all the studies analyzed were observational, with inherent limitations, the causality could not be confirmed.

Applications

Although our systematic review and meta-analysis, in line with previous studies and meta-analyses, reported an association between PPI therapy and the risk for development of CDI, such association remains controversial and a final estimation of the real risk has not been established. Further high-quality, prospective studies are needed to assess whether this association is causal. Until then, clinicians should be aware that long-term PPI therapy may be associated with the risk of CDI, and prescribe the PPIs in the lowest effective dose only to patients with a clear indication.

Terminology

PPIs are a group of potent inhibitors of gastric acid secretion. CDI is a symptomatic infection due to the spore-forming bacterium *C. difficile*.

Peer-review

This manuscript is an interesting, informative and well-presented meta-analysis on PPI therapy and risk of *C. difficile* infection.

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META-ANALYSIS

Outcomes after liver transplantation in accordance with ABO compatibility: A systematic review and meta-analysis

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Abstract

AIM

To evaluate the differences in outcomes between ABO-incompatible (ABO-I) liver transplantation (LT) and ABO-compatible (ABO-C) LT.

METHODS

A systematic review and meta-analysis were performed by searching eligible articles published before November 28, 2016 on MEDLINE (PubMed), EMBASE, and Cochrane databases. The primary endpoints were graft survival, patient survival, and ABO-I-related complications.

RESULTS

Twenty-one retrospective observational studies with a total of 8247 patients were included in this meta-analysis. Pooled results of patient survival for ABO-I LT were comparable to those for ABO-C LT. However, ABO-I LT showed a poorer graft survival than ABO-C LT (1-year: OR = 0.66, 95%CI: 0.57-0.76, $P < 0.001$; 3-year: OR = 0.74, 95% CI 0.64-0.85, $P < 0.001$; 5-yearr: OR =0.75, 95%CI: 0.66-0.86, $P < 0.001$). Furthermore, ABO-I LT was associated with more incidences of antibody-mediated rejection (OR = 74.21, 95%CI: 16.32- 337.45, $P < 0.001$), chronic rejection (OR =2.28, 95%CI: 1.00-5.22, $P = 0.05$), cytomegalovirus infection (OR = 2.64, 95%CI: 1.63-4.29, $P < 0.001$), overall biliary complication (OR = 1.52, 95%CI: 1.01-2.28, $P = 0.04$), and hepatic artery complication (OR = 4.17, 95%CI: 2.26-7.67, $P < 0.001$) than ABO-C LT. In subgroup analyses, ABO-I LT and ABO-C LT showed a comparable graft survival in pediatric patients and those using rituximab, and ABO-I LT showed an

increased acute cellular rejection in cases involving deceased donor grafts.

CONCLUSION

Although patient survival in ABO-I LT was comparable to that in ABO-C LT, ABO-I LT was inferior to ABO-C LT in graft survival and several complications. Graft survival of ABO-I LT could be comparable to that of ABO-C LT in pediatric patients and those using rituximab.

Key words: ABO-incompatibility; Liver transplantation; Graft survival; Patient survival; Complications

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Core tip: This meta-analysis analyzed more than 8000 cases of ABO-incompatible (ABO-I) and ABO-compatible (ABO-C) liver transplantation (LT). Although patient survival was similar, ABO-I LT was inferior to ABO-C LT in graft survival and several ABO-I-related complications. Graft survival of ABO-I LT was comparable to that of ABO-C LT in pediatric patients and those using rituximab.

Lee EC, Kim SH, Park SJ. Outcomes after liver transplantation in accordance with ABO compatibility: A systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(35): 6516-6533 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6516.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6516

INTRODUCTION

In an animal experiment in 1969, Starzl *et al*[1] reported that the liver is a privileged organ that could be transplanted with relatively lower prevalence of acute rejection than those associated with the kidney or heart. Furthermore, in 1979, Starzl *et al*[2] reported 11 cases of successful ABO-incompatible (ABO-I) liver transplantation (LT) without graft rejections[2]. Since then, however, there has been a series of reports of heightened prevalence of antibody-mediated rejection (AMR), lower graft survival, hepatic artery thrombosis (HAT), and cholangitis in ABO-I LT compared to ABO-compatible (ABO-C) LT[3-6]. Nevertheless, the application of various desensitization strategies, such as plasma exchange (PE) (or plasmapheresis), splenectomy, graft local infusion (GLI), mycophenolate mofetil (MMF), rituximab, and intravenous immunoglobulin (IVIG) to ABO-I LT highlight the potential of ABO-I LT as a promising alternative to ABO-C LT, and the introduction of rituximab has brought about substantial improvements in the outcomes of ABO-I LT[7-12]. Currently, its importance is expanding in the East, where the proportion of uses of ABO-I allografts for living donor liver transplantation (LDLT) is higher than that in the West, and particularly in Korea and Japan-two countries that show notably higher proportions of interfamilial organ donation.

However, there are still heated debates with regard to the prevalence of graft survival, patient survival, ABO-I-related complications, such as rejection, infection, biliary stricture, and HAT associated with ABO-I LT and ABO-C LT, with much heterogeneity in different reports. Therefore, considering the fact that cases of ABO-I LT would inevitably rise due to demands for donor organs far outnumbering the supply and increased difficulty of matching appropriate ABO-C liver allografts, a comprehensive analysis of the results from previous reports of LT across the ABO blood group barrier is needed.

MATERIALS AND METHODS

Study selection

Systematic review and meta-analysis were performed in compliance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines[13]. Databases of Medline (Pubmed), EMBASE, and Cochrane library were used to search for relevant articles among publications dated November 28, 2016. Publication year and language were not specified or limited for the search. The following keywords for the database search were used: (ABO OR = blood group OR = blood type) AND (incompatibility OR = mismatch OR = barrier) AND liver transplantation. Title and abstracts of identified articles were screened independently by two investigators (Lee EC , Shim JR), and full-text articles with potential relevance were obtained.

Eligibility criteria

The included studies were articles that compared ABO-I LT and ABO-C LT with a minimum of one outcome of interest. The following types of articles were excluded: abstracts, meeting papers, case reports/series, reviews, meta-analyses, letters, editorial comments, animal studies, single-arm studies, and studies unable to extract data. When there were overlapping cohorts examined by the same institutions, data from the most recent studies were used.

Assessment of methodological quality

Because there was no randomized controlled trial study included in this review, methodological quality was assessed based on a maximum score of 9 for "selection of patients", "comparability", and "outcome of study" as per the Newcastle-Ottawa Quality Assessment Scale for Cohort Studies[14]. All of the studies included in this meta-analysis were assessed by two investigators (Lee EC, Shim JR), and disagreements were resolved by a consensus.

Data extraction

Data for the following items were extracted: first author, publication year, study periods, region, sample size, population, recipient age, donor age, urgent indication, donor type, prescription for ABO-I LT, immunosuppression, graft survival, patient survival, AMR, acute cellular rejection (ACR), CR (chronic rejection), bacterial infection, fungal infection, cytomegalovirus (CMV) infection, overall biliary complication, bile leak, biliary stricture, hepatic artery (HA) complication, hepatic vein (HV) complication, and portal vein (PV) complication. For studies that divided ABO-C LT into ABO-C non-identical LT and ABO-Identical (ABO-Id) LT and reported separate outcomes for each[15-20], the outcomes were combined for the purpose of this meta-analysis. If the required data were not clearly articulated in the selected articles, we requested the original data *via* an email to the corresponding authors. All data were independently investigated and cross-checked by two investigators (Lee EC, Shim JR), and another investigator (Kim SH) provided the final confirmation.

Statistical analysis

This meta-analysis was performed in compliance with the Cochrane guidelines for systematic revie[21]. Categorical variables were analyzed with odds ratios (OR) with 95% confidence interval (CI) using the Mantel-Haenszel method, and continuous variables were analyzed with weighted mean differences (WMD) with 95%CI using an inverse variance method. Heterogeneity among studies was assessed with Higgin's I^2 index[22] or Cochran's Q test[21,23]. The random-effects model was used when I^2 was > 50% or P-value (Cochran's Q test) was < 0.10, and the fixed-effects model was used in all other cases. Heterogeneous results were further examined with a sensitivity analysis using the leave-one-out method and subgroup analyses. Possible publication bias was assessed using funnel plots and Egger's regression test, which evaluates a funnel plot asymmetry[24,25]. If a publication bias with P-value < 0.10 (Egger' s regression test) was detected, the impact on the outcomes of the meta-analysis was assessed after enhancing the symmetry using the trim-and-fill method of Duvall and Tweedie[26]. The presence of publication bias in fewer than 10 studies was considered unreliable as per the Cochrane Handbook for Systematic Reviews[27]. A P-value of < 0.05 was considered statistically significant. Statistical analyses were performed using the Review Manager (RevMan) software version 5.3 (http://tech.cochrane.org/revman) and R package "meta" (https://cran.r-project.org/web/packages/meta).

RESULTS

Search results

A total of 986 citations were found in the primary search using combinations of keywords for database search, of which 762 irrelevant or duplicated citations were excluded. After retrieving the titles and abstracts from the remaining 224 articles, another 186 articles were excluded. From the 38 potentially relevant studies, additional studies were excluded for the following reasons: single-arm studies (n = 9), overlapping cohorts from the same institutions (n = 4), studies investigating multiple desensitization protocols (n = 1), timing of rituximab administration (n = 1), and pathology (n = 1), and unable data extraction (n = 1). As a result, 21 studies were included in this meta-analysis[5,15-20,28-41]. The flow diagram of study selection is shown in Figure 1.

Characteristics of included studies

This meta-analysis included 21 retrospective observational studies that were conducted on a total of 8247 patients[5,15-20,28-41]. Of these patients, 1494 underwent ABO-I LT, while 6753 underwent ABO-C LT. There has been no randomized clinical trial conducted on this topic. Study periods ranged from 1984-2014. For studies involving less than 10% heterogeneity in study population (adult *vs* pediatric), donor type (deceased *vs* living), urgent indication [*i.e.,* fulminant hepatic failure (FHF), acute liver failure (ALF), re-transplantation, and critically ill patients in the intensive care unit], and use of rituximab for ABO-I LT, these parameters were classified according to the majority. One registry study was included in this meta-analysis[35]. Characteristics of the included studies are summarized in Table 1.

Methodological quality assessment

All studies included in this meta-analysis showed a Newcastle-Ottawa Scale (NOS) score ⩾ 6. Four case or propensity score matched studies were included[29,30,35,41]. Quality assessments of included cohort studies are presented in Table 2.

Graft and patient survival

Graft survival and patient survival were reported by 16 and 15 studies, respectively. ABO-I LT showed poorer outcomes than those of ABO-C LT in the pooled results of graft survival (1-year: OR = 0.66, 95%CI: 0.57-0.76, P < 0.001; 3-year: OR = 0.74, 95%CI: 0.64-0.85, P < 0.001; 5-year: OR = 0.75, 95%CI: 0.66-0.86, P < 0.001; 10-year: OR = 0.80, 95%CI: 0.69-0.93, P = 0.004; Figure 2).

There were no differences in 1-, 3-, and 5-year patient survival in accordance with ABO compatibility (1-year: OR = 0.88, 95%CI: 0.67-1.16, P = 0.38; 3-year: OR = 0.89, 95%CI: 0.64-1.23, P = 0.47; 5-year: OR = 0.89, 95%CI: 0.66-1.20, P = 0.45; Figure 3). However, there was a significant difference in the 10-year patient survival between ABO-I and ABO-C groups (10-year: OR = 0.46, 95%CI: 0.28-0.78, P = 0.004; Figure 3). There was no significant

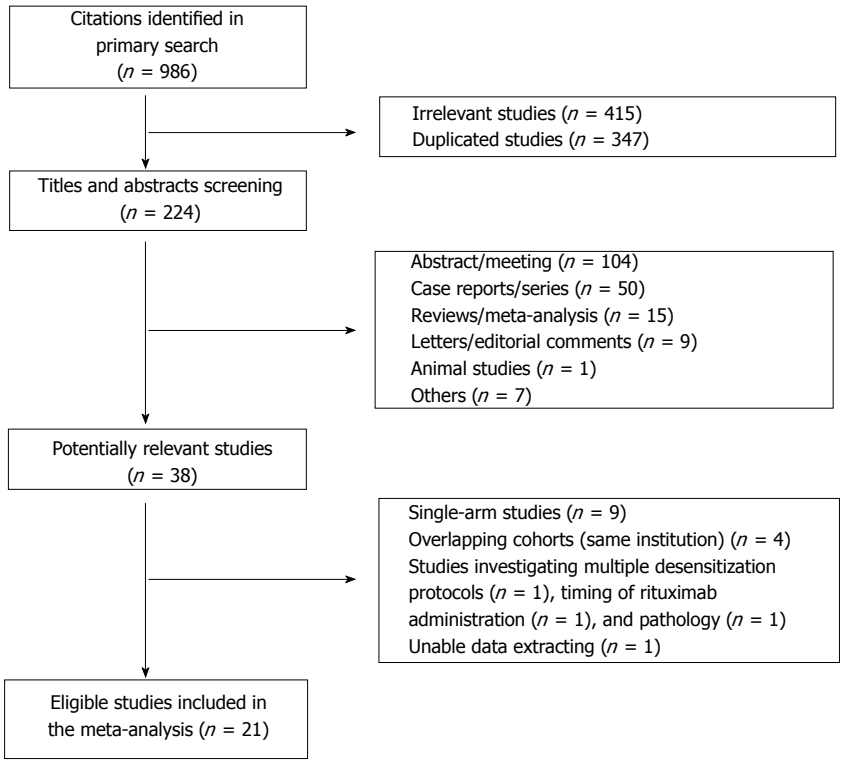


Figure 1 Flow diagram showing the selection of articles for meta-analysis.

heterogeneity in graft and patient survival.

Complications

Rejection: AMR, ACR, and CR were reported by 4, 14, and 4 studies, respectively. Pooled results showed that the risks for AMR (OR = 74.21, 95%CI 16.32-337.45, $P < 0.001$; Figure 4A) and CR (OR = 2.28, 95%CI: 1.00-5.22, $P = 0.05$; Figure 4C) were significantly higher in ABO-I LT than in ABO-C LT, but there was no statistically significant difference in the risk for ACR (OR = 1.23, 95%CI: 0.93-1.62, $P = 0.15$; Figure 4B). There was no significant heterogeneity in the results for rejection.

Infection: Bacterial and fungal infections were each reported by 4 studies, while CMV infection was reported by 5 studies. Although there were no differences in bacterial infection (OR = 0.72, 95%CI: 0.46-1.14, $P = 0.16$; Figure 4D) and fungal infection (OR = 0.66, 95%CI: 0.37-1.18, $P = 0.16$; Figure 4E) in accordance with ABO-compatibility, CMV infection was more prevalent in ABO-I LT than in ABO-C LT (OR = 2.64, 95%CI: 1.63-4.29, $P < 0.001$; Figure 4F). There was no significant heterogeneity in the results for infection.

Biliary: There were no statistically significant differences in overall biliary complication (OR = 1.75, 95%CI: 0.89-3.43, $P = 0.10$; Figure 5A), bile leak (OR = 1.85, 95%CI: 0.46-7.39, $P = 0.39$; Figure 5B), and biliary stricture (OR = 1.37, 95%CI: 0.70-2.70, $P = 0.36$; Figure 5C) in accordance with ABO compatibility. However, there were high heterogeneities in overall biliary complication ($\chi^2 = 15.22$, degree of freedom [d.f.] = 7, $P = 0.03$; $I^2 = 54\%$), bile leak ($\chi^2 = 4.25$, d.f. = 2, $P = 0.12$; $I^2 = 53\%$), and biliary stricture ($\chi^2 = 9.36$, d.f. = 5, $P = 0.10$; $I^2 = 47\%$).

Vascular: HA complication (OR = 4.17, 95%CI: 2.26-7.67, $P < 0.001$; Figure 5D) was significantly more prevalent in ABO-I LT than in ABO-C LT. However, there were no significant differences between ABO-I LT and ABO-C LT in HV complication (OR = 1.60, 95%CI: 0.64-4.00, $P = 0.32$; Figure 5E) and PV complication (OR = 1.83, 95%CI: 0.70-4.76, $P = 0.22$; Figure 5F). There was no severe heterogeneity in vascular complications.

Sensitivity and subgroup analyses

In this meta-analysis, there were high heterogeneities in overall biliary complication, bile leak, and biliary stricture. We performed a sensitivity analysis on these variables using the leave-one-out method and found that omitting the study of Sanchez *et al*[41] with a wide range of CI (overall biliary complication: OR = 85.00, 95%CI: 7.97-906.81; Bile leak: OR = 10.82, 95%CI: 1.17-100.44) eliminated the heterogeneity in overall biliary complication and bile leak. Particularly, post-sensitive analysis results showed that ABO-I LT was associated with higher prevalence of overall biliary complications than ABO-C LT (OR = 1.52, 95%CI: 1.01-2.28, $P = 0.04$; Table 3).

Table 1 Characteristics of the included studies

Ref.	Publication (yr)	Study periods (yr)	Region	Arms	Sample size	Population[3]	Recipient age	Donor age	Urgent Indications[3,4]	Donor type[3]	Prescription for ABO-I LT	Immunosuppression
Song *et al*[28]	2016	2008-2013	South Korea	ABO-I	235	Adult	52.8 ± 8.0	29.2 ± 9.1	No	Living	Rituximab, PE, GLI (±), Splenectomy (±), Cyclophosphamide	Steroids, Basiliximab,Tac, MMF
				ABO-C	1301		51.7 ± 5.9	28.2 ± 7.6				
Kim *et al*[29]	2016	2010-2013	South Korea	ABO-I	472	Adult	50 (22-65)	32 (18-68)	No	Living	Rituximab, PE, GLI (±)	Steroids, Basiliximab, Tac, MMF
				ABO-C	942		51 (20-68)	30 (18-62)				
Kim *et al*[30]	2016	2011-2014	South Korea	ABO-I	252	Adult	51.3 ± 6.7	30.1 ± 11.2	No	Living	Rituximab, PE, IVIG, Preoperative MMF	Steroids, Basiliximab, Tac, MMF
				ABO-C	752		51.1 ± 6.7	28.8 ± 11.3				
Ikegami *et al*[31]	2016	1997-2013	Japan	ABO-I	19	Adult	47.7 ± 15.7	36.6 ± 11.3	No	Living	Rituximab3, IVIG (±), PE, GLI (±), Splenectomy (±), Preoperative MMF (±)	Steroids, Tac (or CsA), MMF
				ABO-C	389		51.7 ± 11.9	37.4 ± 10.5				
Lee *et al*[32]	2015	2006-2013	Taiwan	ABO-I	46	Adult	53.5 (19-67)	NA	No	Living	Rituximab, Plasmapheresis (or PE)	Steroids, Tac, MMF
				ABO-C	340		54.7 (18-70)	NA				
Shen *et al*[33]	2014	2010-2013	China	ABO-I	35	Adult	46.7 ± 12.1	NA	Yes	Deceased	Rituximab, IVIG	Steroids, Basiliximab, Tac, MMF
				ABO-C	66		42.6 ± 10.2	NA				
Heffron *et al*[34]	2010	1998-2008	United States	ABO-I	12	Pediatric	NA	NA	Yes	Deceased	-	Steroids, Daclizumab, Tac, MMF
				ABO-C	21		NA	NA				
Stewart *et al*[35]	2009	1990-2006	United States	ABO-I	1302	Infant	0.3	8.1	No	Deceased	NA	NA
				ABO-C	3902		0.4	8.3				
				ABO-I	1162	Pediatric	9.6	23.9				
				ABO-C	3482		9	16.5				
				ABO-I	5852	Adult	45.7	36				
				ABO-C	17552		50.3	37.9				
Iwamoto *et al*[36]	2008	2000-2007	Japan	ABO-I	15	Adult	NA	NA	No	Living	NA	NA
				ABO-C	37		NA	NA				
Toso *et al*[20]	2007	1991-2005	Canada	ABO-I	14	Adult	42 (17-61)	NA	Yes	Deceased	Lymphocyte-depleting antibodies5, Plasmapheresis (±)	Steroids, Daclizumab, CsA (or Tac), AZA (or MMF, Sirolimus)
				ABO-C1	29		47 (16-62)	NA				
				ABO-Id	65		47 (17-66)	NA				
Saito *et al*[37]	2007	2000-2001	Japan	ABO-I	10	All ages	NA	NA	No	Deceased, Living	NA	NA
				ABO-C	81		NA	NA				
Koukoutsis *et al*[19]	2007	1984-2005	United Kingdom	ABO-I	4	Adult	NA	NA	Yes	Deceased	NA	NA
				ABO-C1	73		NA	NA				
				ABO-Id	203		NA	NA				
Ueda *et al*[18]	2006	1990-2003	Japan	ABO-I	74	Pediatric	NA	NA	No	Living	Steroids pulse weekly, PGE1, CsA - > AZA (1 mo after LT)	Steroids, Tac
				ABO-C1	114		NA	NA				
				ABO-Id	380		NA	NA				
Heffron *et al*[38]	2006	1999-2005	United States	ABO-I	16	Pediatric	6.5 ± 6.2	NA	No	Deceased	Plasmapheresis (±)	Steroids, Daclizumab, Tac, MMF
				ABO-C	122		8.1 ± 6.2	NA				
Bjøro *et al*[17]	2003	1990-2001	Nordic countries	ABO-I	10	All ages	NA	44.8 (22-55)	Yes	Deceased	NA	NA
				ABO-C†	76		NA	42.3 (12-85)				
				ABO-Id	143		NA	41.0 (2-75)				
Chui *et al*[39]	1997	1986-1996	Australia	ABO-I	7	All ages	13 (6-32)	NA	Yes	Deceased	Plasmapheresis (±), Splenectomy (±)	Steroids, CsA, AZA
				ABO-C	36		NA	NA				
Cacciarelli *et al*[16]	1995	1988-1993	United States	ABO-I	14	Pediatric	2.2 ± 1.1	NA	No	Deceased	OKT3 (or ATG, CsA)	Steroids, ATG (or OKT3, CsA), Tac
				ABO-C†	22		4.2 ± 1.0	NA				
				ABO-Id	108		3.7 ± 0.5	NA				

Lo *et al*[40]	1994	1988-1993	United States	ABO-I	29	All ages	NA	NA	Yes	Deceased	ATG (±)	Steroids, CsA (or OKT3), AZA
				ABO-C	196		NA	NA				
Sanchez *et al*[41]	1993	1985-1991	United States	ABO-I	182	Adult	45 (16-61)	NA	No	Deceased	NA	NA
				ABO-C	182		47 (17-59)	NA				
Reding *et al*[15]	1992	1984-1989	Belgium	ABO-I	16	All ages	NA	NA	Yes	Deceased	OKT3 (±)	Steroids, CsA, AZA(±)
				ABO-C†	16		NA	NA				
				ABO-Id	38		NA	NA				
Gugenheim *et al*[5]	1990	1984-1988	France	ABO-I	17	All ages	30 (12-49)	NA	Yes	Deceased	-	Steroids, CsA, AZA
				ABO-C	217		NA	NA				

[1]Compatible, but not identical; [2]Propensity or case matched patients; [3]If there are minority groups that make up less than about 10%, the article is categorized as covering the majority; [4]Such as FHF, ALF, retransplantation, and critically ill patients in the intensive care unit; [5]Documented as "lymphocyte-depleting antibodies", but not clarified exactly. ABO-C: ABO-compatible; ABO-I: ABO-incompatible; ABO-Id: ABO-identical; ATG: anti-thymocyte globulin; AZA: Azathioprine; CsA: Cyclosporin A; GLI: Graft local infusion; IVIG: Intravenous immunoglobulin; LT: Liver transplantation; MMF: Mycophenolate mofetil; NA: Not applicable; OKT3: Muromonab-CD3; PE: Plasma exchange; Tac: Tacrolimus.

Although there were no significant heterogeneities in most comparisons, such as in graft survival, patient survival, and complications, we attempted to minimize potential heterogeneities and detail the subgroup-specific differences through subgroup analyses. Possible confounding factors-the parameters that were speculated to impact the outcomes of this meta-analysis-included study population (adult *vs* pediatric), use of rituximab for ABO-I LT, urgent indication, and donor type (deceased *vs* living). There were no significant differences in most subgroup comparisons.

However, studies that involved pedia-tric patients[16,18,34,35,38] showed better 1-year (OR = 0.88, 95%CI: 0.68-1.15 *vs* OR = 0.59, 95%CI: 0.50-0.69; *P* = 0.01) and 3-year graft survivals (OR = 0.95, 95%CI: 0.71-1.26 *vs* OR = 0.67, 95%CI: 0.57-0.80; *P* = 0.04) after ABO-I LT than those involving adult patients. Furthermore, in such studies, there were no significant differences between ABO-I LT and ABO-C LT in 1-, 3-, 5-, and 10-year graft survivals (1-year: OR=0.88, 95%CI: 0.68-1.15, *P* = 0.35; 3-year: OR = 0.95, 95%CI: 0.71-1.26, *P* = 0.71; 5-year: OR = 0.82, 95%CI: 0.63-1.07, *P* = 0.14; 10-year: OR = 0.71, 95%CI: 0.41-1.23, *P* = 0.22).

Meanwhile, using rituximab in ABO-I LT patients[28-33] showed better 1-year graft survival (OR = 0.88, 95%CI: 0.58-1.33 *vs* OR = 0.44, 95%CI: 0.30-0.66; *P* = 0.02) after ABO-I LT compared to cases not using rituximab[15,16,18, 34,38-40, 42]. Moreover, in such cases, 1-, 3-, and 5-year graft survival of ABO-I LT were not significantly different from those of ABO-C LT (1-year: OR = 0.88, 95%CI: 0.58-1.33, *P* = 0.55; 3-year: OR = 0.81, 95%CI: 0.56-1.18, *P* = 0.28; 5-year: OR = 0.96, 95%CI: 0.66-1.39, *P* = 0.83). On the other hand, when rituximab was not used, incidence of biliary stricture (OR = 1.00, 95%CI: 0.46-2.15 *vs* OR=4.32, 95%CI: 1.18- 15.81; *P* = 0.06) and ACR (OR = 0.86, 95%CI: 0.57-1.30 *vs* OR = 1.61, 95%CI: 1.01-2.58; *P* = 0.048) tended to be higher in ABO-I LT than in ABO-C LT. However, there was no difference in AMR incidence (OR=48.32, 95%CI: 2.31-1011.61 *vs* OR = 245.87, 95%CI: 13.04-4636.62; *P* = 0.45) and patient survival (1-year: OR = 0.88, 95% CI 0.59-1.31 *vs* OR = 0.67, 95%CI: 0.44-1.04, *P* = 0.38; 3-year: OR = 0.90, 95%CI: 0.64-1.27 *vs* OR = 0.80, 95%CI: 0.31-2.06, *P* = 0.82; 5-year: OR = 0.90, 95%CI: 0.61-1.31 *vs* OR = 1.00, 95%CI: 0.20-5.08, *P* = 0.89) in accordance with the use of rituximab.

Studies that involved urgent indica-tions[15,17,19,20,33,34,39,40,42] had worse 1-year graft survival (OR = 0.37, 95%CI: 0.23-0.59 *vs* OR=0.70, 95%CI: 0.61-0.81; *P* = 0.01) but better 5-year patient survival (OR = 2.38, 95%CI: 0.86-6.63 *vs* OR = 0.79, 95%CI: 0.57-1.08, *P* = 0.043) for ABO-I LT than those for ABO-CT when compared to studies without urgent indications.

In addition, compared to cases that involved the use of deceased donor liver allografts, those that involved the use of living donor liver allografts[18,28-32,36] showed lower prevalence of ACR (OR = 0.87, 95%CI: 0.58-1.32 *vs* OR = 1.69, 95%CI: 1.14-2.50; *P* = 0.02) and biliary stricture (OR = 1.00, 95%CI: 0.46-2.15 *vs* OR = 4.49, 95%CI: 1.36-14.87; *P* = 0.04) in ABO-I LT. Results of sensitivity analysis and subgroup analysis are summarized in Table 3.

Publication bias

In this meta-analysis, 1-year patient survival was found to have a potential publication bias with a funnel plot asymmetry (Figure 6A) and a *P* = 0.06 calculated by the Egger's regression test. Other variables did not show a significant publication bias. After adjusting for funnel plot asymmetry using the trim-and-fill method[26] (Figure 6B), ABO-I LT showed a significantly poorer 1-year patient survival than ABO-C LT (OR=0.73, 95%CI: 0.56-0.95, *P* = 0.02; I^2 = 25.8%).

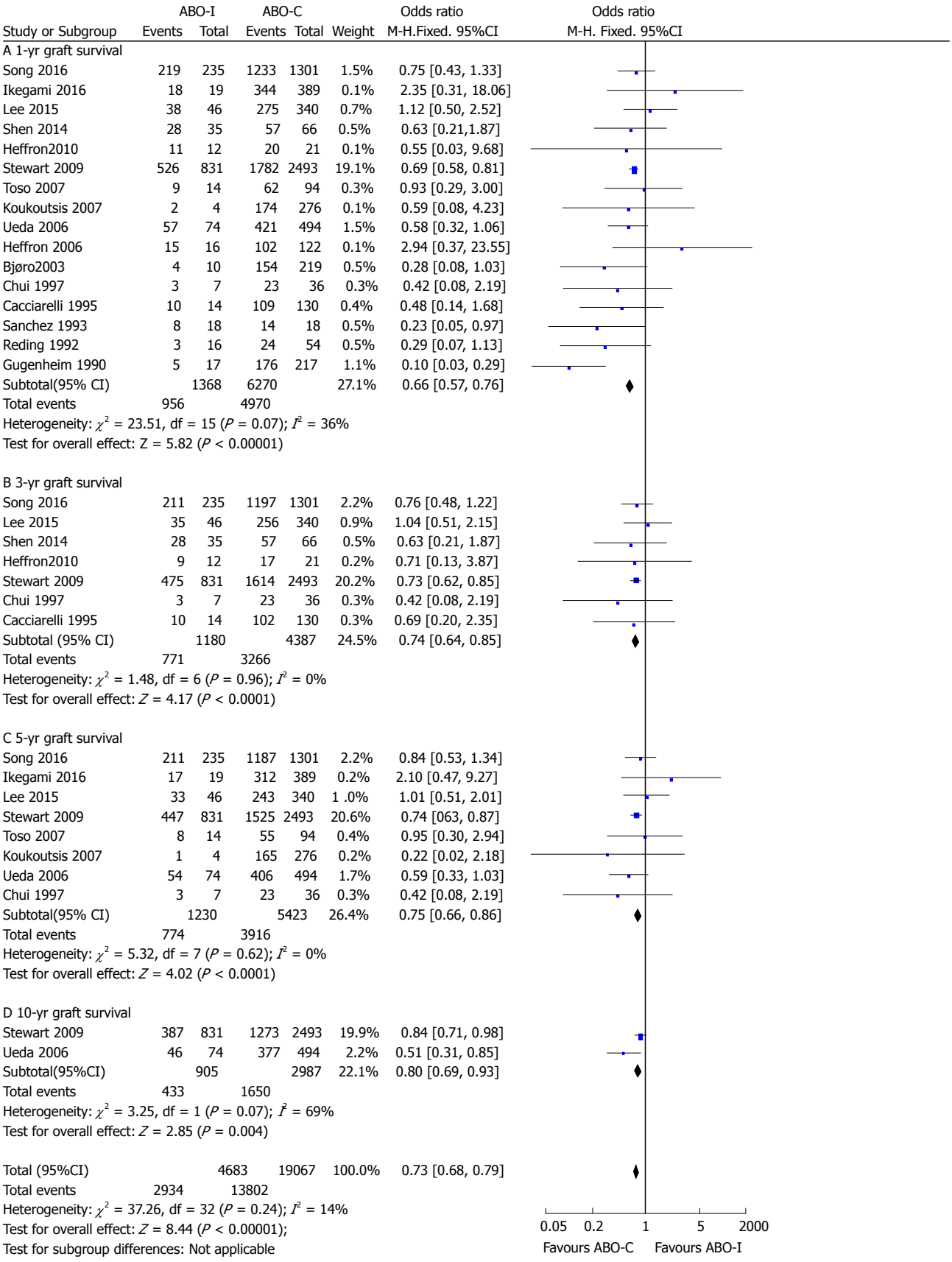


Figure 2 Comparison of graft survival between ABO-incompatible and ABO-compatible liver transplantation. ABO-C: ABO-compatible; ABO-I: ABO-incompatible; LT: Liver transplantation.

Table 2 Modified Newcastle-Ottawa quality assessment scale for cohort studies included in the meta-analysis

Ref.	Selection				Comparability[1]	Outcome			Overall
	Representativeness	Selection	Ascertainment	Incident disease		Assessment	Length of follow-up	Adequacy of follow-up	Quality Score (Maximum 9)
Song *et al*[28]	↓	↓	↓	↑	↑	↓	↓	↓	6
Kim *et al*[29]	↓	↓	↓	↑	↓↓	↓	↓	↓	8
Kim *et al*[30]	↓	↓	↓	↑	↓↓	↓	↓	↓	8
Ikegami *et al*[31]	↓	↓	↓	↑	↑	↓	↓	↓	6
Lee *et al*[32]	↓	↓	↓	↑	↑	↓	↓	↓	6
Shen *et al*[33]	↓	↓	↓	↑	↑	↓	↓	↓	6
Heffron *et al*[34]	↓	↓	↓	↑	↑	↓	↓	↓	6
Stewart *et al*[35]	↓	↓	↓	↑	↓↓	↓	↓	↓	8
Iwamoto *et al*[36]	↓	↓	↓	↑	↑	↓	↓	↓	6
Toso *et al*[20]	↓	↓	↓	↑	↑	↓	↓	↓	6
Saito *et al*[37]	↓	↓	↓	↑	↑	↓	↓	↓	6
Koukoutsis *et al*[19]	↓	↓	↓	↑	↑	↓	↓	↓	6
Ueda *et al*[18]	↓	↓	↓	↑	↑	↓	↓	↓	6
Heffron *et al*[38]	↓	↓	↓	↑	↑	↓	↓	↓	6
Bjøro *et al*[17]	↓	↓	↓	↑	↑	↓	↓	↓	6
Chui *et al*[39]	↓	↓	↓	↑	↑	↓	↓	↓	6
Cacciarelli *et al*[16]	↓	↓	↓	↑	↑	↓	↓	↓	6
Lo *et al*[40]	↓	↓	↓	↑	↑	↓	↓	↓	6
Sanchez *et al*[41]	↓	↓	↓	↑	↓↓	↓	↓	↓	8
Reding *et al*[15]	↓	↓	↓	↑	↑	↓	↓	↓	6
Gugenheim *et al*[5]	↓	↓	↓	↑	↑	↓	↓	↓	6

[1]A maximum of two downward arrows (↓↓) can be given for comparability. ↓ : Consistent with criteria and low risk of bias; ↑ : Not consistent with criteria and high risk of bias.

DISCUSSION

Since the first attempt of ABO-I LT by Starzl *et al*[1,2], poor outcomes after ABO-I LT, including AMR, lower graft survival, HAT, and cholangitis, were insurmountable barriers for expanding the application of transplantation across the ABO blood group barrier, from a few urgent cases to cases of chronic liver disease and liver cancer[3-6]. Extraordinary improvements have been made in the outcomes of ABO-I LT with the introduction of multiple desensitization strategies, such as PE (or plasmapheresis), splenectomy, GLI, rituximab, MMF, and IVIG, as well as with advances of immunosuppression agents[7-12]. However, whether ABO-I LT is comparable to ABO-C LT remains a topic of debate.

This meta-analysis revealed that pooled results of graft survival were poorer in ABO-I LT than in ABO-C LT. However, patient survival did not significantly vary in accordance with ABO compatibility in most cases. The data on 10-year patient survival had low reliability, as they were only reported by one study-although produced based on a long-term follow-up[18].

Meanwhile, the cumulative meta-analysis in order by the median year of study period showed that the cumulative results of graft and patient survival remained consistent since the early 2000s (Supplementary Figures 1 and 2). This is mainly due to the stabilization of the desensitization protocol through the application of PE (or plasmapheresis)[42-44], muromonab-CD3 (OKT3)[42-44], splenectomy[9], PV infusion[10,45], HA infusion[46,47], rituximab[48-50], and IVIG[7,8,51] in ABO-I LT. However, from a different perspective, the fact that ABO-I LT patients showed a poorer graft survival than ABO-C LT patients and that cumulative meta-analysis of graft survival remained mostly unchanged since the early 2000s implies that the current desensitization protocol for ABO-I LT still requires an improvement.

With regard to ABO-I-related complications, the prevalence of AMR, CR, CMV infection, and HA complication was higher in ABO-I LT than in ABO-C LT. Overall biliary complication-after omitting a study of Sanchez *et al*[41] with a wide range of CI in the

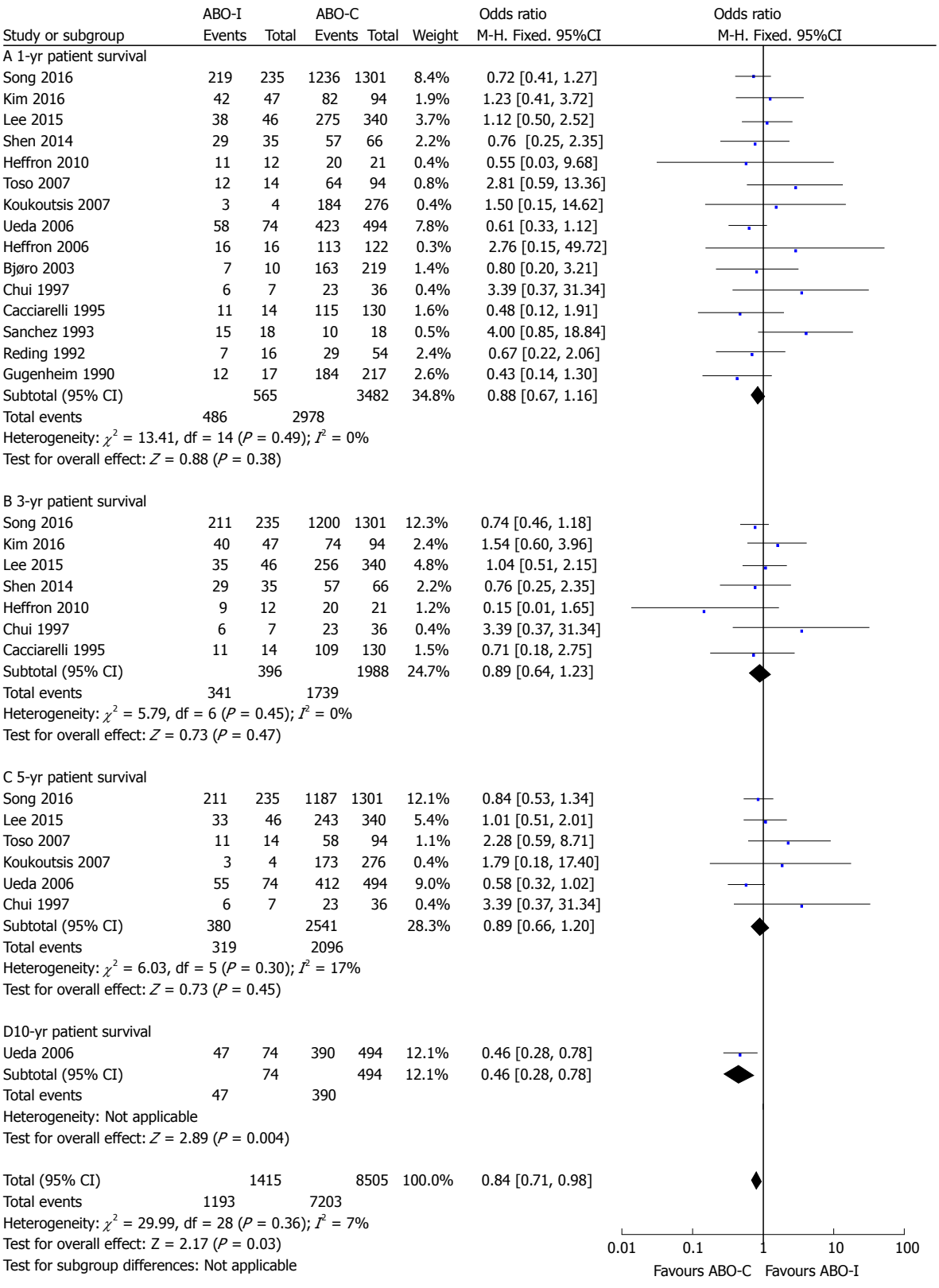


Figure 3 Comparison of patient survival between ABO-incompatible and ABO-compatible liver transplantation. ABO-C: ABO-compatible; ABO-i: ABO-incompatible; LT: Liver transplantation.

sensitivity analysis-was more prevalent in ABO-I LT than in ABO-C LT.

In the subgroup analyses, studies that only involved pediatric patients[16,18,34,35,38], compared to those that did not, showed better 1-year and 3-year graft survivals after ABO-I LT than those after ABO-C LT. Furthermore, in such cases, 1-, 3-, 5-, and 10-year graft survivals after ABO-I LT were comparable to those after ABO-C LT. There were several reports that pediatric ABO-I LT was more successful than

Table 3 Sensitivity analysis and subgroup analysis

Variables	No. of studies	OR = [95%CI]	P^1 value	I^2 (%)
Sensitivity analysis				
Overall biliary complication		1.75 [0.89-3.43]	0.10	54
Omitting Sanchez *et al*[41]	7	1.52 [1.01-2.28]	0.04	0
Omitting Cacciarelli *et al*[16]	7	1.81 [0.87-3.76]	0.11	61
Omitting Heffron *et al*[38]	7	1.86 [0.87-3.95]	0.11	60
Omitting Toso *et al*[20]	7	1.86 [0.84-4.13]	0.13	60
Omitting Iwamoto *et al*[36]	7	1.81 [0.84-3.92]	0.13	61
Omitting Shen *et al*[33]	7	1.89 [0.90-3.96]	0.09	59
Omitting Lee *et al*[32]	7	1.69 [0.72-3.94]	0.23	56
Omitting Kim *et al*[29]	7	2.07 [0.98-4.37]	0.06	50
Bile leak		1.85 [0.46-7.39]	0.39	53
Omitting Sanchez *et al*[41]	2	0.98 [0.38-2.49]	0.96	0
Omitting Kim *et al*[30]	2	2.47 [0.19-31.38]	0.49	77
Omitting Kim *et al*[29]	2	3.62 [0.53-24.77]	0.19	46
Biliary stricture		1.37 [0.70-2.70]	0.36	47
Omitting Sanchez *et al*[41]	5	1.28 [0.62-2.62]	0.51	54
Omitting Lo *et al*[40]	5	1.11 [0.54-2.26]	0.78	42
Omitting Ikegami *et al*[31]	5	1.55 [0.71-3.39]	0.27	49
Omitting Kim *et al*[30]	5	1.47 [0.74-2.95]	0.27	51
Omitting Kim *et al*[29]	5	1.72 [0.91-3.25]	0.09	28
Omitting Song *et al*[28]	5	1.20 [0.43-3.31]	0.73	49
Subgroup analysis				
1-yr graft survival		0.66 [0.57-0.76]	< 0.001	36
Rituximab for ABO-I LT (yes/no)	4 *vs* 7	0.88 [0.58-1.33] *vs* 0.44 [0.20-0.66]	0.02	0 *vs* 49
Living donor (yes/no)	4 *vs* 12	0.79 [0.56-1.13] *vs* 0.64 [0.55-0.74]	0.27	0 *vs* 44
Urgent indication (yes/no)	8 *vs* 8	0.37 [0.23-0.59] *vs* 0.70 [0.61-0.81]	0.01	27 *vs* 8
Pediatric2 (yes/no)	5 *vs* 12	0.88 [0.68-1.15] *vs* 0.59 [0.50-0.69]	0.01	12 *vs* 45
3-yr graft survival		0.74 [0.64-0.85]	< 0.001	0
Rituximab for ABO-I LT (yes/no)	3 *vs* 3	0.81 [0.56-1.18] *vs* 0.60 [0.26-1.41]	0.53	0 *vs* 0
Living donor (yes/no)	2 *vs* 5	0.84 [0.57-1.25] *vs* 0.72 [0.62-0.84]	0.47	0 *vs* 0
Urgent indication (yes/no)	3 *vs* 4	0.59 [0.27-1.31] *vs* 0.74 [0.64-0.86]	0.58	0 *vs* 0
Pediatric2 (yes/no)	3 *vs* 5	0.95 [0.71-1.26] *vs* 0.67 [0.57-0.80]	0.04	0 *vs* 0
5-yr graft survival		0.75 [0.66-0.86]	< 0.001	0
Rituximab for ABO-I LT (yes/no)	3 *vs* 2	0.96 [0.66-1.39] *vs* 0.56 [0.33-0.96]	0.11	0 *vs* 0
Living donor (yes/no)	4 *vs* 4	0.83 [0.61-1.14] *vs* 0.73 [0.63-0.86]	0.46	10 *vs* 0
Urgent indication (yes/no)	3 *vs* 5	0.60 [0.26-1.39] *vs* 0.76 [0.66-0.87]	0.6	0 *vs* 0
Pediatric 2 (yes/no)	2 *vs* 7	0.82 [0.63-1.07] *vs* 0.73 [0.62-0.86]	0.44	42 *vs* 0
10-yr graft survival		0.80 [0.69-0.93]	0.004	69
Rituximab for ABO-I LT (yes/no)	0 *vs* 1	NA *vs* 0.51 [0.31-0.85]	NA	NA
Living donor (yes/no)	1 *vs* 1	0.51 [0.31-0.85] *vs* 0.84 [0.71-0.98]	0.07	NA *vs* NA
Urgent indication (yes/no)	0 *vs* 2	NA *vs* 0.70 [0.44-1.11]	NA	NA *vs* 69
Pediatric 2 (yes/no)	2 *vs* 1	0.71 [0.41-1.23] *vs* 0.81 [0.70-0.98]	0.65	72 *vs* NA
1-yr patient survival		0.88 [0.67-1.16]	0.38	0
Rituximab for ABO-I LT (yes/no)	4 *vs* 7	0.88 [0.59-1.31] *vs* 0.67 [0.44-1.04]	0.38	0 *vs* 0
Living donor (yes/no)	4 *vs* 11	0.79 [0.56-1.13] *vs* 1.04 [0.67-1.61]	0.35	0 *vs* 9
Urgent indication (yes/no)	8 *vs* 7	0.93 [0.56-1.52] *vs* 0.87 [0.62-1.20]	0.83	0 *vs* 21
Pediatric (yes/no)	4 *vs* 11	0.64 [0.38-1.09] *vs* 0.98 [0.71-1.35]	0.18	0 *vs* 5
3-yr patient survival		0.89 [0.64-1.23]	0.47	0
Rituximab for ABO-I LT (yes/no)	4 *vs* 3	0.90 [0.64-1.27] *vs* 0.80 [0.31-2.06]	0.82	0 *vs* 43
Living donor (yes/no)	3 *vs* 4	0.91 [0.64-1.32] *vs* 0.78 [0.38-1.62]	0.71	5 *vs* 15
Urgent indication (yes/no)	3 *vs* 4	0.81 [0.35-1.91] *vs* 0.90 [0.63-1.28]	0.83	43 *vs* 0
Pediatric (yes/no)	2 *vs* 5	0.46 [0.15-1.38] *vs* 0.94 [0.67-1.32]	0.22	18 *vs* 0
5-yr patient survival		0.89 [0.66-1.20]	0.45	17
Rituximab for ABO-I LT (yes/no)	2 *vs* 2	0.89 [0.61-1.31] *vs* 1.00 [0.20-5.08]	0.89	0 *vs* 57
Living donor (yes/no)	3 *vs* 3	0.79 [0.57-1.08] *vs* 2.38 [0.86-6.63]	0.04	0 *vs* 0
Urgent indication (yes/no)	3 *vs* 3	2.38 [0.86-6.63] *vs* 0.79 [0.57-1.08]	0.04	0 *vs* 0
Pediatric (yes/no)	1 *vs* 5	0.58 [0.32-1.02] *vs* 1.04 [0.72-1.48]	0.09	NA *vs* 0
10-yr patient survival		0.46 [0.28-0.78]	0.004	7
Rituximab for ABO-I LT (yes/no)	0 *vs* 1	NA *vs* 0.46 [0.28-0.78]	NA	NA
Living donor (yes/no)	1 *vs* 0	0.46 [0.28-0.78] *vs* NA	NA	NA
Urgent indication (yes/no)	0 *vs* 1	NA *vs* 0.46 [0.28-0.78]	NA	NA
Pediatric (yes/no)	1 *vs* 0	0.46 [0.28-0.78] *vs* NA	NA	NA
ACR		1.23 [0.93-1.62]	0.15	0
Rituximab for ABO-I LT (yes/no)	5 *vs* 5	0.86 [0.57-1.30] *vs* 1.61 [1.01-2.58]	0.048	0 *vs* 0
Living donor (yes/no)	4 *vs* 9	0.87 [0.58-1.32] *vs* 1.69 [1.14-2.50]	0.02	0 *vs* 0
Urgent indication (yes/no)	6 *vs* 7	1.56 [0.96-2.53] *vs* 1.08 [0.77-1.53]	0.23	0 *vs* 22

Pediatric (yes/no)	3 *vs* 10	1.64 [0.82-3.29] *vs* 1.16 [0.85-1.57]	0.37	0 *vs* 0
CR		2.28 [1.00-5.22]	0.05	42
Rituximab for ABO-I LT (yes/no)	2 *vs* 2	6.45 [0.13-333.04] *vs* 2.64 [0.83-8.44]	0.67	80 *vs* 0
Living donor (yes/no)	2 *vs* 2	6.45 [0.13-333.04] *vs* 2.64 [0.83-8.44]	0.67	80 *vs* 0
Urgent indication (yes/no)	2 *vs* 2	2.64 [0.83-8.44] *vs* 6.45 [0.13-333.04]	0.67	0 *vs* 80
Pediatric (yes/no)	0 *vs* 4	NA *vs* 2.28 [1.00-5.22]	NA	NA *vs* 42
AMR		74.21 [16.32-337.45]	< 0.001	12
Rituximab for ABO-I LT (yes/no)	2 *vs* 1	48.32 [2.31-1011.61] *vs* 245.87 [13.04-4636.62]	0.45	53 *vs* NA
Living donor (yes/no)	1 *vs* 3	208.48 [12.49-3479.38] *vs* 35.81 [6.02-212.88]	0.3	NA *vs* 18
Urgent indication (yes/no)	3 *vs* 1	35.81 [6.02-212.88] *vs* 208.48 [12.49-3479.38]	0.3	18 *vs* NA
Pediatric (yes/no)	0 *vs* 4	NA *vs* 74.21 [16.32-337.45]	NA	NA *vs* 12
Bacterial infection		0.72 [0.46-1.14]	0.16	0
Rituximab for ABO-I LT (yes/no)	4 *vs* 0	0.72 [0.46-1.14] *vs* NA	NA	0 *vs* NA
Living donor (yes/no)	3 *vs* 1	0.70 [0.42-1.15] *vs* 0.84 [0.29-2.45]	0.75	0 *vs* NA
Urgent indication (yes/no)	1 *vs* 3	0.84 [0.29-2.45] *vs* 0.70 [0.42-1.15]	0.75	NA *vs* 0
Pediatric (yes/no)	0 *vs* 4	NA *vs* 0.72 [0.46-1.14]	NA	NA *vs* 0
Fungal infection		0.66 [0.37-1.18]	0.16	0
Rituximab for ABO-I LT (yes/no)	3 *vs* 0	0.78 [0.42-1.44] *vs* NA	NA	0 *vs* NA
Living donor (yes/no)	2 *vs* 2	0.65 [0.31-1.33] *vs* 0.63 [0.09-4.40]	0.99	0 *vs* 62
Urgent indication (yes/no)	2 *vs* 2	0.63 [0.09-4.40] *vs* 0.65 [0.31-1.33]	0.99	62 *vs* 0
Pediatric (yes/no)	0 *vs* 4	NA *vs* 0.71 [0.39-1.28]	NA	NA *vs* 0
CMV infection		2.64 [1.63-4.29]	< 0.001	43
Rituximab for ABO-I LT (yes/no)	3 *vs* 0	2.2 [1.23-3.93] *vs* NA	NA	4 *vs* NA
Living donor (yes/no)	3 *vs* 2	2.25 [1.24-4.09] *vs* 6.43 [0.17-242.88]	0.58	4 *vs* 82
Urgent indication (yes/no)	1 *vs* 4	1.45 [0.45-4.74] *vs* 2.77 [1.12-6.86]	0.4	NA *vs* 53
Pediatric (yes/no)	0 *vs* 5	NA *vs* 2.64 [1.63-4.29]	NA	NA *vs* 43
Overall Biliary complication		1.75 [0.89-3.43]	0.1	54
Rituximab for ABO-I LT (yes/no)	3 *vs* 2	1.38 [0.62-3.07] *vs* 1.25 [0.35-4.51]	0.89	51 *vs* 0
Living donor (yes/no)	3 *vs* 5	1.52 [0.74-3.10] *vs* 2.36 [0.63-8.87]	0.57	46 *vs* 66
Urgent indication (yes/no)	2 *vs* 6	1.23 [0.48-3.21] *vs* 2.08 [0.85-5.07]	0.44	0 *vs* 66
Pediatric (yes/no)	2 *vs* 6	1.25 [0.35-4.51] *vs* 1.95 [0.85-4.46]	0.57	0 *vs* 67
Bile leak		1.85 [0.46-7.39]	0.39	53
Rituximab for ABO-I LT (yes/no)	2 *vs* 0	0.96 [0.38-2.46] *vs* NA	NA	0 *vs* NA
Living donor (yes/no)	2 *vs* 1	0.98 [0.38-2.49] *vs* 10.82 [1.17-100.44]	0.051	0 *vs* NA
Urgent indication (yes/no)	0 *vs* 3	NA *vs* 1.85 [0.46-7.39]	NA	NA *vs* 53
Pediatric (yes/no)	0 *vs* 3	NA *vs* 1.85 [0.46-7.39]	NA	NA *vs* 53
Biliary stricture		1.37 [0.70-2.70]	0.36	47
Rituximab for ABO-I LT (yes/no)	4 *vs* 1	1.00 [0.46-2.15] *vs* 4.32 [1.18-15.81]	0.06	52 *vs* NA
Living donor (yes/no)	4 *vs* 2	1.00 [0.46-2.15] *vs* 4.49 [1.36-14.87]	0.04	52 *vs* 0
Urgent indication (yes/no)	1 *vs* 5	4.32 [1.18-15.81] *vs* 1.44 [1.04-1.99]	0.11	NA *vs* 42
Pediatric (yes/no)	0 *vs* 6	NA *vs* 1.52 [1.11-2.08]	NA	NA *vs* 47
HV complication		1.60 [0.64-4.00]	0.32	NA
Rituximab for ABO-I LT (yes/no)	1 *vs* 0	1.60 [0.64-4.00] *vs* NA	NA	NA
Living donor (yes/no)	1 *vs* 0	1.60 [0.64-4.00] *vs* NA	NA	NA
Urgent indication (yes/no)	0 *vs* 1	NA *vs* 1.60 [0.64-4.00]	NA	NA
Pediatric (yes/no)	0 *vs* 1	NA *vs* 1.60 [0.64-4.00]	NA	NA
PV complication		1.83 [0.70-4.76]	0.22	0
Rituximab for ABO-I LT (yes/no)	3 *vs* 1	1.19 [0.31-4.65] *vs* 2.65 [0.29-24.07]	0.55	0 *vs* NA
Living donor (yes/no)	3 *vs* 2	1.19 [0.31-4.65] *vs* 3.27 [0.82-13.07]	0.31	0 *vs* 0
Urgent indication (yes/no)	2 *vs* 3	3.27 [0.82-13.07] *vs* 1.19 [0.31-4.65]	0.31	0 *vs* 0
Pediatric (yes/no)	0 *vs* 5	NA *vs* 1.83 [0.70-4.76]	NA	NA *vs* 0
HA complication		4.17 [2.26-7.67]	< 0.001	0
Rituximab for ABO-I LT (yes/no)	3 *vs* 3	2.52 [0.68-9.27] *vs* 4.43 [0.90-21.87]	0.59	0 *vs* 53
Living donor (yes/no)	4 *vs* 5	3.62 [1.20-10.91] *vs* 4.44 [2.13-9.25]	0.76	0 *vs* 10
Urgent indication (yes/no)	3 *vs* 6	5.50 [2.33-13.00] *vs* 3.30 [1.39-7.83]	0.41	0 *vs* 0
Pediatric (yes/no)	1 *vs* 8	0.47 [0.03-8.56] *vs* 5.26 [2.73-10.14]	0.11	NA *vs* 0

[1]*P* value for overall effect or test for differences in subgroup analysis; [2]Stewart *et al*[35] reported graft survival rates of pediatric and adults, respectively. ABO-I: ABO-Incompatible; ACR: Acute cellular rejection; AMR: Antibody-mediated rejection; CMV: Cytomegalovirus; CR: Chronic rejection; HA: Hepatic artery; HV: Hepatic vein; LT: Liver transplantation; PV: Portal vein.

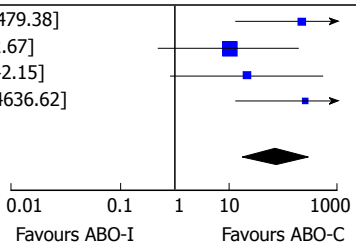
adult ABO-I LT[52,53]. Egawa *et al*[52] reported that an advanced recipient age for ABO-I LDLT is associated with poor outcomes, including graft and patient survivals, intrahepatic biliary complications, and hepatic necrosis. Maternal anti-ABO antibodies (Ab) begin to disappear from week two after birth, and neonates begin to produce their own reservoir of anti-ABO Ab from weeks 8-12 after birth, which reaches a level similar to that of adults by age 5-10[54,55]. Thus, younger pediatric patients may be immunologically immature, showing lower anti-ABO Ab levels and immature complement system[56,57], which could be a possible explanation for our result of pediatric graft survival.

A

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	17	235	0	1301	22.2%	208.48 [12.49, 3479.38]
Shen 2014	2	35	0	66	50.9%	9.93 [0.46,272.67]
Toso 2007	1	14	0	94	19.2%	21.00 [0.81, 542.15]
Gugenhenim 1990	6	17	0	217	7.6%	245.87 [13.04, 4636.62]
Total (95% CI)		301		1678	100.0%	74.21 [16.32, 337.45]
Total events	26		0			

Heterogenity χ^2 = 3.39, df = 3 (P = 0.34); I^2 = 12%

Test for overall effect: Z = 5.57 (P < 0.00001)

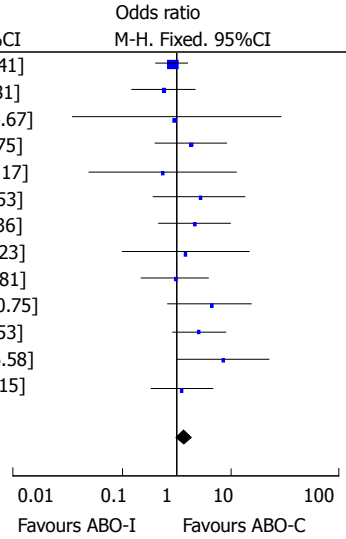


B

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	19	235	122	1301	39.4%	0.85 [0.51, 1.41]
Kim 2016	6	47	17	94	11.3%	0.66 [0.24, 1.81]
Kim 2016	0	25	1	75	0.9%	0.97 [0.04, 24.67]
Ikegami 2016	4	19	58	389	4.9%	1.52 [0.49, 4.75]
Shen 2014	1	35	3	66	2.3%	0.62 [0.06, 6.17]
Heffron 2010	6	12	7	21	2.9%	2.00 [0.47, 8.53]
Toso 2007	8	14	41	94	5.2%	1.72 [0.55, 5.36]
Koukoutsis 2007	2	4	121	276	2.0%	1.28 [0.18, 9.23]
Heffron 2006	6	16	47	122	7.8%	0.96 [0.33, 2.81]
Cacciarelli 1995	11	14	73	130	3.5%	2.86 [0.76, 10.75]
Lo 1994	20	29	104	196	9.5%	1.97 [0.85, 4.53]
Sanchez 1993	11	18	5	18	2.2%	4.09 [1.01, 16.58]
Gugenheim 1990	7	17	82	217	8.0%	1.15 [0.42, 3.15]
Total (95%CI)		485		2999	100.0%	1.23 [0.93, 1.62]
Total events	101		156			

Heterogenity χ^2 = 10.61, df = 12 (P = 0.68); I^2 = 0%

Test for overall effect: Z = 1.44 (P = 0.15)

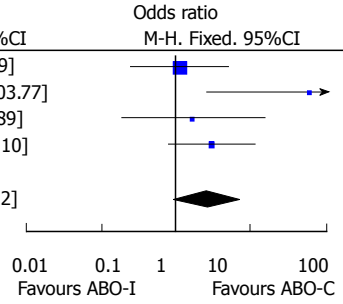


C

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	2	235	10	1301	52.8%	1.11 [0.24, 5.09]
Ikegami 2016	1	19	0	389	0.8%	63.16 [2.49, 1603.77]
Lo 1994	1	29	4	196	17.3%	1.71 [0.18, 15.89]
Gugenheim 1990	3	17	14	217	29.1%	3.11 [0.80, 12.10]
Total (95% CI)		300		2103	100.0%	2.28 [1.00, 5.22]
Total events	7		28			

Heterogenity χ^2 = 5.17, df = 3 (P = 0.16); I^2 = 42%

Test for overall effect: Z = 1.96 (P = 0.05)

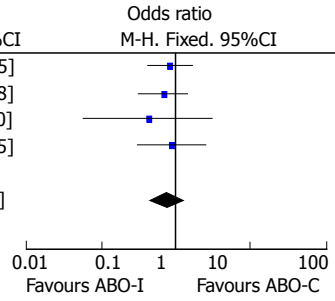


D

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	9	235	61	1301	38.7%	0.81 [0.40, 1.65]
Kim 2016	13	47	35	94	36.3%	0.64 [0.30, 1.38]
Ikegami 2016	1	19	47	389	8.9%	0.40 [0.50, 3.10]
Shen 2014	6	35	13	66	16.1%	0.84 [0.29, 2.45]
Total (95% CI)		336		1850	100.0%	0.72 [0.46, 1.14]
Total events	29		156			

Heterogenity χ^2 = 0.58, df = 3 (P = 0.90); I^2 = 0%

Test for overall effect: Z = 1.42 (P = 0.16)

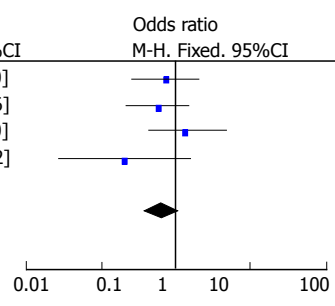


E

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	4	235	30	1301	29.6%	0.73 [0.26, 2.10]
Kim 2016	6	47	19	94	36.2%	0.58 [0.21, 1.56]
Shen 2014	5	35	7	66	13.6%	1.40 [0.41, 4.80]
Toso 2007	1	14	26	94	20.5%	0.20 [0.03, 1.62]
Total (95%CI)		331		1555	100.0%	0.66 [0.37, 1.18]
Total events	16		82			

Heterogenity χ^2 = 2.81, df = 3 (P = 0.42); I^2 = 0%

Test for overall effect: Z = 1.40 (P = 0.16)



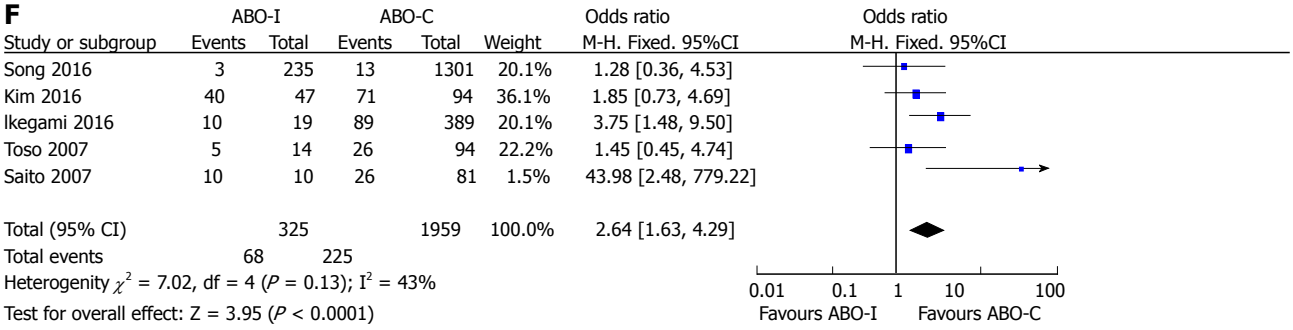


Figure 4 Comparison of rejection and infection between ABO-incompatible and ABO-compatible liver transplantation. A: AMR; B: ACR; C: CR; D: Bacterial infection; E: Fungal infection; F: CMV infection. ABO-C: ABO-Compatible; ABO-I: ABO-incompatible; ACR: Acute cellular rejection; AMR: Antibody-mediated rejection; CMV: Cytomegalovirus; CR: Chronic rejection; LT: Liver transplantation.

In this meta-analysis, cases that used rituximab in ABO-I LT patients[28-33] showed better 1-year graft survival after ABO-I LT than those that did not use rituximab[5,15,16,18,34,38-40]. Furthermore, in such cases, 1-, 3-, and 5-year graft survivals of ABO-I LT were comparable to those of ABO-C LT. On the other hand, biliary stricture and ACR tended to be more prevalent after ABO-I LT when rituximab was not used. There were no differences in AMR and patient survival in accordance with the use of rituximab.

Rituximab is a chimeric human anti-CD20 monoclonal antibody, which destroys B cells via antibody-dependent cell-mediated cytotoxicity, direct antigen-antibody reaction, and complement-dependent cytotoxicity[58,59]. Since its first introduction as a prophylactic in Japan in 2002, multiple centers have used rituximab during ABO-I LT, which is considered to have contributed to the dramatic improvements in the outcomes of ABO-I LT[9,11,60,61]. In this meta-analysis, it was noted that using rituximab improved graft survival while reducing incidences of biliary stricture and ACR after ABO-I LT. However, its effects on AMR and patient survival were rather unclear, and we speculate this to be a result of excluding some studies from the subgroup analyses for lack of clear descriptions of desensitization methods in ABO-I LT[17,19,20, 35-37,41].

Meanwhile, studies that involved urgent indications, such as FHF, ALF, re-transplantation, and critically ill patients in the intensive care unit[5,15,17,19,20,33,34,39,40], showed worse 1-year graft survival but better 5-year patient survival in ABO-I LT than in ABO-C LT when compared to studies that did not involve urgent indications. Further, there were no significant differences in 3- and 5-year graft survivals between the two types of LT. This may be due to the fact that studies that only examined recipients with urgent indications mostly involved relatively lower prevalences of chronic liver disease and liver cancer but more advanced disease severity and inadequate desensitizations before ABO-I LT.

Shaked *et al*[62] showed that there were no differences in biopsy-proven ACR and graft loss by rejection between LDLT and deceased donor liver transplantation (DDLT). However, our subgroup analysis showed that ACR was less prevalent after ABO-I LT in cases that only used living donor liver grafts[18,28-32,36] than in cases that did not. In other words, there were no differences in ACR in accordance with ABO compatibility in cases of LDLT, but incidences of ACR increased in cases of ABO-I LT using deceased donor liver grafts. It could be assumed that compared to DDLT, LDLT has immunological advantages resulting from high genetic similarities between organ donor and recipient and short cold ischemic times[63,64].

Further, biliary complications, such as bile leak and biliary stricture, are known to be more prevalent in LDLT with inherent weakness arising from a small duct size, possible multiplicity of bile duct, and cutting liver parenchyma, compared to those in DDLT[65-68]. However, our analysis revealed that using only living donor liver grafts[18,28-32,36] resulted in fewer cases of biliary stricture in ABO-I LT. One of the possible reasons is that studies only involving deceased donor grafts in our meta-analysis of biliary stricture[40,41] were published at least 20 years earlier than the studies involving living donor allografts[28-31]. Further, some of them involved urgent indications[40], which would have resulted in the use of markedly different desensitization and immunosuppression methods and surgical techniques from those employed today.

Meanwhile, in this meta-analysis, a potential publication bias was detected in the 1-year patient survival. Possible sources of asymmetry in the funnel plot would most definitely include small study effects, but poor methodological quality, true heterogeneity, artifactual, and chance could be other sources as well[21,25,69-72].

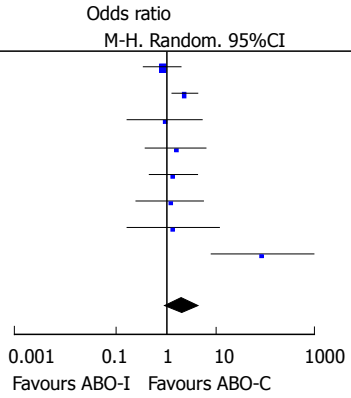
This review has some limitations. First, it was based on non-randomized controlled trials because it is practically impossible to randomly allocate patients into either ABO-C LT or ABO-I LT group. Second, some articles lacked clear descriptions about patient demographics and study design, such as age, enrollment criteria, graft type, and desensitization and immunosuppression methods. Third, some results showed heterogeneity and potential publication bias.

A

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Random. 95%CI
Kim 2016	9	47	21	94	18.2%	0.82 [0.34, 1.97]
Lee 2015	23	46	101	340	21.4%	2.37 [1.27, 4.41]
Shen 2014	2	35	4	66	9.5%	0.94 [0.16, 5.40]
Iwamoto 2008	4	15	7	37	12.2%	1.56 [0.38, 6.38]
Toso 2007	6	14	33	94	15.0%	1.39 [0.44, 4.34]
Heffron 2006	2	16	13	122	10.6%	1.20 [0.24, 5.87]
Cacciarelli 1995	1	14	7	130	7.0%	1.35 [0.15, 11.86]
Sanchez 1993	15	18	1	18	6.2%	85.00 [7.97, 906.81]
Total (95% CI)		205		901	100.0%	1.75 [0.89, 3.43]
Total events	62		187			

Heterogenity Tau^2 = 0.45 χ^2 = 15.22, df = 7 (P = 0.03); I^2 = 54%

Test for overall effect: Z = 1.63 (P = 0.10)

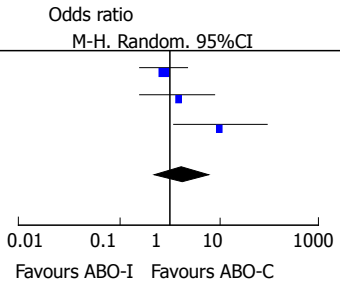


B

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Random. 95%CI
Kim 2016	5	47	12	94	44.8%	0.81 [0.27, 2.46]
Kim 2016	2	25	4	75	31.2%	1.54 [0.27, 8.98]
Sanchez 1993	7	18	1	18	24.0%	10.82 [1.17, 700.44]
Total (95% CI)		90		187	100.0%	1.85 [0.46, 7.39]
Total events	14		17			

Heterogenity Tau^2 = 0.80 χ^2 = 4.25, df = 2 (P = 0.12); I^2 = 53%

Test for overall effect: Z = 0.87 (P = 0.39)

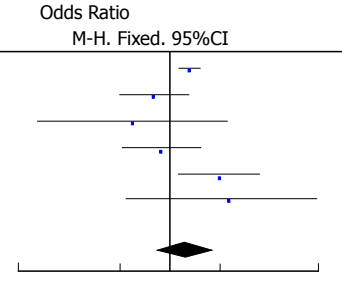


C

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Random. 95%CI
Song 2016	46	235	156	1301	37.1%	1.79 [1.24, 2.57]
Kim 2016	5	47	16	94	20.4%	0.58 [0.20, 1.70]
Kim 2016	0	25	4	75	4.7%	0.31 [0.02, 5.99]
Ikegami 2016	3	19	78	389	17.1%	0.75 [0.21, 2.63]
Lo 1994	4	29	7	196	16.5%	4.32 [1.18, 15.81]
Sanchez 1993	2	18	0	18	4.3%	5.61 [0.25, 125.45]
Total (95% CI)		373		2073	100.0%	1.37 [0.70, 2.70]
Total events	60		261			

Heterogenity Tau^2 = 0.29 χ^2 = 9.36, df = 5 (P = 0.10); I^2 = 47%

Test for overall effect: Z = 0.91 (P = 0.36)

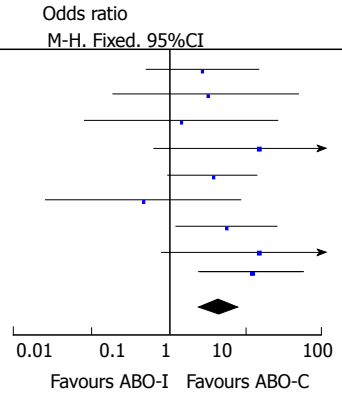


D

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds rati0 M-H. Fixed. 95%CI
Song 2016	2	235	4	1301	15.6%	2.78 [0.51, 15.28]
Kim 2016	1	25	1	75	6.2%	3.08 [0.19, 51.20]
Ikegami 2016	0	19	6	389	8.0%	1.51 [0.08, 27.83]
Iwamoto 2006	2	15	0	37	3.2%	13.89 [0.63, 308.13]
Toso 2007	4	14	9	94	21.5%	3.78 [0.98, 14.54]
Heffron 2006	0	16	7	122	22.8%	0.47 [0.03, 8.56]
Lo 1994	3	29	4	196	11.9%	5.54[1.17, 26.15]
Sanchez 1993	5	18	0	18	4.6%	15.07 [0.77, 296.44]
Gugenheim 1990	3	17	4	217	6.2%	11.41 [2.32, 56.05]
Total (95% CI)		388		2449	100.0%	4.17 [2.26, 7.67]
Total events	20		35			

Heterogenity: χ^2 = 5.88, df = 8 (P = 0.66); I^2 = 0%

Test for overall effect: Z = 4.58 (P < 0.00001)

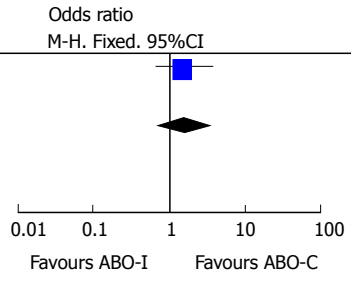


E

Study or subgroup	ABO-I Events	ABO-I Total	ABO-C Events	ABO-C Total	Weight	Odds ratio M-H. Fixed. 95%CI
Song 2016	6	235	21	1301	100.0%	1.60 [0.64, 4.00]
Total (95%CI)		235		1301	100.0%	1.60 [0.64, 4.00]
Total events	6		21			

Heterogenity: Not applicable

Test for overall effect: Z = 1.00 (P < 0.32)



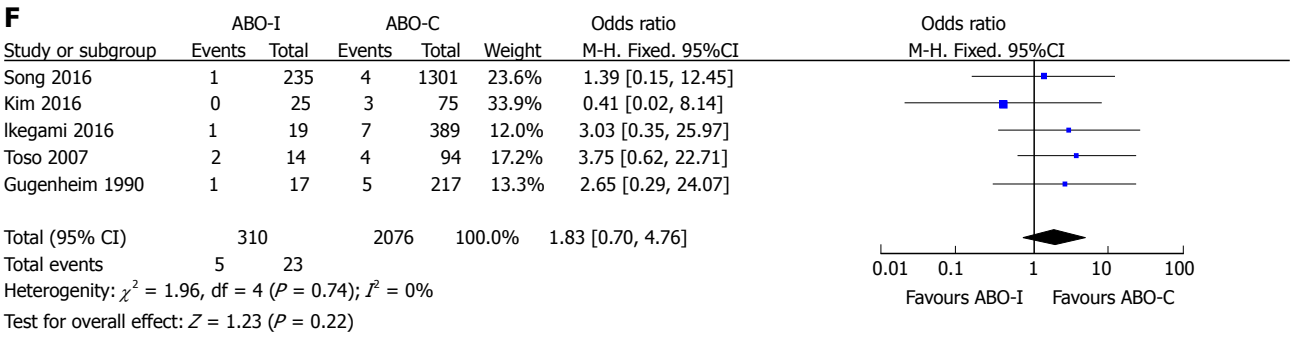


Figure 5 Comparison of biliary and vascular complications between ABO-Incompatible and ABO-Compatible liver transplantation. A: Overall biliary complication; B: Bile leak; C: Biliary stricture; D: HA complication; E: HV complication; F: PV complication. ABO-C: ABO-compatible; ABO-I: ABO-incompatible; HA: Hepatic artery; HV: Hepatic vein; LT: Liver transplantation; PV: Portal vein.

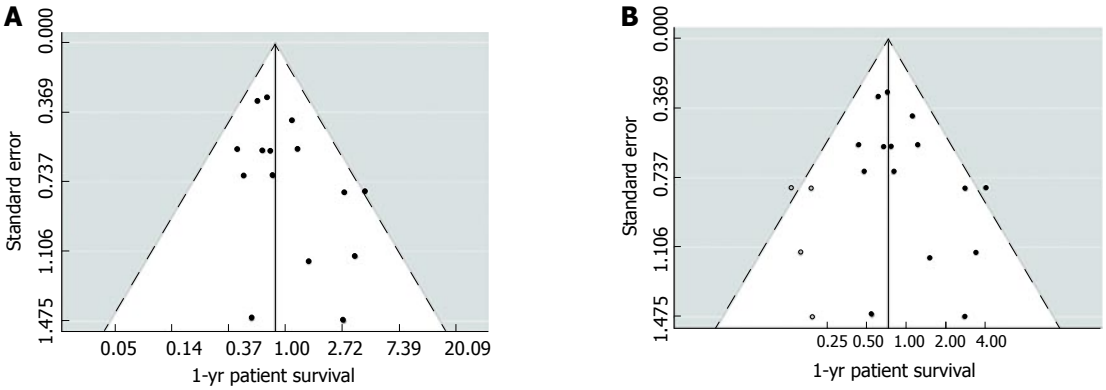


Figure 6 (A) Funnel plot and (B) Adjusted funnel plot using the Trim-and-Fill method of studies reporting on 1-yr patient survival after ABO-incompatible liver transplantation *vs* ABO-Compatible liver transplantation. Closed circles represent observed published studies; open circles represent imputed unpublished studies. ABO-C: ABO-compatible; ABO-I: ABO-incompatible; LT: Liver transplantation.

This meta-analysis is the largest review that integrating more than 8000 cases of ABO-I LT and ABO-C LT. It revealed that ABO-I LT is associated with poorer graft survival and higher prevalence of AMR, CR, CMV infection, overall biliary complication, and HA complication than those of ABO-C LT. There were no significant differences in patient survival, ACR, bacterial infection, fungal infection, bile leak, biliary stricture, and HV and PV complications in accordance with ABO compatibility. In our subgroup analysis, graft survival in ABO-I LT was found to be comparable to that in ABO-C LT in pediatric patients. Use of rituximab was associated with better graft survival in ABO-I LT. In cases of DDLT, there was a higher incidence of ACR after ABO-I LT. Although substantial improvements and advances have been made in liver transplantations across the ABO blood group barrier, persistent limitations call for further endeavors to achieve better outcomes.

ACKNOWLEDGMENTS

The authors wish to thank Jae Ryong Shim, MD for his contribution to this article.

COMMENTS

Background

Increased ABO-incompatible (ABO-I) liver transplantation (LT) is inevitable due to reduced organ donation and difficulty in finding suitable ABO-compatible (ABO-C) allografts. In particular, the importance of ABO-I LT is increasing in Asian countries where the use rate of ABO-I liver allograft is higher than that of Western countries due to the large number of organ donations in the family in living donor liver transplantation.

Research frontiers

Outcomes after LT in accordance with ABO compatibility is still controversial. Therefore, it is necessary to evaluate the possibilities and the limitations of ABO-I LT by meta-analysis integrating outcomes of previous reports comparing ABO-I and ABO-C LT.

Innovations and breakthroughs

ABO-I LT is comparable to ABO-C LT in terms of patient survival, but is inferior in graft survival, antibody-mediated rejection, chronic rejection, cytomegalovirus infection, overall biliary complication, and hepatic artery complication. However, in pediatric patients and those using rituximab, the graft survival of ABO-I LT was comparable to that of ABO-C LT.

Applications

The authors performed a meta-analysis of outcomes after liver transplantation in accordance with ABO compatibility. In this way, the possibilities and the limitations of ABO-I LT can be clarified.

Terminology

ABO-I transplantation is an assignment method for organ transplantation, which allows the use of available organs more efficiently regardless of the ABO blood type, which cannot otherwise be used due to hyperacute rejection.

Peer-review

This meta-analysis is the largest review article of more than 8000 cases of ABO-I and ABO-C LT. The authors concluded that ABO-I LT, although patient survival was similar, was inferior to ABO-C LT in graft survival and several ABO-I-related complications. The article is well written and of highly clinical implications.

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CASE REPORT

Case of colonic intussusception secondary to mobile cecum syndrome repaired by laparoscopic cecopexy using a barbed wound suture device

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Abstract

A 27-year-old man with recurrent right lower quadrant pain was admitted to our hospital. Ultrasonography and computed tomography examination of the abdomen revealed a target sign in the ascending colon, which was compatible with the diagnosis of cecal intussusception. The intussusception was spontaneously resolved at that time, but it relapsed 6 mo later. The patient underwent a successful colonoscopic disinvagination; there was no evidence of neoplastic or inflammatory lesions in the colon and terminal ileum. The patient underwent laparoscopic surgery for recurring cecal intussusception. During laparoscopy, we observed an unfixed cecum on the posterior peritoneum (*i.e.* a mobile cecum). Thus, we performed laparoscopic appendectomy and cecopexy with a lateral peritoneal flap using a barbed wound suture device. The patient's post-operative course was uneventful, and he continued to do well without recurrence at 10 mo after surgery. Laparoscopic cecopexy using a barbed wound suture device is a simple and reliable procedure that can be the treatment of choice for recurrent cecal intussusception associated with a mobile cecum.

Key words: Colonic intussusception; Adult; Mobile cecum; Cecopexy; Barbed wound suture device

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Core tip: Colonic intussusception secondary to mobile cecum is uncommon, and the surgical procedure is controversial. As the main cause of intussusception comprises neoplasms, most patients with colonic intussusception undergo intestinal resection. In our case, the cause of colonic intussusception was only the mobile cecum. Thus, we performed laparoscopic cecopexy using a barbed wound suture device, after which the patient made steady progress. This surgical procedure benefits from its good cosmetic outcomes and reduced invasiveness. In addition, a barbed wound suture device is useful for laparoscopic cecopexy, as it is easy to handle and results in a shorter operation time.

Yamamoto T, Tajima Y, Hyakudomi R, Hirayama T, Taniura T, Ishitobi K, Hirahara N. Case of colonic intussusception secondary to mobile cecum syndrome repaired by laparoscopic cecopexy using a barbed wound suture device. *World J Gastroenterol* 2017; 23(35): 6534-6539 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6534.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6534

INTRODUCTION

Mobile cecum syndrome is characterized by chronic right lower quadrant pain without evidence of appendicitis or other etiological factors. Although abnormal mobility of the cecum and ascending colon has been estimated to occur in 10%-20% of the population, intussusception or volvulus of the cecum secondary to a mobile cecum is uncommon[1,2]. Approximately one-third of the intussusceptions of the intestinal tract occur as a result of surgery-induced changes, such as adhesion, submucosal edema, and intestinal motility disorders; colonic intussusceptions in adults are usually caused by malignant or benign tumors[3]. Therefore, surgical interventions, such as intestinal resection through either an open or laparoscopic approach, have been performed for the majority of adult patients with colonic intussusception[4]. We describe herein a rare case of colonic intussusception secondary to mobile cecum syndrome, which was successfully treated with laparoscopic cecopexy using a barbed wound suture device.

CASE REPORT

A 27-year-old man with recurring abdominal pain in the right lower quadrant was re-admitted to our hospital. The patient had a 3-year history of mild ulcerative colitis, but he had been free from abdominal symptoms and continued to do well without medications. On admission, abdominal ultrasonography (Figure 1A) and contrast-enhanced computed tomography (CT) (Figure 1B) showed a target sign in the ascending colon with dilatation of the cecum and ileum, which was compatible with the diagnosis of cecal intussusception. The patient had no previous surgical history, and there was no evidence of intraabdominal tumors or inflammatory conditions in these imaging evaluations. Thereafter, the intussusception spontaneously resolved.

The patient was discharged a few days after admission, but the abdominal pain recurred 6 mo later. Abdominal CT scan again revealed cecal intussusception. An emergency colonoscopy was performed to reduce the intussusception, in which the cecum had advanced into the ascending colon. An edematous colonic mucosa restricted to the lead point of intussusception was identified (Figure 1C). No other inflammatory changes or neoplastic lesions were detected in the colon and terminal ileum, even after reduction of the intussusception. The patient was thus diagnosed with recurrent cecal intussusception that was likely due to a mobile cecum, and underwent an elective laparoscopic surgery.

The patient was placed in the supine position under general anesthesia. A 20-mm long skin incision was made on the umbilicus, and a 12-mm trocar was inserted. After creating a pneumoperitoneum with carbon dioxide at an intra-abdominal pressure of 10 mmHg, additional 5-mm and 12-mm trocars were placed at the left upper quadrant and the middle of the lower abdomen, respectively. Upon laparoscopy, no evidence of ischemia, inflammation, or caliber change in the gastrointestinal tract, including the cecum, appendix and terminal ileum, was found. A linear indentation running along the minor axis of the large bowel was identified in the middle portion of the ascending colon, which left a trace of intussusception (Figure 2A). The cecum was easily mobilized from the right lower quadrant to the upper abdominal cavity, as it was not fixed to the posterior parietal peritoneum (Figure 2B).

The findings confirmed that the recurrent cecal intussusception was associated with an abnormal fixation of the cecum and ascending colon to the parietal peritoneum (*i.e.* a mobile cecum). Laparoscopic appendectomy and cecopexy were performed. After the appendectomy, an approximately 10-cm long incision was made in the right parietal peritoneum along the ascending colon for the cecopexy (Figure 3A); then, the cecum and ascending colon were fixed to the incision line of the parietal peritoneum with a continuous suture technique, using an absorbable barbed wound suture device (V-Loc™ 180; Medtronic, Tokyo, Japan) (Figure 3B). The operation time was 87 min, with less than 10 g of blood loss. The patient's postoperative course was uneventful, and he was discharged 4 d after surgery. The patient continued to do well without recurrence at 10 mo after surgery.

DISCUSSION

Intestinal intussusception is the leading cause of gastrointestinal obstruction in children. However, it is

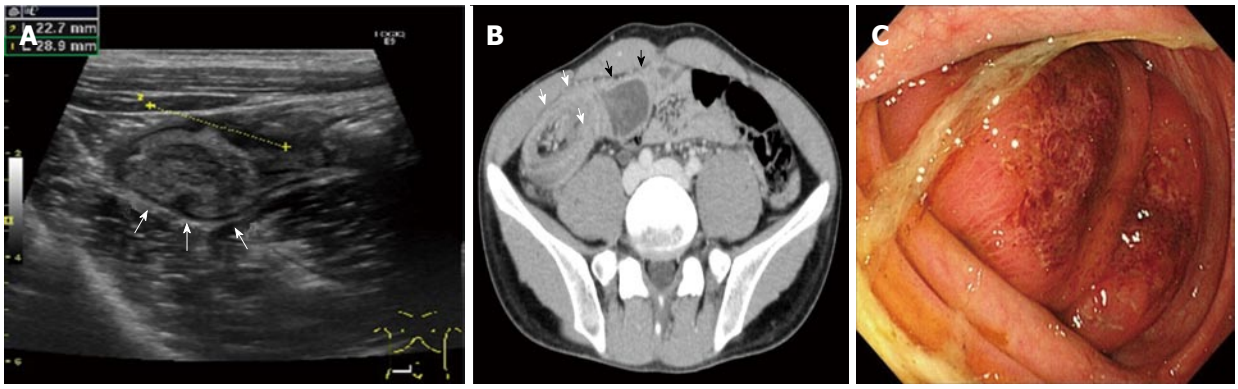


Figure 1 Target sign on abdominal ultrasonography (A) and contrast-enhanced computed tomography scan (B). The so-called bowel-within-bowel configuration, in which the layers of the bowel are duplicated, thereby forming concentric rings, is seen (white arrows). Dilatation of the terminal ileum is observed (black arrows). C: A colonoscopic view of the intussusception. Edematous colonic mucosa was identified on the lead point of intussusception. No neoplastic lesion was detected, even after reduction of intussusception.

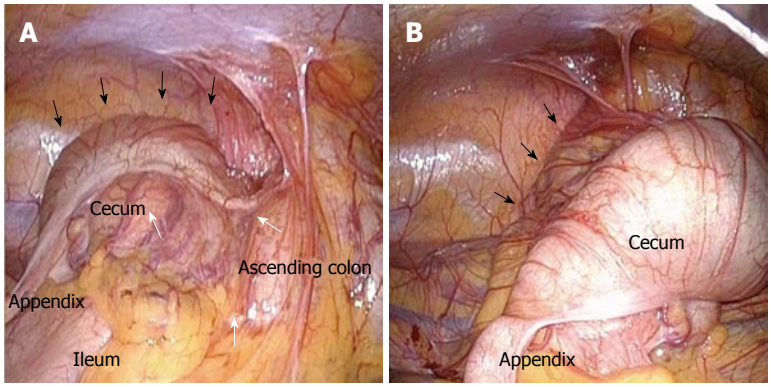


Figure 2 Laparoscopic view. A: A linear indentation leaving a trace of intussusception was found in the middle portion of the ascending colon (white arrows). The cecum was not attached to the retroperitoneum (black arrows); B: The cecum was easily moved to the upper abdomen. No fixation of the cecum was observed (black arrows).

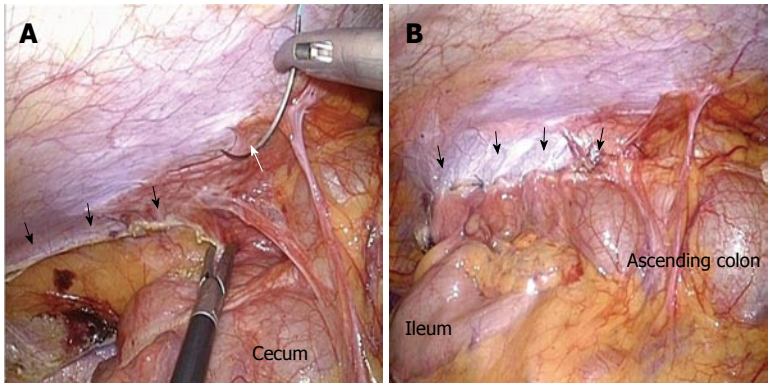


Figure 3 Laparoscopic cecopexy. A: An approximately 10-cm long incision (black arrows) was made on the right parietal peritoneum along the ascending colon; B: The cecum and ascending colon were stitched to the parietal peritoneum with continuous sutures using an absorbable barbed wound suture device (black arrows).

rarely encountered in adults, accounting for 1%-5% of all cases of intestinal obstruction[5]. Although the exact mechanism of bowel intussusception remains unclear, one leading theory is that intestinal motility disorder due to intraluminal lesions or inflammation induces invagination[6,7]. The common site of intussusception is the junction between the free intestine and the portion fixed to the retroperitoneum or at postoperative adhesions[7,8]. Non-malignant lesions, such as benign or inflammatory neoplasms, Meckel's diverticulum and appendix, are the most common cause of intussusception in the small bowel, with only 30% of cases due to malignant neoplasms[6,9]. In contrast, malignant lesions are responsible for most cases of colonic intussusceptions[7,10].

In the present case, neither malignant or benign

Table 1 Colonic intussusception associated with mobile cecum in adults: reported cases from 2005 to 2016

Ref.	Age	Sex	Clinical symptoms	Repeated symptoms	Episode duration	Diagnostic modalities	Characteristic finding	Etiology of intussusception / location	Operative procedure
Hamdi *et al*[16]	85	F	Abdominal pain, diarrhea	Yes	3 mo	Barium enema, CT	Target mass	Tumor /cecum	Resection
Drnovsek *et al*[15]	65	M	Abdominal pain, rectal bleeding	No	12 h	CT	Target sign	Tubulovillous adenoma /cecum	Right hemicolectomy
Kuzmich *et al*[14]	62	M	Abdominal pain, weight loss	Yes	2 mo	US	Target sign	Submucosal lipoma /ileocecal valve	Right hemicolectomy
Frydman *et al*[13]	22	F	Rectal prolapse	No	1 d	CT	Target sign	Villous adenoma /cecum	Right hemicolectomy
Present case	27	M	Right lower quadrant pain	Yes	7 mo	US, CT	Target sign	None	Laparoscopic cecopexy

CT: Computed tomography; US: Ultrasonography.

tumors nor inflammatory conditions were identified upon colonoscopic, ultrasonographic and CT examinations. Laparoscopically, other than the unfixed cecum and ascending colon on the posterior peritoneum, no notable finding (*e.g.,* abdominal adhesions or mesenteric lymph node swelling) was observed, thereby indicating that the cecal intussusception occurred secondary to the mobile cecum in our patient.

Mobile cecum is not uncommon, with up to 25% to 64% of cadavers exhibiting a mobile cecum. Nevertheless, colonic intussusception related to mobile cecum is rare in adults[11,12]. We performed a review of the literature from 1995 to 2016 using the PubMed database with the search terms "intussusception," "mobile cecum" and "adult," which yielded only 4 cases of colonic intussusception associated with mobile cecum[13-16]. Moreover, all of these cases had some lead lesions as a cause of intussusception, such as villous adenoma and submucosal lipoma in the cecum or ileocecal valve (Table 1).

Meanwhile, our case showed no evidence of neoplastic lesions or inflammatory conditions in the colon and terminal ileum, except edematous colonic mucosa identified at the lead point of intussusception. In terms of the cause of intussusception, our patient represented a rare case of colonic intussusception associated with a mobile cecum. The mucosal edema identified on the lead point of intussusception might be a cause or an effect of the intussusception in our patient. It is undeniable that the mucosal edema might have been induced by ulcerative colitis because the patient had a history of mild ulcerative colitis; however, he had been free from ulcerative colitis-related specific symptoms and required no medications for the treatment.

The presenting symptoms of colonic intussusception are nonspecific, such as abdominal pain, nausea and emesis, and a definitive diagnosis can be made only in 33% of patients prior to surgery[17]. The typical target sign or a sausage-shaped mass on a CT scan is used to confirm the diagnosis of intussusception[17, 18]. In our patient, cecal intussusception was diagnosed on the basis of the target sign on abdominal ultrasonography and CT examination. Ultrasonography is a noninvasive and readily available imaging modality and easy to perform in routine clinical practice, thus, it was useful in the diagnostic workup of colonic intussusception in our patient.

In the treatment of colonic intussusception, colonoscopic reduction is worth considering because it is less invasive than surgery, although its efficacy remains controversial[9]. In adult patients, surgical resection is generally required because most cases have intraluminal neoplasms as a cause of intussusception[3,7,19]. In the present case, we performed an elective laparoscopic cecopexy after endoscopic reduction of cecal intussusception because the patient had neither intraluminal lesions nor mesenteric lymphadenopathy.

Cecopexy or right hemicolectomy can be performed to treat mobile cecum syndrome with abdominal symptoms. Some studies have recommended only colectomy because there is a possibility of recurrence of the abdominal pain due to volvulus or intussusception after performing a cecopexy[20,21]. Meanwhile, other reports have described cecopexy as a good treatment option for patients with a mobile cecum, which is associated with a low rate of disease and symptom recurrence[22,23]. Given the absence of any recurrence in our case, laparoscopic cecopexy seems to be a useful treatment of choice for cecal intussusception secondary to mobile cecum without neoplastic lesions.

Open and laparoscopic surgery can be performed for mobile cecum syndrome, and the benefits of laparoscopic surgery, in terms of cosmetic outcomes and less invasiveness, have been reported[24,25]. In the present case, we performed an elective laparoscopic cecopexy after colonoscopic reduction of cecal intussusception because the patient was generally stable and had no neoplastic lesions that could have possibly caused cecal intussusception. In addition, a barbed wound suture device was used to fix the cecum to the posterior peritoneum. Previous studies have reported various benefits of using barbed sutures, including knotless suturing, shorter stitching time and increased convenience for the surgeon[26,27]. In our case, a barbed suture device was useful for laparoscopic cecopexy, as it was easy to handle and resulted in a shorter operation

time.

Laparoscopic cecopexy is a useful strategy to treat colonic intussusception secondary to a mobile cecum without any other pathologic disorders. A barbed suture device is also useful in performing a laparoscopic cecopexy in terms of its ease in handling and shorter stitching time.

COMMENTS

Case characteristics

A 27-year-old man with recurring abdominal pain in the right lower quadrant.

Clinical diagnosis

The patient's abdomen was slightly distended and hard. Tenderness of the right lower quadrant was detected with a palpable mass when intussusception occurred.

Differential diagnosis

Cecal volvulus or intra-abdominal tumors.

Laboratory diagnosis

Laboratory data were within normal limits.

Imaging diagnosis

Abdominal ultrasonography and computed tomography scan showed a target sign in the ascending colon with dilatation of the cecum and ileum, which was compatible with the diagnosis of cecal intussusception.

Pathological diagnosis

There was no evidence of malignancy or any other inflammatory disease.

Treatment

Laparoscopic cecopexy using a barbed wound suture device.

Related reports

Past studies have recommended only colectomy to treat the mobile cecum, given the high rate of recurrence after cecopexy. Recently, some case reports have described a cosmetic benefit and reduced invasiveness of cecopexy without recurrence of cecal intussusception.

Term explanation

A mobile cecum, which is defined as the unfixed cecum and ascending colon on the posterior peritoneum, is not uncommon. However, colonic intussusception related to mobile cecum is rare in adults.

Experiences and lessons

Colonic intussusception secondary to a mobile cecum is uncommon, and the surgical procedure for treatment is controversial. In this case, we performed laparoscopic cecopexy using a barbed wound suture device. This surgical procedure produces a good cosmetic outcome, while a barbed wound suture device is useful for laparoscopic cecopexy, as it is easy to handle and results in a shorter operation time.

Peer-review

The paper is an interesting Case Report of colonic intussusception secondary to mobile cecum syndrome and is suitable for publication.

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CASE REPORT

Acquired amegakaryocytic thrombocytopenia previously diagnosed as idiopathic thrombocytopenic purpura in a patient with hepatitis C virus infection

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Author contributions: Ichimata S and Kobayashi M performed the histological evaluation, analyzed the serum sample, and drafted the manuscript; Honda K analyzed the serum sample; Shibata S and Matsumoto A evaluated clinical data; Kanno H performed the histological evaluation, analyzed the serum sample, and critically reviewed the manuscript.

Institutional review board statement: This case report was exempt from the Institutional Review Board standard at Shinshu University School of Medicine.

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

Conflict-of-interest statement: The authors have no conflict of interest.

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Abstract

We report the first case of a patient with hepatitis C virus (HCV) infection and idiopathic thrombocytopenic purpura (ITP), who later developed acquired amegakaryocytic thrombocytopenia (AAMT), with autoantibodies to the thrombopoietin (TPO) receptor (c-Mpl). A 64-year-old woman, with chronic hepatitis C, developed severe thrombocytopenia and was diagnosed with ITP. She died of liver failure. Autopsy revealed cirrhosis and liver carcinoma. In the bone marrow, a marked reduction in the number of megakaryocytes was observed, while other cell lineages were preserved. Therefore, she was diagnosed with AAMT. Additionally, autoantibodies to c-Mpl were detected in her serum. Autoantibodies to c-Mpl are one of the causes of AAMT, acting through inhibition of TPO function, megakaryocytic maturation, and platelet formation. HCV infection induces several autoantibodies. HCV infection might also induce autoantibodies to c-Mpl, resulting in the development of AAMT. This mechanism may be one

of the causes of thrombocytopenia in patients with HCV infection.

Key words: Hepatitis C virus; Acquired amegakaryocytic thrombocytopenia; Anti-thrombopoietin receptor (c-Mpl) autoantibodies; Idiopathic thrombocytopenic purpura; Thrombocytopenia

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Core tip: Thrombocytopenia occurs frequently in patients with hepatitis C virus (HCV) infection. Acquired amegakaryocytic thrombocytopenia (AAMT) is one of the causes of severe thrombocytopenia. The exact mechanisms of AAMT have not been fully elucidated. However, patients with autoantibodies to thrombopoietin receptor (c-Mpl) develop AAMT. Similarly, autoantibodies are sometimes generated in patients with HCV infection. Here, we report the first case of a patient with HCV infection who later developed AAMT with autoantibodies to c-Mpl. AAMT with autoantibodies to c-Mpl may be one of the causes of thrombocytopenia in patients with HCV infection.

Ichimata S, Kobayashi M, Honda K, Shibata S, Matsumoto A, Kanno H. Acquired amegakaryocytic thrombocytopenia previously diagnosed as idiopathic thrombocytopenic purpura in a patient with hepatitis C virus infection. *World J Gastroenterol* 2017; 23(35): 6540-6545 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6540.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6540

INTRODUCTION

Thrombocytopenia occurs frequently in patients with chronic hepatitis C. The causes of thrombocytopenia in patients with chronic hepatitis C are multiple, such as hypersplenism, immunological processes, and decreased thrombopoietin (TPO) level[1,2].

Acquired amegakaryocytic thrombocytopenia (AAMT) is one of the causes of severe thrombocytopenia and is characterized by a marked reduction in the number of megakaryocytes, with preserved hematopoiesis of the other lineages in the bone marrow[3]. The exact mechanisms of AAMT have not been fully elucidated. However, the defect of TPO receptor (c-Mpl) expression due to *c-Mpl* gene mutation is the major cause of congenital amegakaryocytic thrombocytopenia[4]. Furthermore, patients with systemic lupus erythematosus (SLE) and systemic sclerosis who have autoantibodies to c-Mpl develop AAMT[5,6]. TPO, produced mainly by hepatocytes, binds to c-Mpl on hematopoietic stem cells and megakaryocytes, and promotes all stages of platelet production, from the proliferation and differentiation of megakaryocytes to megakaryocytic maturation and platelet formation[1]. Thus, autoantibodies to c-Mpl may be one of the causes of AAMT, through inhibiting TPO function. AAMT with autoantibodies to c-Mpl has not previously been reported in patients with hepatitis C virus (HCV) infection.

Here, we report the first case of a patient with HCV infection and idiopathic thrombocytopenic purpura (ITP), who later developed AAMT with autoantibodies to c-Mpl.

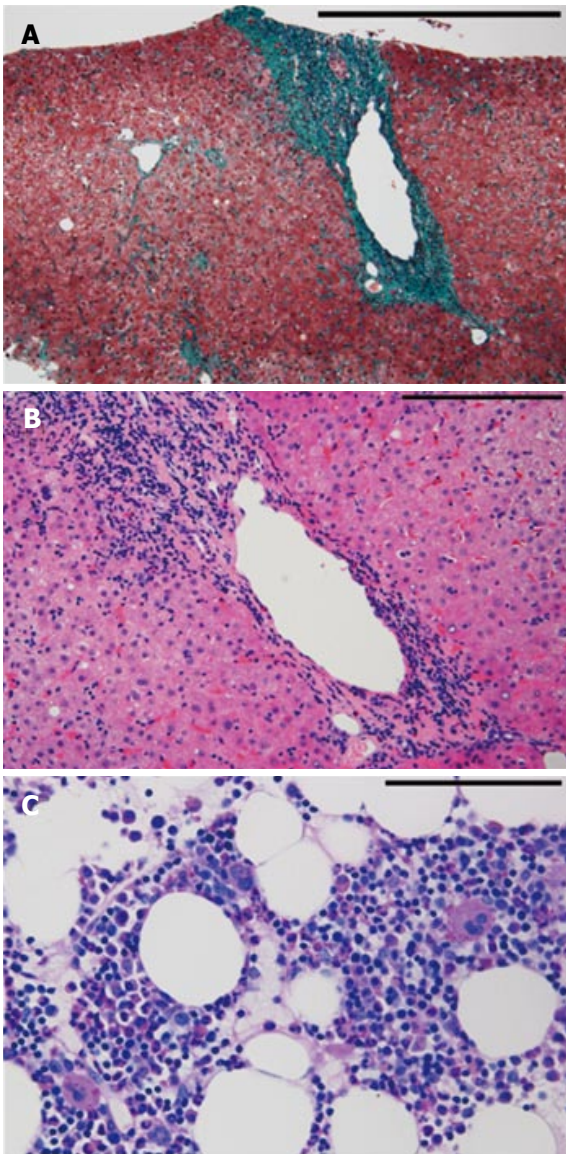


Figure 1 Histopathological features of liver biopsy specimen and clot section of bone marrow aspirate. A: The liver biopsy specimen shows fibrous portal expansion. There is no fibrous bridging (Elastica-Goldner staining, scale bar; 500 μm); B: Mild piecemeal necrosis, mild intralobular degeneration and focal necrosis, and moderate portal inflammation are observed (H and E staining, scale bar; 200 μm); C: The clot section of bone marrow aspirate shows normal numbers of megakaryocytes and other cell lineages are preserved (Periodic Acid Schiff staining, scale bar; 100 μm).

CASE REPORT

A 64-year-old woman was admitted with the chief

Table 1 Laboratory data on last admission

CBC			Chemistry		
WBC	$8.21 \times 10^3/\mu L$	TP	6.4 g/dL	Na	129 mEq/L
Neutrophils	89%	Alb	2.4 g/dL	K	4.8 mEq/L
Lymphocytes	7%	BUN	29.5 mg/dL	Cl	96 mEq/L
RBC	$3.64 \times 10^6/\mu L$	Cre	1.13 mg/dL	Glu	178 mg/dL
Hemoglobin	10 g/dL	AST	78 U/L	CRP	1.08 mg/dL
HCT	30%	ALT	55 U/L	NH_3	63 μg/dL
Platelets	$41 \times 10^3/\mu L$	γ-GT	88 U/L	HCV-Ab	12.8 COI
		T-bil	3.88 mg/dL	HCV (RT-PCR)	5.2 L.IU/mL
Coagulation		D-bil	2.72 mg/dL	T-AFP	571.4 ng/mL
PT	17.2 s	ALP	402 U/L	AFP L3	42.2 ng/mL
APTT	39.3 s	LD	273 U/L	PIVKA2	15 mAU/mL
Fibrinogen	123 mg/dL	AMY	63 U/L		
D-dimer	5 μg/mL	ChE	27 U/L		

AFP L3: Alpha-fetoprotein L3 isoform; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMY: Amylase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; CBC: Complete blood count; ChE: Cholinesterase; Cre: Creatinine; CRP: C-reactive protein; D-bil: Direct bilirubin; Glu: glucose; HCV-Ab: Hepatitis C antibody; HCT: Hematocrit; HCV (RT-PCR): Hepatitis C RNA (reverse transcriptase polymerase chain reaction); LDH: Lactate dehydrogenase; PIVKA2: Protein induced be vitamin K absence 2; PT: Prothrombin time; RBC: Red blood cells; T-AFP: Total alpha-fetoprotein; T-Bi: Total bilirubin; TP: Total protein; WBC: White blood cell; γ-GT: γ-glutamyltransferase.

complaint of dyspnea. She had a past history of post-transfusion hepatitis approximately forty years beforehand, and subsequently she was diagnosed with HCV infection (genotype 1b, high). At the age of 41, she developed thrombocytopenia (platelets count: $12.2 \times 10^4/\mu L$). At that time, she received interferon therapy, but the HCV infection persisted. At the age of 51 and 52, liver biopsy and bone marrow aspirations were performed, respectively. Liver biopsy specimens revealed periportal mild inflammatory cell infiltration and fibrosis (Modified Histological Activity Index: activity was 5/18, fibrosis was 1/6; Figure 1A and B). The clot section of the bone marrow aspirate showed no significant change, and the number of megakaryocytes was within the normal range. Although the platelet-associated IgG (PA IgG) was not measured, she was diagnosed with ITP (Figure 1C). At the age of 61, liver cancer was detected, using computed tomography and magnetic resonance imaging, and she received transcatheter arterial chemoembolization (TACE) on several occasions. On the most recent admission, her liver cancer was found to be enlarged and ascites and pleural effusion had increased. Laboratory data are shown in Table 1. Her laboratory data indicated hepatic dysfunction, remnants of liver cancer and thrombocytopenia. On day 15 of her admission, her general condition deteriorated, and she died of liver failure. An autopsy was performed.

At autopsy, she showed generalized jaundice and purpura in the anterior chest wall. Ascites (2600 mL) and pleural effusion (left: 100 mL, right: 3400 mL) were observed. Liver weight was 660 g, indicating severe atrophy. The cut surface of the liver showed diffuse micronodular cirrhosis with a dark green nodule (15 mm × 15 mm) in the left lobe, and a yellow, partly reddish or green, lesion with an irregular margin (70 mm × 50 mm) in the right lobe (Figure 2A). Spleen weight was 240 g, indicating mild enlargement. Varicose veins were observed in the lower esophagus, stomach, and rectum.

Histopathologically, liver specimens showed diffuse small regenerative nodules with fibrous septum and septal mild mononuclear cell infiltration (Figure 2B). The right lobe lesion was mainly composed of two components: hepatocellular carcinoma with bile production (Figure 2C) and adenocarcinoma with mucin production (Figure 2D). Therefore, the diagnosis of combined hepatocellular-cholangiocarcinoma was made. There were no viable tumor cells in the left lobe lesion, compatible following TACE treatment for liver carcinoma. Microscopic metastases were observed in both lungs. Bone marrow specimens showed slight hypocellularity (30%-40%), with a myeloid to erythroid ratio: 3 to 1, and a marked reduction in the number of megakaryocytes, < 1 megakaryocyte/10 high-power fields (Figure 2E). Immunostaining for CD41 revealed scattered small megakaryocytes (Figure 2F). Other lineages of hematopoietic cells were preserved, and myelofibrosis, dysplasia, and metastatic lesions were not observed. Spleen specimens showed mild congestion without extramedullary hematopoiesis. Characteristic histopathological findings of the spleen in patients with ITP, such as an increase of secondary follicles with well-delineated germinal centers, an expansion of a follicular marginal zone of the white pulp and a diffuse proliferation of foamy histiocytes, were not obvious in this patient. There was no definite lesion in the thyroid.

Next, we evaluated serum TPO levels at the time of her last admission using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, United States) according to the manufacturer's protocol. The serum TPO level of the patient was 54 pg/mL, and the serum TPO levels of two healthy individuals without HCV infection were 27

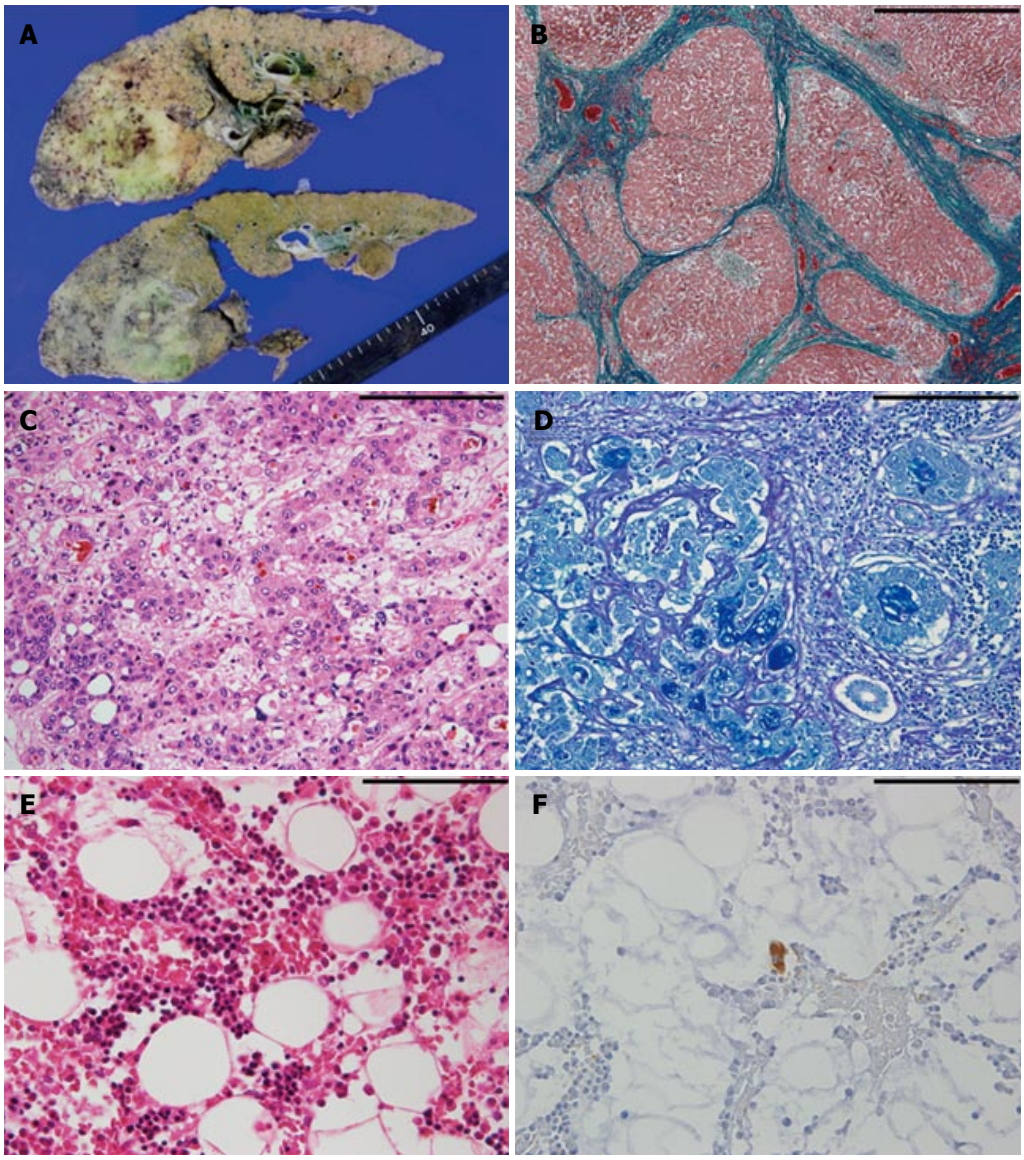


Figure 2 Macroscopic and histopathological features of autopsy specimens. A: The cut surface of the liver shows diffuse micronodular cirrhosis with a yellow-green lesion in the right lobe; B: The non-tumorous liver shows diffuse small regenerative nodules with fibrous septum (Elastica-Goldner staining, scale bar; 1000 μm); C and D: Histopathological findings of combined hepatocellular-cholangiocarcinoma; C: hepatocellular carcinoma component (H and E staining) and D: adenocarcinoma component (Alcian Blue-Periodic Acid Schiff staining) (C and D, scale bar; 200 μm); E: In the bone marrow, no megakaryocytes are observed (H and E staining); F: A small megakaryocyte is identified through immunostaining for CD41 (E and F, scale bar; 100 μm).

pg/mL and 37 pg/mL (mean 32 pg/mL). In addition, the presence or absence of anti-TPO receptor (c-Mpl) autoantibodies was determined, using Human anti-thrombopoietin receptor (C-MPL) autoantibodies IgG ELISA kit (CUSABIO, Wuhan, China), according to the manufacturer's protocol. The serum sample of the patient at the time of her last admission was positive for anti-c-Mpl antibodies, compared to the negative results of the sera of two healthy individuals without HCV infection.

DISCUSSION

In the current case, AAMT associated with autoantibodies to c-Mpl was considered to be one of the major causes of her severe thrombocytopenia. Hypersplenism and paraneoplastic autoimmunity were considered to be restrictive causes of her thrombocytopenia because she showed thrombocytopenia before development of her liver cirrhosis and liver cancer. In addition, her serum TPO level was preserved at the time of her last admission. Yet ITP was one of the causes of her thrombocytopenia. However, characteristic histopathological findings of the spleen in patients with ITP were not obvious in this patient at the time of autopsy.

It is unclear when autoantibodies to c-Mpl started to be produced in this patient. The patient showed a normal number of megakaryocytes at least ten years before her death, that is, ten years after the onset of thrombocytopenia. This finding is compatible with ITP. Therefore, autoantibodies to c-Mpl might have

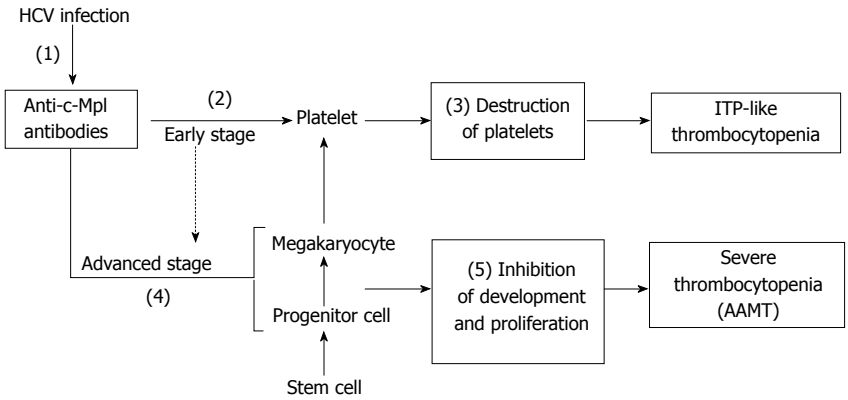


Figure 3 A schema of the pathogenesis of the current case. Hepatitis C virus infection may cause the generation of anti-c-Mpl antibodies (1). At first, most of the generated antibodies would be absorbed with the c-Mpl on platelets because platelets are the largest component in the megakaryocyte lineage (2). These antibody-attached platelets are destroyed in the spleen (3), therefore, idiopathic thrombocytopenic purpura (ITP)-like clinical manifestations are observed (Early stage). Following a sufficient reduction of platelets, these antibodies begin to bind to the c-Mpl on the megakaryocytes and its progenitor cells in the bone marrow (4). Attached antibodies block the functions of thrombopoietin, causing inhibition in the development and proliferation of the megakaryocyte lineage (5). Thus, severe reduction of megakaryocytes in the bone marrow occurs, that is, acquired amegakaryocytic thrombocytopenia (AAMT) (Advanced stage).

developed after the diagnosis of ITP and the patient subsequently developed AAMT. It is possible that autoantibodies to c-Mpl had already been produced at the time of the ITP diagnosis. Kuwana *et al*[5] reported that autoantibodies to c-Mpl are detected in approximately 8% of ITP patients. Thus, patients with autoantibodies to c-Mpl might develop ITP at an early stage, and then develop AAMT during the course of the disease. This hypothesis is summarized in Figure 3. The receptor c-Mpl is expressed in the megakaryocytic lineage from late progenitors to platelets, and platelets display high-affinity receptors for TPO[7]. Therefore, even if autoantibodies to c-Mpl had been produced at an early stage, most of the autoantibodies would have been absorbed with c-Mpl on platelets, and the proliferation and differentiation of megakaryocytes would not have been severely impaired. Thus, the number of megakaryocytes in bone marrow would be relatively well preserved and the patient's bone marrow may show a histopathology compatible with ITP. After a sufficient reduction in the number of platelets, AAMT then could then develop because autoantibodies to c-Mpl start to bind to c-Mpl on megakaryocytes and progenitor cells, inhibiting their development and maturation. Hoffman *et al*[8]. described a patient with ITP who later developed AAMT associated with antibodies that suppressed the colony formation of megakaryocytes[8].

HCV infection might have induced autoantibodies to c-Mpl in the current patient. Autoantibodies to c-Mpl are not detected in healthy controls[5]. In patients with HCV infection, several autoantibodies are produced, such as anti-nuclear antibodies, anti-smooth muscle antibodies, organ-specific autoantibodies, and anti-platelet antibodies[1]. In addition, anti-platelet IgG antibodies are detected in 26.3% of patients with HCV infection, showing a higher prevalence compared to healthy controls[9]. These mechanisms may play a role in the development of AAMT, with autoantibodies to c-Mpl in patients with HCV infection. However, interferon therapy induces several autoantibodies to multiple organ systems, such as anti-thyroid antibodies, auto-antibodies indicative of autoimmune hepatitis, and anti-platelet autoantibodies[10], and exhibits side effects of developing autoimmune diseases. Autoimmune thrombocytopenia sometimes occurs both during and after interferon therapy[11]. Thus, interferon therapy might have induced autoantibodies to c-Mpl in the current patient. We consider that interferon therapy did not play a significant role in the induction of autoantibodies to c-Mpl here because the patient's bone marrow had shown a normal number of megakaryocytes for ten years at least, following interferon therapy.

The current case provides a new perspective on thrombocytopenia in patients with HCV infection. AAMT with autoantibodies to c-Mpl may be one of the causes of thrombocytopenia in these patients. Some patients with HCV infection-associated thrombocytopenia, for whom thrombopoietin receptor agonists have a weak effect, might have this condition. Further investigation will be necessary, especially concerning the relationship between AAMT with autoantibodies to c-Mpl and HCV infection.

AAMT with autoantibodies to c-Mpl can be one of the causes of thrombocytopenia in patients with chronic HCV infection.

COMMENTS

Case characteristics

A 64-year-old woman with hepatitis C virus (HCV) infection and idiopathic thrombocytopenic purpura presented with dyspnea.

Clinical diagnosis

Liver failure due to chronic hepatitis C.

Differential diagnosis
Heart failure and renal failure.

Laboratory diagnosis
Anemia, thrombocytopenia, decreased albumin, elevated bilirubin, liver dysfunction, elevated alpha-fetoprotein.

Imaging diagnosis
Computed tomography revealed liver cirrhosis with a right lobe mass, bilateral pleural effusions and ascites.

Pathological diagnosis
Liver cirrhosis, combined hepatocellular-cholangiocarcinoma in the liver and microscopic metastases in both lungs, and acquired amegakaryocytic thrombocytopenia (AAMT) in the bone marrow.

Related reports
There are a limited number of reports describing AAMT with autoantibodies to thrombopoietin receptor (c-Mpl) in patients with systemic lupus erythematosus and systemic sclerosis.

Term explanation
AAMT is characterized by a marked reduction in the number of bone marrow megakaryocytes and occurs, in part, through autoantibodies to c-Mpl.

Experiences and lessons
In patients with HCV infection, several autoantibodies are produced. Autoantibodies to c-Mpl may also be produced and AAMT may occur in patients with HCV infection. Thus, AAMT with autoantibodies to c-Mpl may be one of the causes of thrombocytopenia in patients with HCV infection.

Peer-review
The case record is correctly described and documented. The authors describe the first case of AAMT associated with HCV infection.

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LETTERS TO THE EDITOR

Novel endoscopic management of buried bumper syndrome in percutaneous endoscopic gastrostomy: The Olympus HookKnife

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Abstract

Buried bumper syndrome (BBS) is an uncommon but serious complication of percutaneous endoscopic gastrostomy. It involves the internal fixation device, or "bumper", migrating into the gastric wall and subsequent mucosal overgrowth. We described a case series of four patients with BBS treated with a novel endoscopic technique using a HookKnife between June 2016 and February 2017. The HookKnife is a rotating L-shaped cutting wire designed for hooking tissue and pulling it away from the gastric wall towards the lumen. The technique was successful in all four cases with no complications. Each patient was discharged on the day of treatment. The HookKnife is a manoeuvrable, safe and effective device for endoscopic removal of buried bumpers and could avoid surgery in a high risk group of patients. To our knowledge this technique has not been described previously. We suggest that this technique should be added to the treatment algorithms for managing BBS.

Key words: Buried bumper syndrome; Percutaneous endoscopic gastrostomy; HookKnife

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Core tip: This letter to the editor describes a case series of four patients who underwent a novel endoscopic

technique for managing buried bumper syndrome using the Olympus HookKnife. This technique was successful in all four patients and no complications were recorded. We propose that this technique may be a safe and effective treatment for buried bumper syndrome in percutaneous endoscopic gastrostomy feeding.

Wolpert LE, Summers DM, Tsang A. Novel endoscopic management of buried bumper syndrome in percutaneous endoscopic gastrostomy: The Olympus HookKnife. *World J Gastroenterol* 2017; 23(35): 6546-6548 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i35/6546.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i35.6546

TO THE EDITOR

We enjoyed Cyrany *et al*[1] article on "Buried bumper syndrome : A complication of percutaneous endoscopic gastrostomy". Buried bumper syndrome (BBS) is an uncommon but serious complication of percutaneous endoscopic gastrostomy (PEG) with an incidence of 0.3%-2.4% per PEG patient year[1]. Symptoms include inability to insert the PEG tube, loss of patency and leakage from the PEG.

Here, we describe a case series of four patients with BBS treated with a novel endoscopic technique using a HookKnife (Olympus EndoTherapy) (Figure 1). The HookKnife is a rotating L-shaped cutting wire designed for hooking tissue and pulling it away from the gastric wall towards the lumen[2] and intended for use in colonic polypectomies. Other applications have been described in the literature; Oyama *et al*[3] describe a technique whereby the HookKnife was used to endoscopically resect lymph node metastases of gastric and oesophageal mucosal cancer.

Three patients were referred to our centre by their general practitioner due to inflammation around the PEG site and an inability to advance the PEG tube. The fourth patient was referred after their original PEG had stopped functioning and a new PEG had been inserted by the medical team. Patients were selected for treatment following referral for suspected BBS which was then evaluated by endoscopy. The patients ranged in age from 28 to 61 and had PEG placement for multiple sclerosis, Huntington's disease, cerebral palsy and tetraplegia. Two patients underwent the procedure in theatre under general anaesthetic whilst the other two underwent the procedure in endoscopy under sedation.

In each case the external PEG tubing was cut short to around 5 cm from the abdominal wall. A 15 mm through-the-scope dilation balloon was passed externally via the PEG tubing and inflated to dilate the mucosal orifice. A mucosal orifice was visible in all the patients in this case series. If a mucosal orifice cannot be clearly visualised then it may be found using the balloon catheter tip inserted externally. If there is no orifice then the mucosa can be cut over the bumper to create an orifice. After dilating the mucosal orifice using the TTS balloon the HookKnife was deployed to incise the gastric mucosa over the buried bumper by hooking the tissue, pulling it towards the lumen and then cutting it using diathermy under direct vision. The balloon was then re-inflated inside the tubing at the level of the PEG bumper to stiffen the PEG system (Figure 2), and both the tubing and balloon catheter clamped together using artery forceps. Short, but firm, controlled pressure was applied towards the gastric lumen to force the bumper into the stomach. The PEG was then removed using a standard technique. In one case a new PEG had already been placed prior to the procedure. In the other 3 cases, a new PEG was placed during the procedure. There were no complications and all four patients were discharged on the day of treatment.



Figure 1 The Olympus HookKnife[2]-A rotating L-shaped cutting wire designed to hook mucosal tissue and pull it towards the lumen. It was originally designed for use in colonic polypectomies. In Oyama *et al*[3] the HookKnife was deployed to resect lymph node metastases of gastric and oeseophageal mucosal cancers.

The HookKnife is a manoeuvrable, safe and effective device for endoscopic removal of buried PEG bumpers. There are several benefits of the HookKnife technique. First, compared with techniques that rely on incising the mucosa towards the gastric wall, such as the needle knife technique and papillotome technique discussed in Cyrany *et al*[1],the HookKnife allows elevation of the gastric mucosa away from the bumper as the mucosal tissue can be hooked using the knife and pulled towards the lumen[2] . This additional level of control allows a more precise incision, more accurate evaluation of the depth of the bumper migration and reduces the risk of inadvertent gastric perforation as the mucosa is cut by pulling it towards the lumen as opposed to cutting towards the gastric wall. Second, the HookKnife could avoid surgery and could therefore be suitable for high risk patients. As this technique can be performed as a day case it avoids the need for an overnight stay in hospital. Finally, none of the patients in this case series experienced bleeding, which was a frequent complication in patients who

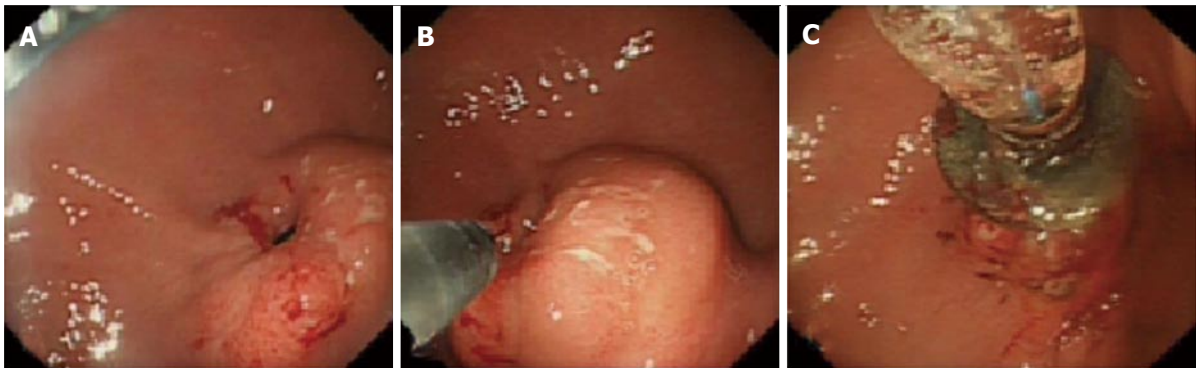


Figure 2 Endoscopic removal of a buried percutaneous endoscopic gastrostomy bumper using the HookKnife technique. A: Visualisation of the buried bumper. The gastric mucosa has overgrown the internal percutaneous endoscopic gastrostomy (PEG) fixation device leaving a small mucosal orifice. A through-the-scope balloon can be used to dilate the mucosal orifice; B: View of the Olympus HookKnife being deployed. Radial incisions are made by hooking the gastric mucosa and pulling it towards the lumen; C: A through-the-scope balloon has been inserted externally to stiffen the PEG system. Pressure is applied to the balloon to force the bumper into the lumen.

underwent the needle knife technique[1]. Adequate haemostasis was achieved by using the knife on a coagulation setting of 10-30 watts and by not pulling the hook too quickly. To our knowledge, this technique has not been described previously and we suggest that this technique should be added to the treatment algorithms for the management of BBS[4]. However, as only a small number of cases are included in this series larger numbers of patients may need to be included to establish whether the HookKnife is a safe and effective method of endoscopic removal of buried bumpers. A possible limitation of this method of BBS removal is that it relies on a subjective assessment of the depth that the bumper is buried.

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REVIEW

Treatment options for alcoholic and non-alcoholic fatty liver disease: A review

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Abstract

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health problems worldwide. These two diseases have similar pathological spectra, ranging from simple steatosis to hepatitis to cirrhosis and hepatocellular carcinoma. Although most people with excessive alcohol or calorie intake display abnormal fat accumulation in the liver (simple steatosis), a small percentage develops progressive liver disease. Despite extensive research on understanding the pathophysiology of both these diseases there are still no targeted therapies available. The treatment for ALD remains as it was 50 years ago: abstinence, nutritional support and corticosteroids (or pentoxifylline as an alternative if steroids are contraindicated). As for NAFLD, the treatment modality is mainly directed toward weight loss and co-morbidity management. Therefore, new pathophysiology directed therapies are urgently needed. However, the involvement of several inter-related pathways in the pathogenesis of these diseases suggests that a single therapeutic agent is unlikely to be an effective treatment strategy. Hence, a combination therapy towards multiple targets would eventually be required. In this review, we delineate the treatment options in ALD and NAFLD, including various new targeted therapies that are currently under investigation. We hope that soon we will be having an effective multi-therapeutic regimen for each disease.

Key words: Alcoholic liver disease; Non-alcoholic fatty

liver disease; Treatment options; Glucocorticoids; Liver transplantation

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Core tip: Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health problems worldwide. Despite extensive research on understanding the pathophysiology of both these diseases there are still no targeted therapies available. In this review, we delineate the treatment options in ALD and NAFLD, including various new targeted therapies that are currently under investigation.

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INTRODUCTION

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are serious health issues whose incidences are on the rise with each passing decade. Alcohol is responsible for approximately 4% of all deaths annually and 5% of all disabilities worldwide[1]. The Centers for Disease Control and Prevention in 2013 have estimated that in the United States acute deaths from alcohol attributable causes have outnumbered deaths from chronic diseases (44000 to 35000) (http://apps.nccd.Cdc.gov/ardi/homepage.aspx). Motor vehicle accidents have been considered the leading cause of acute death from alcohol-attributable injuries. While the incidence and prevalence of NAFLD is on the rise with each passing decade. Presently, 25%-35% and 5%-15% of the general population of Western and Asian countries, respectively, are affected by this disease[2]. This proportion is even higher in people with type 2 diabetes (60%-70%), and in those who are obese or morbidly obese (75%-92%) compared to the general population[3-5]. The prevalence of obesity in the United States has increased from 10% to 60% of the total population in the last three decades and is considered to be one of the main factors for the increasing prevalence of NAFLD[6].

The risk factors for both these diseases are well known. Patients with ALD consume an excessive amount of alcohol while NAFLD patients are usually obese; have insulin resistance and/or metabolic syndrome. Available data from various studies show that NAFLD may be the hepatic manifestation of metabolic syndrome[7]. The spectrum of both diseases ranges from benign steatosis to hepatitis to cirrhosis and hepatocellular carcinoma. Most patients with NAFLD or ALD have hepatic steatosis which is usually asymptomatic; only 20%-35% of these patients' progress to steatohepatitis or cirrhosis[8]. The role of genetic polymorphism, mainly the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene (rs738409 variant), has also recently been shown to be a risk factor for progression to advanced liver disease in both NAFLD and ALD that could explain why only a subset of patients who are chronic alcohol abusers or have high caloric intake present with progressive liver injury. Evidence from recent studies has shown this variability may be related to *PNPLA3* variant (rs738409). The single nucleotide polymorphism rs738409 variant within the *PNPLA3* causes a substitution of methionine for isoleucine at position 148. The GG phenotype of this PNPLA3 variant, rs738409, predicts a greater risk of progression to cirrhosis and HCC than the GC and CC phenotype which have shown to have a smaller risk for progression[9-12]. Despite an increased understanding of the pathophysiology and risk factors for ALD and NAFLD, we still do not have an appropriate therapeutic regimen for either disease.

The treatment options of ALD have not changed in the last four decades, and abstinence is still the cornerstone of treatment. This is supported by nutrition therapy and steroids[13,14]. Unfortunately, alcoholic hepatitis, which is the most serious manifestation of ALD, has a short term mortality of up to 50% in patients who are unresponsive to corticosteroid treatment[15]. Furthermore, limited treatment options are available for patients who are steroid non-responders or have contraindications to steroid usage (upper gastrointestinal bleed, impaired renal functions and sepsis). While the treatment for NAFLD is mainly directed toward attenuating the risk factor such as gradual weight loss by lifestyle modification with a focus on nutrition and exercise[16,17], other therapies utilizing insulin sensitizers (thiazolidinediones) and antioxidants (vitamin E) also have been found to be useful. However, their long-term safety and adverse effects have not been rigorously evaluated.

Thus, effective and safe therapeutic regimens are needed for these liver diseases. In this review, we present the current therapies as well as upcoming potential new approaches and treatment strategies for both diseases.

ALD TREATMENT

General management

For the last 50 years, abstinence has remained the primary therapy for ALD treatment. However, serious symptoms develop with the abrupt cessation of alcohol. Treating the alcohol withdrawal syndrome is thus extremely important and requires administration of fluid, calories, vitamins and minerals. Unstable patients need to be admitted to a critical care unit and airway protection is often required in patients with hepatic encephalopathy. Table 1 summarizes the treatment options and potential new options for ALD

Table 1 Treatment options for alcoholic liver disease and alcoholic steatohepatitis

General management
Abstinence
Nutritional support
Glucocorticosteroids
Pentoxifylline
Anti-TNF therapy
Antioxidants
Liver transplantation
Potential new therapies
Probiotics and antibiotics
S-adenosylmethionine
Betaine
Targeting various chemokines and interleukins
Endocannabinoids antagonists
Osteopontin inhibition
Stem cell therapy

and ASH (alcoholic steatohepatitis).

Alcohol withdrawal syndrome: This syndrome is characterized by symptoms that occur 6-24 h after abrupt cessation of alcohol in patients who drink consistently and excessively. Long acting benzodiazepines like chlordiazepoxide or diazepam are administered for prevention of seizures while intermediate acting benzodiazepines like lorazepam are recommended in withdrawal patients who are elderly or have had recent head trauma or liver or respiratory failure[18]. Antiepileptic like carbamazepine can also be used as a benzodiazepine substitute for preventing seizures. Antipsychotics like haloperidol can be used if patients have excess agitation or psychotic symptoms[18]. Alcoholics are usually malnourished and deficient in vitamins, especially vitamin B1 (thiamine), thus putting them at risk of developing Wernicke encephalopathy, so all such patients should be given thiamine[19]. Parenteral thiamine is preferred over oral thiamine because in addition to impaired gastrointestinal absorption in alcoholics, oral thiamine has poor bioavailability that does not allow for attaining a sufficient concentration in cerebrospinal fluid. However, parenteral thiamine has a short half-life, thus multiple high-dosing is required to achieve sufficient concentration for active and passive diffusion through the blood brain barrier[20-22]. An IV dose of 500 mg (three times daily for two consecutive days) is recommended, followed by 500 mg of IV or IM thiamine for five more days if a response to the therapy is seen[23-25]. Another dosing regimen is 500 mg of IV thiamine (three times daily for 2-3 d), followed by 250 mg of IV thiamine for the next 2-3 d. The intravenous regimen is then followed by oral thiamine treatment indefinitely[26,27]. Thiamine should be given before fluids containing glucose to prevent neurological damage.

Abstinence: The first step towards treatment requires the patient to accept the fact that they are dependent and addicted to alcohol. Abstinence can resolve alcoholic fatty liver disease and can improve the survival rate of cirrhotic or decompensated liver failure patients. Thus, motivating the patients to abstain from alcohol and follow the proper treatment regime are major steps. However, preventing relapse in such patients has always been a big challenge. Patients can participate in Alcoholics Anonymous group meetings for self-control and motivation. Psychological support by an addiction specialist can also help in maintaining sobriety. Recognizing and treating any associated psychiatric conditions can be helpful in such patients[28]. Pharmacotherapy also helps in maintaining sobriety; drugs like naltrexone and acamprosate assist in reducing alcohol intake in heavy drinkers[29,30]. Topiramate has found to be effective in decreasing craving and withdrawal symptoms in alcoholics[31]. Disulfiram, an acetaldehyde dehydrogenase inhibitor, is also being used. It causes accumulation of serum acetaldehyde, which produces unpleasant sensations of nausea, vomiting, abdominal pain and dizziness. Such sensations deter patients from consuming alcohol[32]. Baclofen, a γ-amino butyric acid agonist, has also been found effective in promoting abstinence[33]. Most of these drugs (disulfiram, acamprosate, naltrexone, topiramate and baclofen), are used only for treatment of alcohol dependence[34] but are not FDA approved for ALD treatment. Of these, only baclofen has been studied for its safety and efficacy in clinical trials that compared placebo and baclofen treatment for ALD patients. In this trial, baclofen (10 mg or 20 mg twice daily) was more effective than placebo in maintaining abstinence. A 53% reduction in the number of drinks per day in the 10 mg baclofen group ($P < 0.0001$) and a 68% reduction in the number of drinks per day in the 20 mg baclofen group was reported, thus demonstrating a dose response effect of baclofen[35]. Naltrexone and disulfiram should be avoided in patients with liver problems because of hepatotoxicity[36]. Moreover, naltrexone should also be avoided in patients with renal failure due to its active tubular secretion[37]. Acamprosate, topiramate and baclofen were found to be safe in such patients[33,38,39]. Another drug which can help to maintain abstinence and reduce craving is metadoxine (MTD). Although not available in the United States, MTD is approved for use in several European countries. Apart from sustaining abstinence, oral MTB is also useful in acute ethanol intoxication since it is rapidly absorbed and augments alcohol metabolism by enhancing acetaldehyde dehydrogenase activity[39]. In a clinical trial with ASH patients, MTX improved liver function tests in 1 mo compared to the placebo[40]. In another trial, improved 3-6 mo survival of severely ill ASH patients who received the combination therapy with MTX compared to those that received monotherapy with either steroids or pentoxifylline (PTX) was observed. The survival rate was higher of the MTD-treated groups at

3 mo (PTX + MTD 59.4% *vs* PTX 33.3%, $P = 0.04$; steroids + MTD 68.6% *vs* steroids 20%, $P = 0.0001$) and at 6 mo (PTX + MTD 50% *vs* PTX 18.2%, $P = 0.01$; steroids + MTD 48.6% *vs* steroids 20%, $P = 0.003$) than in the non-MTD treated groups. Furthermore, the patients receiving MTD maintained greater abstinence than those not on MTD (74.5% *vs* 59.4%, $P = 0.02$)[41]. Smoking and obesity are independent risk factor for the progression of ALD[42,43]. Hence, lifestyle modifications like weight loss and smoking cessation are also helpful. Hepatitis C virus (HCV) is another independent risk factor for ALD progression due to synergistic deleterious effects of both agents in promoting liver injury and HCC development[44,45]. The main mechanisms of this effect are that both alcohol and HCV alter cellular immunity, increase free radical oxidative damage, and in the case of alcohol exposure, promote replication of HCV. Thus, the combination often result in the presence of advanced liver disease with severe histological features at a much younger age and a decreased survival[46,47]. It has been reported that alcoholic patients with HCV infection have a 30 fold increased risk of getting cirrhosis[48] and two to eight fold increased risk of all-cause mortality compared with those without the HCV infection[49,50]. Thus, all ALD patients should be screened for HCV before starting treatment and all HCV patients should be advised to stop or reduce alcohol consumption[51].

Nutritional support: Most patients with ALD are malnourished, and disease severity often correlates with the degree of malnutrition[52]. Most of the complications of ALD are strongly associated with protein calorie malnutrition[53]. Thus, nutrition support is one of the important steps in ALD treatment. Vitamins (like folate, vitamin B6, vitamin B12[54,55], vitamin A and thiamine[56] and minerals (like selenium, zinc, copper, and magnesium) are often found to be altered in ALD and some believe that these alterations play a role in initiation and progression of liver injury[57]. Especially, zinc levels are decreased in ALD patients and in animal models, and its supplementation has been shown to improve ALD[58]. A major study has also shown that enteral nutrition reduces infectious complications and improves 1-year mortality in such patients[59,60].

The American College of Gastroenterology and the American Association for the Study of Liver Diseases guidelines recommend 1.2 to 1.5 g/kg per d of protein intake and 35 to 40 kcal/kg per d of body weight for energy intake in patients with ALD[61]. This type of malnourished patient is often predisposed to infections so empiric antibiotic treatment is also advised.

Glucocorticosteroids: There have been various clinical trials on the use of corticosteroids for treating ALD patients[62-64]. Despite mixed outcomes, corticosteroids are overall beneficial for survival of these patients. Unfortunately, 40% of patients are unresponsive to corticosteroid with virtually no other treatment options. Hence, new target oriented therapies are critically required for the management of such patients[15].

A meta-analysis which pooled data from 3 randomized control trials, found that patients with modified DF $\geqslant$ 32 or MELD score $\geqslant$ 21 treated with prednisolone at 40 mg/d for 28 d and then a tapered dose over 2-4 wk (Class I, level A), conferred a 28-d survival benefit of glucocorticoids (85%) *vs* placebo (65%), with mortality decreasing from 35% in controls to 15% in patients on steroids[64]. Early changes in bilirubin levels (at day 7 of treatment) and the Lille score were used to predict the prognosis following steroid administration[65]. A Lille's score greater than 0.45 on the 7th d after initiation of the treatment indicated that the patient was unresponsive to steroid therapy and predicted a lower survival rate of 25% at 6 mo. Recently this score has been re-classified as complete responders (score $\leqslant$ 0.16), partial responders (score 0.16-0.56), and null responders (score $\geqslant$ 0.56), and is associated with the 28-d survival rate of 91%, 79% and 53%, respectively, with $P < 0.0001$[13]. Steroids have been found to have a significant beneficial effect in complete and partial responders but not in null responders, hence discontinuation of steroid therapy is recommended for non-responders[66]. In addition to non-responders, steroids are generally avoided in patients with active infection, gastrointestinal bleeding, chronic hepatitis B virus infection or hepatorenal syndrome (HRS) because of adverse effects in these patient populations[67]. Steroids are relatively contraindicated in severe AH patients with coexistent sepsis. Thus, such patients may be treated with second line drug PTX[68]. Patients should also be screened for any infection before starting steroids and for infective complications while on steroids. Occurrence of sepsis and infective complications while the patient is on steroids is a poor prognostic sign[69]. It has been reported that patients infected after initiation of steroids had a significant lower 2-mo survival than patients with no infection (46.4% ± 6.9% *vs* 77.5% ± 3.2%, $P < 0.00001$). Thus, it is very important to differentiate infection at admission from that which occurs after starting the steroid treatment, as survival rates differ significantly. Overall, infection was more common in steroid null responders than responders[70].

PTX: Steroids are generally used as the first line of treatment in severe alcoholic hepatitis patients with DF $\geqslant$ 32, except in those with renal failure or HRS or contraindication to steroids[71]. PTX (400 mg 3 times per day for 28 d) is a substitute in such cases (Class I, level B). PTX decreases pro-inflammatory cytokines like TNF-α, has anti-fibrotic properties[72] and confers a mortality benefit by reducing the incidence of HRS[73]. A pilot study in ASH patients using PTX demonstrated reduced mortality and HRS incidence when compared to patients given a placebo[74]. These findings were later confirmed in a double-blind placebo controlled trial, where PTX decreased the 28-d mortality compared to

placebo (24.5% *vs* 46%). Also, 50% of those who died in the PTX group developed HRS, while 91.7% who died in the placebo group developed HRS, confirming that PTX reduces the incidence of HRS in such patients[75]. A study in ASH patients comparing PTX and prednisolone have shown a better survival rate of 35.29% in the PTX group *vs* 14.71% on steroids. This reduced mortality was presumably because of a decrease in incidence of HRS and gastrointestinal bleeding in the PTX group. However, this study was underpowered[76]. To date, no other study has shown any additional survival benefit of the combined PTX and corticosteroid treatment[77,78]. A recently conducted randomized, multicenter, double-blind trial (STOPAH) across 65 hospitals in the United Kingdom that recruited more than a thousand patients revealed no impact of PTX on survival or disease progression in severe non-alcoholic steatohepatitis (NASH) patients in comparison to placebo[79,80]. However, because of a lack of other treatment options, PTX is being used in some centers.

Anti-TNF therapy: Intestinal gut permeability is increased in chronic alcoholics that promotes the translocation of gut luminal antigens especially endotoxin to reach the liver and enhance TNF-α production[81]. TNF-α has been found to correlate with disease severity in severe alcoholic hepatitis patients[82], and also play a vital role in alcohol induced liver injury in various animal models of alcoholic liver injury[83]. Further, mice deficient in TNF receptor 1 do not develop liver injury when administered alcohol[83]. Based on these considerations, various human studies were undertaken using anti-TNF therapy. While initial studies were found to be promising, the results could not be duplicated in larger clinical trials. A large randomized controlled trial comparing prednisolone alone with a combination of prednisolone and infliximab had to be stopped before completion because of an increase in infection rate in the prednisolone and infliximab combination group[84]. Further, patients had to be screened for tuberculosis and nocardia infection prior to participation in the study, thus limiting its clinical utility[85].

Antioxidants: Alcohol causes oxidative stress by increasing reactive oxygen species (ROS), and decreasing endogenous antioxidant levels[86]. But to date, all trials examining antioxidants (such as lecithin, β-carotene, vitamin C, vitamin E, allopurinol, desferrioxamine, and N-acetylcysteine) either alone or in combination with steroids have been disappointing[87,88].

Liver transplantation: Liver transplantation remains the definitive therapy for end stage decompensated cirrhosis due to ALD. Severe alcoholic hepatitis patients nonresponsive to steroids have a 3 mo mortality rate of 70% and with HRS the mortality rate is ⩾ 90% unless the patients get liver transplantation[89,90]. At present, there are very few options for treating severe alcoholic hepatitis patients who are non-responsive to steroids and have a Lille score > 0.56. Thus, liver transplantation remains the only hope for such patients, but the issue of transplantation in alcoholics has always remained controversial. Concerns include the risk of recidivism, poor compliance with postoperative care, and ALD being a self-inflicted disease[91]. Recidivism following transplantation is a major challenge, which occurs at a rate of 10%-50%[92,93]. A meta-analysis reviewing factors responsible for recidivism found 3 major variables: a poor social support system, a family history of alcohol abuse/dependence and pre-transplant abstinence of 6 mo or less[94]. Thus, we need a multidisciplinary approach including the presence of an Alcohol Addiction Unit which can significantly contribute in reducing alcohol relapse after transplantation. Also, there should be psychological evaluation for any mental illness to determine patient suitability for transplantation.

Most transplant programs require the patients to undergo a 6-mo period of abstinence prior to transplantation[95]. Studies over the years have provided data both for and against the 6-mo abstinence rule. One report suggested that the 6-mo period of abstinence would allow the liver to recover with medical treatment and possibly there would be no need for transplantation[96]. Another study revealed that some recovery in liver function can take place within 3 mo of abstinence while many patients may die during the 6 mo of waiting period. This led to the suggestion of possibly reducing the period of abstinence to 3 mo[97]. Yet another study has also challenged the 6-mo abstinence rule by showing beneficial effects of early liver transplantation in steroid non-responding severe alcoholic hepatitis patients. In this study patients (with Lille score of 0.88) after 13 d of being unresponsive to steroids were put on the transplant list and it was found that the 6-mo survival rate was higher in patients who received early transplantation than those who did not (77% *vs* 23%, $P < 0.001$)[98].

However, patients who have received liver transplantation show a high incidence of de novo cancer[99,100], lymphoproliferative disorder and skin cancer. In some cases, squamous cell carcinoma of the oropharynx or esophagus has also been detected, likely due to the cumulative effects of smoking and post-transplant immunosuppressive drugs. Also, liver transplantation due to ALD is associated with a high rate of cardiovascular complications[101].

Potential new therapeutic options in ALD

Advances in basic science have helped to gain better insights into the pathophysiology of ALD that have provided new treatment options as discussed below.

Role of probiotics and antibiotics: Healthy intestinal flora is critically important for our well-being.

An alcohol-induced change in the gut microflora plays a major role in the pathogenesis of alcoholic hepatitis. Equally important in liver disease progression is the alcohol-induced increased intestinal permeability that allows for the gut luminal antigens, including endotoxin/LPS (component of the cell wall of gram negative bacteria), to reach the liver and promote the synthesis and secretion of several inflammatory cytokines[102]. Various studies have proposed the use of probiotics in restoring the normal bowel flora in patients with ALD[103]. In a study performed on patients with ALD it was shown that using probiotics (Bifidobacterium or Lactobacillus) for 4 wk enhances and normalizes neutrophil phagocytic capacity and helps in reducing endotoxin-driven elevation in cytokine levels[104]. A similar study revealed significant improvement in AST, ALT and γ-glutamyl transferase levels in ALD patients administered probiotics (Bifidobacterium or Lactobacillus) for 5 d[105]. Rifaximin, a biochemical derivative of Rifamycin, the drug for hepatic encephalopathy, given for 28 d in a clinical trial decreased systemic endotoxin levels[106]. Indeed, blood LPS levels help in predicting response to steroids and mortality of alcoholic hepatitis patients[107]. Thus, modifying the gut microbe flora by probiotics and antibiotics could be a potential therapeutic approach for treating ALD which is being actively pursued.

Role of S-adenosylmethionine and betaine: S-adenosylmethionine (SAM) is a key methyl donor that is involved in many methylation reactions critical for normal liver function. SAM also acts as an antioxidant by activating the pathway for GSH synthesis. Decreased SAM levels have been reported in ALD patients; thus, elevating SAM levels could be a potential therapy. Various animal studies have shown liver injury can be reversed by preventing a decrease in SAM levels[108]. Also, SAM administration decreases oxidative stress and hepatic stellate cell activation[109]. A randomized controlled trial using SAM or placebo for 2 years in alcohol cirrhotic patients found that the mortality and liver transplantation rates were higher in the placebo group than in the SAM group (29% *vs* 12%)[110]. Thus, there is need for long-term, high quality trials in the future to establish its effectiveness. Along the same line as SAM, betaine treatment has been very effective in improving liver injury in various animal models[111,112]. By remethylation homocysteine to generate methionine, betaine not only removes the toxic metabolites homocysteine and S-adenosylhomocysteine, but also generates SAM and normalizes the methylation potential[113]. Betaine is hepato-protective and prevents alcohol-induced steatosis, oxidative stress, apoptosis and abnormal protein accumulation[111,112], and breakdown of sulphur containing amino acid[114]. Clinical trials using betaine should be conducted.

Role of targeting various chemokines and interleukins: Chemokines play a pivotal role in the pathogenesis of alcoholic hepatitis. Studies have shown that various chemokines and their subfamily members, including CXCL5, CXCL6, CXCL10 and CCL20, are notably high in ASH livers compared to normal control livers and higher levels correlate with worse prognosis and outcomes[115,116]. Of these, CCL20 is the most elevated chemokine in ASH livers that attracts lymphocytes, monocytes, Th17 (Helper T17) cells, and dendritic cells. The consequent production of more chemokines and inflammatory mediators ultimately causes heavy neutrophilic infiltration and liver damage[117,118]. Additional studies in the future are required to determine if targeting CCL20 and other chemokines can be an effective and safe therapeutic approach for ALD patients.

IL-8 is one of the most important chemoattractant of neutrophils, which further causes hepatic infiltration as well as increased portal pressure[115]. A higher level of IL-8 in alcoholic hepatitis patients is associated with worse prognosis[115]. A therapeutic approach towards counteracting IL-8 levels should be considered as it will decrease neutrophil infiltration of the liver and prevent progressive liver damage.

IL-22 plays a critical role in bacterial infections and tissue repair. It is a part of the IL-10 family which decreases the production of various pro-inflammatory cytokines[119]. IL-22 has been found to have anti-apoptotic, antimicrobial, antioxidant and anti-steatotic effects, thus it can be used as a therapeutic option in ALD patients. It has been found that levels of T helper cells producing IL-22 correlate with improvement in alcoholic hepatitis patients[120]. Recombinant IL-22 administration showed improvement of liver injury in ethanol-fed mice[121] and in an animal model of acute hepatitis while blocking the IL-22 receptor led to worsening of the disease[122]. Thus, up regulating IL-22 levels can be a potential therapy for ALD.

IL-17 increases chemotaxis of neutrophils and various other chemokines and its levels are found to be increased in alcoholic hepatitis[123]. Secukinumab, an anti-IL-17 monoclonal antibody has shown favorable results in clinical trials of rheumatoid arthritis, psoriasis and uveitis[124]. Up until now, no study has been done in patients with liver disease using this monoclonal antibody, which can be a potential therapy.

Role of endocannabinoids: Endocannabinoids signalling through cannabinoid receptors, CB-1 and CB-2, has been implicated in the pathogenesis of ALD[125]. Studies using animal models of alcoholic liver injury revealed that CB1-deficient mice are resistant, whereas CB2-deficient mice are more susceptible to fatty liver damage[126,127]. These findings suggested that therapy targeting CB1 and CB2 receptors should be utilized as an alternative for the management of ALD.

Role of osteopontin: There is substantial evidence

Table 2 Treatment options for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Lifestyle changes	Weight loss
	Dietary changes
	Exercise
Insulin sensitizers	Thiazolidinedione's
	Metformin
Lipid lowering agents	Statins
	Ezetimibe
Hepatoprotective agents	UDCA
Antioxidants	Vitamin E
Incretin analogues	GLP-1 agonists
	DPP-IV inhibitors
Anti-inflammatory agents	PTX
Others	Probiotics
	Angiotensin receptor blockers
	Endocannabinoid antagonists
	Bariatric surgery
	Liver transplantation
Potential new therapeutic options	Caspases inhibitors
	ASK1 inhibitors
	p38 MAPK inhibitors
	PPAR- alpha and delta agonists
	FXR agonists
	NOX-1/4 inhibitors
	Galectin-3 antagonists
	Acetyl CoA carboxylase inhibitors
	FGF-21 and FGF-19 analogues
	CCR2 and CCR5 inhibitors
	SCD-1 inhibitors
	Lysyl oxidase-like 2 inhibitors
	Sirtuins

GLP-1: Glucagon-like peptide 1; DPP-IV: Dipeptidyl-peptidase IV; PTX: Pentoxifylline; PPAR: Peroxisome proliferator activated receptor; NOX: NADPH oxidase; FGF-21: Fibroblast growth factor 21; SCD1: Stearoyl coenzyme A desaturase 1.

suggesting that osteopontin (OPN) plays a notable role in wound healing in response to injury in many organs[128]. It is an extracellular matrix protein with pro-fibrogenic properties, and is found to be highly expressed in alcoholic hepatitis patients[129]. One study demonstrated attenuation of alcohol mediated liver disease in mice lacking OPN[130]. More studies should be conducted to assess OPN as a potential therapeutic target.

Stem cell therapy: Hematopoietic stem cell transplant ion is an evolving field. Though limited research has been performed in this area, it could be a promising therapeutic approach in the future. Recent studies have suggested that stem cell transplantation may reduce liver inflammation and improve fibrosis in patients with liver cirrhosis[131]. Mesenchymal stem cells (MSC) directly inhibit the activation of hepatic stellate cells and may also induce apoptosis of hepatic stellate cells[132]. They have also been reported to stimulate proliferation of endogenous hepatocytes[133,134]. A pilot study performed on 12 patients with ALD to assess the regenerative capacity of the liver after infusion of bone marrow derived-MSC through the hepatic artery showed improvement in the histological grading with an overall decrease in TGF-β, type 1 collagen and smooth muscle actin[135]. A significant improvement in the Child-Pugh score and albumin levels was noted in another similar study on 9 cirrhotic patients given bone marrow derived stem cells *via* the portal vein[136]. Liver function was also reported to be better after stem cell therapy in cirrhotic patients[137]. Results of these studies are encouraging and stem cell therapy could serve as a potential breakthrough treatment for ALD. However, the benefits and safety of stem cells should be examined in a large sized RCT.

NAFLD TREATMENT

Like ALD there is no effective treatment to date for NAFLD. In the absence of a proven effective therapy, we must follow a multi-disciplinary approach in NAFLD treatment, where a combination of drugs and factors are taken into consideration to counter multiple pathological risk factors involved in NAFLD. These are summarized in Table 2 and are further discussed below.

Weight loss, dietary modification and changes in lifestyle

Treatment is mainly directed towards weight loss and risk factor reduction, as most patients are obese or have metabolic syndrome[138]. A weight loss of 3%-5% reduces steatosis while a ⩾ 5%-7% drop in weight has been shown to resolve NASH. Greater reductions in weight (*i.e.*, ⩾ 10%) may also improve hepatic fibrosis. Weight loss is mainly due to diet modification and exercise. However, the shortcoming of this approach is the lack of adherence and non-compliance with time[139]. Various studies have shown the benefit of weight loss in NAFLD[140]. Dietary modification also plays a key role since a carbohydrate-rich diet, especially with high fructose, is the major cause of obesity, insulin resistance and NAFLD development[141]. Thus, sugar consumption should be kept below 10% of total caloric intake in a day and a fructose-rich diet should be avoided in such patients. Food rich in omega-3 fatty acid should be included and those rich in saturated fat and omega-6 fatty acid should be excluded from the diet[142]. An omega-3 fatty acid rich diet promotes fatty acid oxidation and decreases fatty acid synthesis, thus improving the lipid profile. Fish and fish oil consumption should be promoted as they are rich in omega-3 fatty acid[143]. Thus, diet and moderate exercise are preferred methods of natural weight loss. A study also revealed that a combination of diet changes and exercise lowered ALT levels better in NAFLD patients than insulin sensitizers or other hypoglycaemic drugs[144]. Weight loss is also beneficial as it improves the cardiovascular risk profile[145]. Nevertheless, it should be noted that weight loss should be gradual, as very rapid weight loss has been associated with a worsening of steatohepatitis and an increased risk for liver failure[146] and gallstones[147].

Apart from natural weight loss, drugs like Orlistat and Sibutramine are also being used for controlling weight. Orlistat is a lipase inhibitor, that prevents fat absorption in the liver and intestine, thus causing weight loss. Sibutramine on the other hand is a serotonin reuptake antagonist which suppresses appetite. Both agents have shown to reduce serum transaminase levels and hepatic steatosis[148,149].

Insulin sensitizers

Since NAFLD is closely associated with obesity and metabolic syndrome, and both conditions cause insulin resistance, treatment strategies invariably include agents which enhance insulin sensitivity.

Thiazolidinedione: Thiazolidinediones (TZDs) are peroxisome proliferator activated receptor (PPAR)-γ agonists, which improve hepatic and peripheral insulin sensitivity[150] *via* increasing plasma adiponectin levels[151]. In addition, adiponectin is also shown to have anti-fibrotic and anti-inflammatory properties. Thus, multiple factors involved in pathogenesis of NAFLD such as high insulin resistance, low adiponectin levels and high pro-inflammatory cytokines are all targeted by these drugs. First generation TZDs (troglitazone) have shown improvement in steatohepatitis but had to be stopped due to hepatotoxicity[152]. However, it paved the way for second generation TZDs (rosiglitazone and pioglitazone) which are not hepatotoxic and showed improvement in insulin resistance, hepatic steatosis and aminotransferases levels[153,154]. A long-term therapy with second generation TZDs may be required as their benefits tend to reverse on discontinuation; however long term therapy is associated with various adverse effects like congestive heart failure, weight gain, peripheral oedema, anaemia and osteoporosis[155,156]. Also, it has been found that TZD therapy alone without nutrition and lifestyle changes is often not effective[154]. Thus, we need additional studies on a larger population with a combination of other drugs to find safe and efficacious treatment options.

Metformin: Metformin, a hypoglycaemic drug, is used for treatment of type 2 diabetes mellitus. Metformin improves hepatic and peripheral insulin resistance by decreasing hepatic gluconeogenesis, lipogenesis and glucose reabsorption from the gut and increasing fatty acid oxidation[157]. While metformin does not cause weight gain as TZDs, it can cause some minor gastrointestinal adverse effects and sometimes lactic acidosis is seen in patients with renal impairment. Various studies have documented an improved insulin sensitivity, cholesterol and aminotransferase levels in NASH patients on metformin but the results are mixed when assessing biopsy-guided improvement in steatosis and NASH activity score (NAS)[158]. Thus, while its effectiveness as a monotherapy is debatable, metformin could be a part of a multi-therapeutic regimen for the management of NAFLD patients.

Lipid lowering agents

NAFLD is often associated with obesity and metabolic syndrome which is characterized by hypercholesterolemia and hypertriglyceridemia. Therefore, the use of lipid lowering agents could be beneficial. While, clofibrate did not show any beneficial effect on the liver tests or the histological scores[159], gemfibrozil showed improvement in ALT levels in NAFLD patients compared to the placebo[160]. Statins have also been tried but have shown variable effects. Nevertheless, lipid-lowering agents should be given as most NAFLD patients are hyperlipidemic and thus have a high risk of developing cardiovascular issues.

Ezetimibe, a drug that inhibits the reabsorption of lipids from the intestine, reduces serum TNF-α levels[161], hepatic lipid content and ALT levels in a mouse model of NAFLD[162]. Human studies for this drug are awaited.

UDCA

This drug has hepatoprotective properties and has been studied in various clinical trials for NAFLD treatment. Initial small studies revealed an improvement in liver enzyme levels and hepatic steatosis[159], but a subsequent RCT showed no improvement in liver histology or aminotransferases[163]. Thus, UDCA is not approved as a monotherapy but is part of a drug combination regime in various trials on NAFLD in progress.

Vitamin E

ROS generation plays an important part in the progression of NASH[164]. Vitamin E and C decrease oxidative stress and thus have been evaluated in patients with NASH. Various clinical trials with vitamin E have revealed an improvement in liver test functions and reduction in oxidative stress markers but significantly less improvement in the histological grading of the disease has been noted[165,166]. A recent trial using a combination of vitamins E and C for 6 mo showed that these were no better than placebo for treating patients with NASH[167]. One study with a three arm trial involving placebo, UDCA and Vitamin E/UDCA combination showed improvement in histology only in the Vitamin E/UDCA combination arm[168]. Another trial comparing a combination of pioglitazone and vitamin E with vitamin E alone over a period of 6 mo showed a decrease in serum ALT in both groups, but a significant histological improvement was only seen in the combination group[169]. A meta-analysis involving high-dose vitamin E supplementation has shown an increase in all-cause mortality and cardiovascular deaths, thus decreasing the enthusiasm for vitamin E therapy[170].

Incretin analogues

Glucagon-like peptide 1 agonists: Glucagon-like peptide 1 (GLP-1) is an incretin hormone that is

produced by intestinal mucosa L cells. GLP-1 has a short half-life, as it is rapidly degraded by dipeptidyl-peptidase IV (DPP-IV). GLP-1 agonists are resistant to DPP-IV and are useful since they lower blood glucose levels by decreasing glucagon secretion, delay gastric emptying and stimulate pancreatic β cells to increase insulin secretion. Furthermore, these agonists have a central appetite suppressive effect and promote weight loss which are favorable outcomes for obese NAFLD/NASH patients[171]. In an obese mouse model, it has shown to improve insulin sensitivity and reduce hepatic steatosis[172].

In various clinical trials, liraglutide has proven to be an effective therapeutic drug for type 2 diabetics producing a good glycemic control and significant weight loss in such patients. Since diabetes is an important component of metabolic syndrome and associated NAFLD development, the effective glycemic control and weight loss makes liraglutide a suitable therapeutic option for NAFLD[173]. In a phase 2 clinical trial study (LEAN study) with 52 NASH subjects using liraglutide compared to placebo, 39% of patients using liraglutide *vs* 9% using placebo attained the primary endpoint (histological resolution of NASH without worsening of fibrosis). Two (9%) of 23 patients in the liraglutide group *vs* eight (36%) of 22 patients in the placebo group had progression of fibrosis. The trial was designed using A'Hern's single-group method, which required eight (38%) of 21 successes in the liraglutide group for the effect of liraglutide to be considered clinically significant. The liraglutide treatment group has also shown improved insulin sensitivity, reduced hepatic glucose production and lipogenesis (ClinicalTrials.gov-NCT01237119). Thus, liraglutide was safe, well-tolerated, and led to histological resolution of NASH, warranting longer term studies in such patients[174,175].

DPP-IV inhibitor: DPP-IV inactivates both incretin hormones (GIP, GLP-1), therefore DPP-IV inhibitors are used in the treatment of type 2 diabetes[176]. NASH patients exhibit higher DPP-IV expression[177]. A cross sectional study on type 2 diabetics without any evident liver disease and NAFLD patients revealed a strong positive correlation of serum DPP-IV activity and insulin resistance with liver enzymes only in NAFLD patients [178]. Serum DPP-IV activity was not increased in the type 2 diabetics with no evidence of liver disease. This led the authors to postulate that the increased serum DPP-IV reported in earlier studies in type 2 diabetics may have been due to some un-diagnosed liver disease and that the excess DPP-IV found in the serum of NAFLD patients is of hepatic origin. They further suggested that serum DPP-IV should be considered as a potential liver disease biomarker[178]. This supposition was also corroborated by another study which analyzed human liver biopsy specimens and showed a strong correlation of DPP-IV expression with stages of fatty liver and NASH[179].

Furthermore, a DPP-IV inhibitor like sitagliptin, decreases hepatic steatosis and serum transaminases levels when given to diabetic NAFLD patients[180,181]. In a recent randomized, double-blind, placebo controlled study, sitagliptin was shown to be safe but no more effective than placebo in improving hepatic steatosis and fibrosis in NAFLD patients. However, in comparison to sitagliptin, an increase in hyaluronic acid levels and increase in FIBROSpect II index (measure of liver fibrosis) was reported in the placebo arm[182]. Another RCT comparing sitagliptin to placebo also revealed no improvement in fibrosis score or NAS after 24 wk of therapy, but reported that sitagliptin increased adiponectin and decreased γ-glutamyl transferase levels[183]. There have been only a few clinical trials with sitagliptin till date. However, despite the lack of convincing evidence, the efficacy of sitagliptin in improving liver fibrosis in NAFLD cannot be ruled out. This is because not only were the trials underpowered but were possibly not long enough to evaluate its effectiveness. Hence, stagliptin effect should be assessed in clinical trials of longer duration with larger number of enrolled patients with NAFLD/NASH.

PTX

PTX can be of potential benefit in NAFLD due to its effects on reducing free radical oxidative stress, TNF-α levels, and potential anti-fibrotic properties[184]. In some trials, PTX has shown improvement in steatosis, lobular inflammation and ballooning degeneration in comparison to baseline, but improvement was not clinically significant when compared to placebo[185]. In a small RCT on NASH patients, 400 mg PTX given three times per day for a period of 1 year decreased hepatic steatosis, inflammation and NAS by ⩾ 2 points and modestly reduced fibrosis[186]. This favorable response was due to a reduction in free-radical-mediated lipid peroxidation[187]. In two recent small RCT evaluating the role of PTX has also shown beneficial effect by improving liver enzymes and histology in NAFLD patients[188,189]. In a recent meta-analysis it was found that only PTX and OCA improve fibrosis in NASH patients[190]. Therefore, further studies are warranted to determine its role in NAFLD/NASH treatment.

Others

Probiotics: Like alcoholic patients, NAFLD patients also exhibit gut bacterial overgrowth, enhanced gut permeability and increased paracellular leakage of gut luminal antigens, factors that promote NASH development. Thus, probiotics can be a therapeutic option for NASH patients[191,192]. In a RCT, improvement in liver enzymes was noted in NAFLD patients on Lactobacillus bulgaricus and Streptococcus thermophilus treatment compared to placebo[193]. In another study, patients randomized to a combination of Bifidobacterium longum with fructo-oligosaccharides plus lifestyle

modification (diet and exercise) or lifestyle modification alone for 24 wk[194], showed a significant decrease in steatosis, TNF-α, AST and NAS in the combination treatment group. Thus, probiotics could also be a part of a combination therapy for NAFLD patients.

Angiotensin receptor blockers: NAFLD is often associated with metabolic syndrome and hypertension is an important component of metabolic syndrome. Thus, angiotensin receptor blockers can be a part of combination therapy regimen of NAFLD. A small pilot study of patients with NASH showed improvements in necro-inflammation and fibrosis with losartan (an angiotensin II receptor antagonist) treatment[195]. Larger studies are required to explore their potential in the management of NAFLD.

Endocannabinoid antagonists: CB1 and CB2 are two receptors which mediate endocannabinoid (EC) activity. The CB1 receptor is mainly expressed in the brain and liver, while CB2 is mainly expressed in the immune cells. These receptors are found to be upregulated in various liver diseases[196]. Anandamide, a highly potent endogenous agonist, has been shown to promote diet-induced obesity and hepatic steatosis in mice *via* acting on the CB-1 receptors.[197] Conversely, CB-1 knockout or rimonabant (CB-1 receptor antagonist)-treated high-fat diet fed mice have less steatosis and weight gain than controls[198]. However, while rimonabant was effective in promoting weight loss of obese patients in many clinical trials, it also caused intolerable adverse effects like depression, anxiety and increased suicidal tendencies that has led to its discontinuation for routine use. Thus, novel cannabinoid type 1 receptor blockers with selectivity for peripheral receptors are required which can have favorable metabolic benefits but decreased psychiatric adverse effects[199,200].

Bariatric surgery: Steady weight loss with exercise and lifestyle modification has been found to increase insulin sensitivity and improve liver histology of NAFLD patients. But the rapid weight loss induced by bariatric surgery increases the risk of developing hepatic failure especially in the cirrhotic patients[201,202]. Bariatric surgery is mostly done in non-cirrhotic NAFLD patients who are morbidly obese. It is, however, not recommended as a primary mode of treatment in such patients as there is still a risk of developing liver failure postoperatively.

Liver transplantation: NAFLD patients with end-stage decompensated liver disease should be considered for liver transplantation. But this is not a permanent cure as NAFLD has been shown to recur in post-transplant liver[203]. This is because transplantation does not correct the multifactorial pathway alteration(s) responsible for NAFLD/NASH development. Therefore, the goals of therapy before and after transplant should be always towards weight management, proper diet consumption and adequate control of glucose and lipids.

Potential new therapeutic options in NAFLD

With advancement in the field of technology, especially bioinformatics and biogenetics, new therapies are currently being tried for managing NASH, some of which are reviewed below.

Caspase inhibition/emricasan: Caspases are enzymes which are required for completion of various apoptotic pathways and for stimulation of various cytokines and therefore, can be a potential therapeutic target. Various animal studies in the past have supported this approach[204,205]. Emricasan, a pan-caspase protease inhibitor, has been shown to inhibit apoptosis, inflammation and fibrosis in a preclinical model of NASH. A preliminary report of a phase Ⅱ clinical trial showed significantly decreased serum ALT and cCK18 levels in NAFLD patients[206]. The therapeutic effects of this drug have also been examined in various other fibrotic liver diseases where it has been shown to reduce hepatic venous pressure gradient (HVPG). A phase Ⅱ trial on NASH patients with fibrosis (ClinicalTrials.gov Identifier: NCT02686762) is ongoing to evaluate the efficacy of emricasan (10 mg and 100 mg/d for 72 wk) to improve fibrosis without worsening of NASH (primary endpoint) and to assess histological improvement or resolution of NASH (secondary endpoint).

ASK1 inhibitors/ASK1-I: Apoptosis signal regulating kinase 1/ASK1 is a MAP3 kinase (mitogen activated protein 3 kinase) which induces apoptosis and fibrosis when activated by stimuli like hyperglycaemia, TGF-β and ROS. This enzyme has been shown to be activated in patients with NASH. GS 4997, a first-in-class, oral small molecule ASK1 inhibitor, given to animals with established NASH showed a significant reduction in hepatic steatosis, fibrosis, body weight, fasting blood glucose, insulin resistance, lipogenesis, cholesterol biosynthesis, plasma AST/ALT levels, and soluble/insoluble collagen and many metabolic parameters of NASH[207-209]. GS-4997 is currently being investigated in a phase Ⅱ clinical trial of patients with NASH (ClinicalTrials.gov Identifier: NCT02466516).

p38 MAPK inhibitors: Chronic inflammation is one risk factor that contributes to progression of NAFLD. p38 mitogen activated kinases (p38 MAPK) is a stress kinase whose activation has been shown to promote inflammation[210-212]. In mammals, four p38 MAPK isoforms have been identified: p38a, b, c and d. p38 MAPK isoforms -c and -d have recently been shown to contribute to the development of steatosis and NASH in various models of NAFLD by regulating T-cell activation, neutrophil recruitment and macrophage production of TNF-α[213,214]. Studies have shown higher liver expression of p38 protein in obese individuals

with steatosis. Thus, deletion of p38 -c and -d in the myeloid cells prevents neutrophil migration to the liver, protecting these animals against diet induced steatosis and inflammation[215]. Therefore, p38 MAPK can be an effective potential target for NAFLD therapy.

PPAR-α and -δ agonists (Elafibranor): PPAR-α is mainly expressed in liver and is principally involved in lipid metabolism, while PPAR-δ is found in various tissues of the body and is involved in fatty acid oxidation and insulin sensitivity. In various animal models, PPAR has been shown to be hepato-protective *via* its effect on decreasing lipid accumulation, inflammation and fibrosis[216-218]. In a RCT (clinicaltrials.gov NCT01694849), a daily dose of 80 or 120 mg Elafibranor or placebo was given to non-cirrhotic NASH patients for 52 wk[219]. The primary endpoint of this study (i.e. resolution of NASH without worsening of fibrosis), was not met. However, it was found that patients with an initial NAS of ⩾ 4 on 120 mg/d of drug showed significant improvement in hepatic inflammation. Nevertheless, Elafibranor efficacy was described as sub-optimal. Another study is in phase 3 clinical trial to clarify Elafibranor's (GFT505) effectiveness (ClinicalTrials.gov-: NCT02704403) to improve the histological grade and reduce all-cause mortality and liver-related outcomes in patients with NASH and fibrosis.

Farnesoid X receptor/FXR agonists (Obeticholic acid): Obeticholic acid (OCA) is a Farnesoid X receptor agonist. It is a synthetic derivative of natural bile acid chenodeoxycholic acid (CDCA), with potency 100 times more than CDCA. Farnesoid X receptor is a nuclear hormone receptor which regulates bile, cholesterol, glucose and lipid metabolism[220,221]. These receptors act *via* multiple pathways; they inhibit hepatic lipogenesis, gluconeogenesis, glycogenolysis and maintain cholesterol balance and improve insulin sensitivity[222,223]. In various animal models, OCA has shown anti-inflammatory and anti-fibrotic properties and also improves insulin resistance and hepatic steatosis[224]. In an animal model, OCA was shown to reduce hepatic inflammation and fibrosis and also decreased intrahepatic vascular resistance and improved portal hypertension[225]. Also in an animal model with advanced cirrhosis, treatment with OCA was shown to reduce gut bacterial translocation from 78.3% to 33.3% ($P < 0.01$) indicating its effect in maintaining intestinal barrier integrity. Thus, it can be used as an option to prevent bacterial infection in such patients[226]. In a small pilot trial of diabetic patients with NAFLD, it was shown to decrease weight and serum γ-glutamyl transferase levels as well as an improvement in liver fibrosis[227]. The multicenter trial (FLINT trial:NCT01265498) showed a decrease in NAS, an improvement in hepatic steatosis, and a small decrease in liver fibrosis in non-cirrhotic NAFLD patients on a daily dose of 25 mg OCA compared to placebo[228]. A phase 3, double blind RCT multicenter study is ongoing to evaluate the safety and efficacy of OCA in NASH patients (ClinicalTrials.gov Identifier: NCT02548351). The effect of OCA on liver histology in non-cirrhotic NASH patients with stage 2 or 3 fibrosis will be compared to placebo. 2065 patients are randomized in 1:1:1 to receive 10 mg OCA, 25 mg OCA or placebo. An interim analysis is to be done at 18 mo and the study is expected to end in 6 years. However, an increase in total cholesterol and triglycerides with a decrease in high density lipoprotein was also seen in the OCA group when compared to placebo[228]. Two phase I studies conducted in healthy individuals given OCA for 14-20 d also reported decreased HDL and increased LDL cholesterol, regardless of the dose of OCA (5, 10 or 25 mg daily)[229,230]. These pro-atherogenic effects can be a concern for NAFLD patients that already have a high risk for cardiovascular adverse events because of dyslipidemia. Therefore, combination therapies with FXR agonist and agents that prevent atherosclerosis are warranted. Apart from OCA, various other FXR agonists such as GW4064, PX20606, GS-9674 and INT-767 are being tested. GW4064, PX20606 and GS-9674 are synthetic non-steroidal FXR agonists. INT-767 is a dual agonist for FXR and TGR5 (the transmembrane G-protein bile acid receptor) while BAR502 is a dual agonist for FXR and GPBAR1 receptors. In various animal models, these agonists have been shown to improve NASH histological features, steatosis and fibrosis [231-234]. Thus, clinical trials are anticipated for these agents as well.

NOX-1/4 inhibitors: NADPH oxidase (NOX), is an enzyme which catalyzes the production of ROS[235]. In various animal models these enzymes are expressed on hepatic stellate cells and promote liver fibrosis and inflammation[236]. In a murine model, NOX 1/4 inhibitor (GKT137831) has been found to decrease ROS production and fibrotic gene expression, thus decreasing liver inflammation and fibrosis[235]. Therefore, these agents can have a beneficial effect in decreasing liver fibrosis in NASH patients but require further studies.

Galectin-3 antagonists: Galectins are proteins that bind to terminal galactose residues on glycoproteins[237]. They are usually expressed in immune cells and are at very low levels in the body but their levels are increased during inflammation and fibrosis[238,239]. Galectin-3 knockout mice show reduced hepatic fibrosis after liver injury. GR-MD-02, a galectin-3 inhibitor, has shown a decrease fibrosis, hepatic steatosis and collagen deposition in various animal models with NASH[240]. A Phase Ⅱ clinical trial for evaluation of the safety and efficacy of GR-MD-02 for the treatment of liver fibrosis and associated portal hypertension in patients with NASH cirrhosis is currently underway (ClinicalTrials.gov Identifier: NCT02462967). This study has enrolled

subjects with portal hypertension and biopsy proven NASH cirrhosis (excluding subjects with medium and large varices and those with decompensated cirrhosis). The expected primary completion date is October 2017 while the study is expected to complete in February 2018.

Acetyl CoA carboxylase inhibitor: Malonyl coenzyme A plays a key role in fatty acid metabolism that maintains a balance between lipogenesis and lipid oxidation[241]. It promotes fatty acid synthesis, and inhibits β-oxidation of lipids. Malonyl CoA is generated from acetyl CoA and the key enzyme regulating this process is acetyl CoA carboxylase (ACC). Therefore, inhibiting ACC prevents fatty acid synthesis and promotes its oxidation. In a murine model of NAFLD, inhibition of ACC has been shown to decrease hepatic steatosis, lipogenesis and increase insulin sensitivity and fatty acid oxidation[241]. Chronic administration of ND-630 (ACC isozyme 1 and 2 inhibitor) to diet-induced obese rats and Zucker diabetic fatty rats caused a reduction in hepatic steatosis, lowered haemoglobin A1C (0.9% reduction) and improved insulin sensitivity[242]. Also in a crossover, randomized, double-blind trial, administration of a single dose of NDI-010976 (a highly potent and selective inhibitor of both ACC1 and ACC2) to overweight/obese subjects inhibited *de novo* lipogenesis in a dose dependent manner[243]. Together, all these results suggest its usefulness in treating metabolic syndrome, type 2 diabetes mellitus, and fatty liver disease. Thus, large long term clinical trials in humans are needed.

FGF-21 and FGF-19 analogues: FGF-21 (fibroblast growth factor 21) is a hormone which is secreted mainly from the liver. It is a starvation-induced peptide hormone with pleiotropic effects whose levels are mainly increased during fasting[244,245]. While FGF-21 concentrations are elevated in human subjects with NAFLD, a lack of FGF-21 worsened the metabolic disorders in an animal model of NASH[246]. Conversely, treatment with FGF-21 analogue (BMS-986036) was found to improve insulin sensitivity, hepatic steatosis and decrease lipogenesis[247]. In another animal model of NASH, LY240531 (a FGF-21 variant) was shown to increase fatty acid oxidation by enhancing hepatic mitochondrial oxygen consumption. Also, various inflammatory markers and AST and ALT levels were reduced, suggesting an attenuation of liver injury[248]. BMS-986036 is currently being evaluated in a phase Ⅱ trial of NASH patients (ClinicalTrials.gov Identifier: NCT02413372).

FXR activation in terminal ileum by bile acid promotes FGF-19 secretion which, in turn, decreases bile acid synthesis and gluconeogenesis[245]. It also results in the activation of the FGFR4 receptor which has a proliferative impact on hepatocytes, thus raising the potential for tumorigenesis[249]. NGM-282, a variant of FGF-19 has been shown to decrease bile acid synthesis and gluconeogenesis without having a tumorigenic effect[245]. In a preliminary pre-clinical study, NGM-282 was shown to improve hepatic steatosis and histological features of NASH in an animal model[250].

CCR2 and CCR5 inhibitor (cenicriviroc): CCR2 and CCR5 are chemokine receptors which are mainly expressed in various immune cells like monocytes, macrophages, Kupffer cells, natural killer cells, T cells and stimulate hepatic stellate cells thus promoting fibrosis. These receptors can be inhibited by cenicriviroc (CVC) which is an inhibitor of the CCR2 and CCR5 receptors. CVC has been shown to decrease fibrosis and inflammation in various animal models of diet-induced NASH or substance-induced NASH[251-254]. There is an ongoing trial (ClinicalTrials.gov Identifier: NCT02217475) with CVC to examine its efficacy in NASH patients with fibrosis. It will compare shorter *vs* longer CVC treatment and assess correlations between decreased inflammation and fibrosis[255].

SCD-1 inhibitors (aramchol): Aramchol is a synthetic lipid molecule which decreases hepatic fat accumulation by decreasing lipogenesis and increasing fatty acid oxidation by inhibiting stearoyl coenzyme A desaturase 1 (SCD1) enzyme[256]. This drug was found to decrease liver fat content significantly in 60 NAFLD patients who were given 100 or 300 mg of this drug daily for 3 mo; the effect of the drug on fibrosis was not determined[256]. A phase Ⅱ clinical trial of this drug is ongoing on NASH patients with fibrosis (ClinicalTrials.gov Identifier: NCT02279524).

Lysyl oxidase-like 2 inhibitor (simtuzumab): Lysyl oxidase-like 2 inhibitor is an enzyme which causes cross linkage of collagen, thus preventing its degradation[257]. This enzyme has been found to promote fibrosis in liver diseases of various etiologies. A monoclonal antibody (simtuzumab) to this enzyme has been studied in various animal models and has shown to decrease fibrosis[258]. Two big trials are ongoing to examine the efficacy of this drug in decreasing fibrosis and preventing progression to cirrhosis in such patients (ClinicalTrials.gov Identifier: NCT01672866 and NCT01672879).

Sirtuins: Sirtuins (SIRTs) are information regulator proteins. There are various types of SIRTs found in mammals. SIRT-1, a member of this family of proteins, has anti-inflammatory effects and increases insulin secretion and sensitivity[259]. A decreased liver expression of SIRT-1 was observed in an animal model of NAFLD[260]. Since SIRT-1 activator (resveratrol) was shown to improve hepatic steatosis and insulin sensitivity[261], SIRT-1 could be a potential target for treatment of NAFLD patients' in future clinical studies.

Betaine: A phase Ⅱ clinical trial of betaine in patients with a clinical diagnosis of NAFLD is close to completion.

This trial (ClinicalTrials.gov Identifier: NCT03073343) will evaluate the effect of two doses of oral betaine in reducing ALT levels in NAFLD patients with and without diabetes.

CONCLUSION

Both ALD and NAFLD are chronic liver diseases with similar spectrums from simple steatosis to cirrhosis with basic differences only in their etiology. Despite understanding much of the pathophysiology of both diseases, there is still no effective treatment for either disease. The treatment for ALD basically relies on alcohol abstinence, nutritional support, lifestyle modifications, steroids and symptomatic treatment of complications of cirrhosis while for NAFLD, the focus of treatment is on weight loss, exercise and the use of insulin sensitizers. Removal of the cause would be the most efficient way of treating both diseases. However, the involvement of several inter-related pathways in the pathogenesis of these diseases indicates that a single therapeutic agent is unlikely to be an effective treatment strategy. Hence, a combination therapy towards multiple targets would eventually be required. Future areas of research also include the safety, efficacy, and ethical considerations of liver transplant in severe ASH for patients who are not responding to medical therapy. Various new target-oriented therapies are under investigation for both diseases and hopefully soon we will be having an effective multi-therapeutic regimen for each disease.

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REVIEW

Nonalcoholic fatty liver disease: Evolving paradigms

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Abstract

In the last years new evidence has accumulated on nonalcoholic fatty liver disease (NAFLD) challenging the paradigms that had been holding the scene over the previous 30 years. NAFLD has such an epidemic prevalence as to make it impossible to screen general population looking for NAFLD cases. Conversely, focusing on those cohorts of individuals exposed to the highest risk of NAFLD could be a more rational approach. NAFLD, which can be diagnosed with either non-invasive strategies or through liver biopsy, is a pathogenically complex and clinically heterogeneous disease. The existence of metabolic as opposed to genetic-associated disease, notably including "lean NAFLD" has recently been recognized. Moreover, NAFLD is a systemic condition, featuring metabolic, cardiovascular and (hepatic/extra-hepatic) cancer risk. Among the clinico-laboratory features of NAFLD we discuss hyperuricemia, insulin resistance, atherosclerosis, gallstones, psoriasis and selected endocrine derangements. NAFLD is a precursor of type 2 diabetes (T2D) and metabolic syndrome and progressive liver disease develops in T2D patients in whom the course of disease is worsened by NAFLD. Finally, lifestyle changes and drug treatment options to be implemented in the individual patient are also critically discussed. In conclusion, this review emphasizes the new concepts on clinical and pathogenic heterogeneity of NAFLD, a systemic disorder with a multifactorial pathogenesis and protean clinical manifestations. It is highly prevalent in certain cohorts

of individuals who are thus potentially amenable to selective screening strategies, intensive follow-up schedules for early identification of liver-related and extrahepatic complications and in whom earlier and more aggressive treatment schedules should be carried out whenever possible.

Key words: Nonalcoholic fatty liver disease; Biomarkers; Clinical correlates; Diagnosis; Epidemiology; Genetics; Liver histology; Management; Metabolic Syndrome; Pathogenesis; Screening; Type 2 diabetes

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Core tip: Nonalcoholic fatty liver disease (NAFLD) is a pandemic disease. Recent evidence highlights new concepts in clinical and pathogenic heterogeneity of NAFLD, a systemic disorder with a multifactorial pathogenesis and protean clinical manifestations. Other than the classical obese phenotype of NAFLD, a lean though metabolically abnormal variant has been recognized. Simple steatosis is no more considered a benign condition; insulin resistance is necessary but not sufficient for the disease progression, and NAFLD is not only a mere hepatic manifestation of metabolic syndrome, but may forerun the development of metabolic syndrome and cardio-renal complications. Several non-invasive diagnostic tests are now available and new drug treatment options are coming.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) features excess intrahepatic ectopic triglyceride deposition[1] in patients who are free of competing etiologies of liver disease[2]. It is the most frequent liver disease and is associated with a wide spectrum of hepatic disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC)[3]. Moreover, frequent co-morbidities of NAFLD include specific cardio-renal-metabolic conditions and increased hepatic/extrahepatic cancer risk[4].

NAFLD is a pandemic disease worldwide, which has been paralleling the ongoing epidemics of obesity, type 2 diabetes (T2D), and metabolic syndrome (MetS)[4]. The prevalence of NAFLD approaches 25%-30% in the Europe and United States general populations; but this figure surges to 80%-90% in selected cohorts of dysmetabolic individuals[5-7]. Patients with NAFLD have an increased risk of premature cardiovascular as well as of liver-related mortality[8]. Of concern, up to 50% of cases NAFLD-HCC may occur in the absence of cirrhosis, a circumstance which will often worsen the outcome[9,10].

Knowledge accumulated since 1980, when Ludwig named and described NASH[11], has challenged most of the paradigms that had been holding the scene over the previous 30 years. For example, NAFLD is now considered a multifaceted complex systemic condition[12,13] which exhibits a sexual dimorphisms[14] and follows a variable hepatic and extra-hepatic course[15,16]. In addition, recent evidence suggests that simple steatosis may progress to NASH and advanced liver fibrosis[17] challenging the previous dogma that "steatosis is a benign condition".

Insulin resistance (IR) is often associated with NAFLD but it is "necessary but not sufficient" for the disease to progress[18]. Moreover, NAFLD may occur in lean individuals[19,20] and it is not only a mere "hepatic manifestation of MetS", but may forerun the development of MetS[21,22]. NAFLD is associated with secretion of diabetogenic hepatokines and inflammatory biomarkers that increase the risk of incident T2D by adversely affecting glucose homeostasis[23]. Of concern, NAFLD and T2D are linked by a "vicious circle" which carries to accelerated worsening of liver disease[24], more difficult metabolic control and earlier appearance of T2D complications[25,26].

The historical "two-hit" pathogenic theory[27] has been replaced by a multi-factorial model, which more incisively recapitulates the complexity of the pathogenesis of the disease[28] by emphasizing the myriads of pathways leading to the same hepatic phenotype[29]. A better definition of the role played by genetics in the development of NAFLD[30] has led to the notion that, probably, two different NAFLD types exist: NAFLD associated with and NAFLD dissociated from MetS, and that each may differentially affect cardio-metabolic risk[31,32]. In addition, the declining gradient of risk of NAFLD in Hispanic patient populations compared to non-Hispanic whites and African Americans may potentially be accounted for by polymorphisms in genes that control metabolism[33]. The impressive amount of data implicating gut microbiota in the development of NAFLD may well illustrate the role of diet in increasing NAFLD risk[34-36] and significantly impact our understanding of NAFLD pathogenesis and treatment[4].

Over time, the diagnostic approach has also undergone substantial changes. Non-invasive physical and chemical biomarkers are now available[37]. Liver biopsy is reserved to selected NAFLD cases in clinical practice and is more extensively performed and repeated to evaluate novel drug agents[3,4,38,39]. Finally, new histological classifications have recently been proposed[40] and the pros and cons of each system must be carefully evaluated[41].

Within the complex frame of pathogenic and clinical heterogeneity of different NAFLD populations

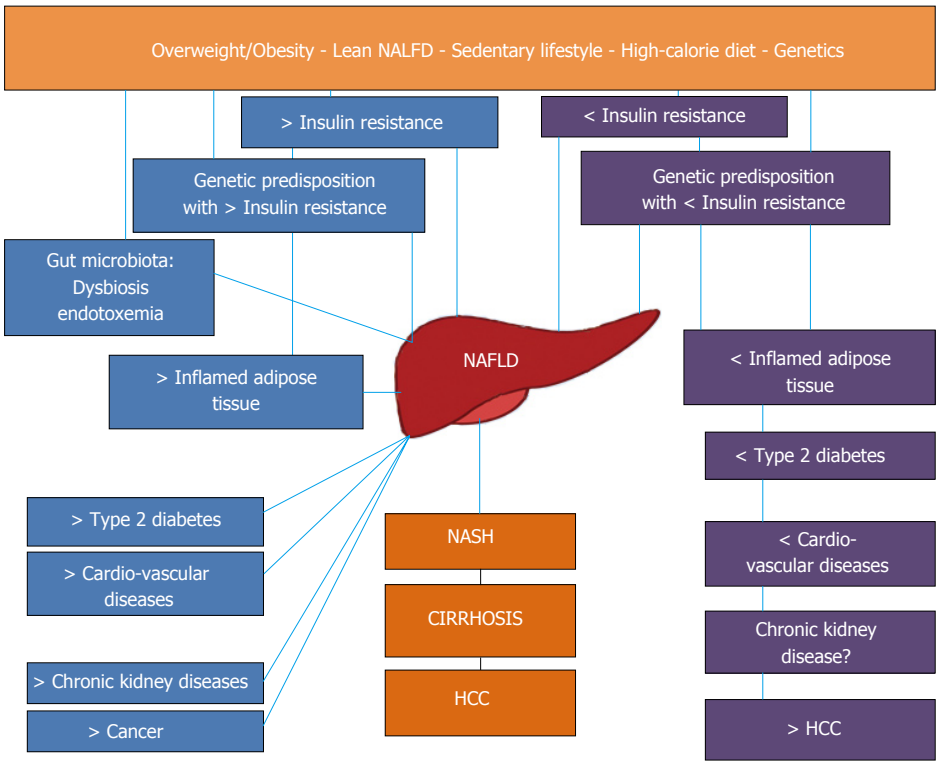


Figure 1 Nonalcoholic fatty liver disease as a pathogenically and clinically heterogeneous condition. Schematic representation of the pathogenic and clinical heterogeneity of different NAFLD populations. Left: "Metabolic" NAFLD is associated with adipose tissue dysfunction and IR and may progress towards hepatic and extrahepatic complications. Right: "Genetic" NAFLD seems to be disconnected from adipose tissue dysfunction and IR, is associated with an increased risk of liver disease progression but is probably spared from extrahepatic complications. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

synthesized in Figure 1, this review aims at disseminating the most updated paradigms on NAFLD and the possible implications for both clinical practice and research purposes.

NAFLD: TO SCREEN OR NOT TO SCREEN. PRINCIPLES OF EPIDEMIOLOGY

An ongoing debate regards whether any screening campaigns should be ever conducted to identify NAFLD individuals[38,42]. Those supporting screening would argue that NAFLD patients are prone to excess liver-related morbidity/mortality and cardiovascular risk and thus in need of intensive follow-up schedules; those against would reply that there is no point in identifying NAFLD, a condition for which no licensed drug treatment schedule is available. At any rate, given the overwhelming prevalence of NAFLD in the general population worldwide, any sensitive approach should best be conducted based on our understanding of which specific cohorts of individuals are exposed to the highest risk of disease.

In the general population, the prevalence of NAFLD has been reported to widely range from 6.3% to 51% related to the different population/ethnicity evaluated as well as to the diagnostic methods utilized to assess the amount of intrahepatic fat content[43]. In particular, the diagnostic technique used to capture cases of NAFLD has undoubtedly affected the results. For example, liver enzymes are far less sensitive than ultrasonographic scanning; in its turn, MR spectroscopy is the most sensitive although this technique is expensive and not universally available[3]. Based on meta-analytic data, Younossi *et al*[6] have reported that, in Europe and in United States, the overall prevalence of NAFLD in the general population approximates 25%. However, there are areas of the world, such as south America and middle East, wherein the average prevalence peaks > 30%; conversely, Africa has the minimum prevalence of NAFLD worldwide[6]. These impressive data clearly imply that no society worldwide can invest so many resources as to screen the general population for NAFLD. It is of interest, therefore, that modifiers of risk might assist health authorities as well as practicing physician in identifying individuals who are particularly prone to developing NAFLD. For example: male gender, middle age and Hispanic ethnicity, all reinforce the risk of NAFLD and, conversely, young women of Afro-American descent have the lowest risk of this disease[7]. Further to the above physiological modifiers, a set of metabolic modifiers have also been identified. They include arterial hypertension and the MetS (whose presence doubles the prevalence of NAFLD up to 50% of individuals); obesity, dyslipidemia and T2D[7]. In

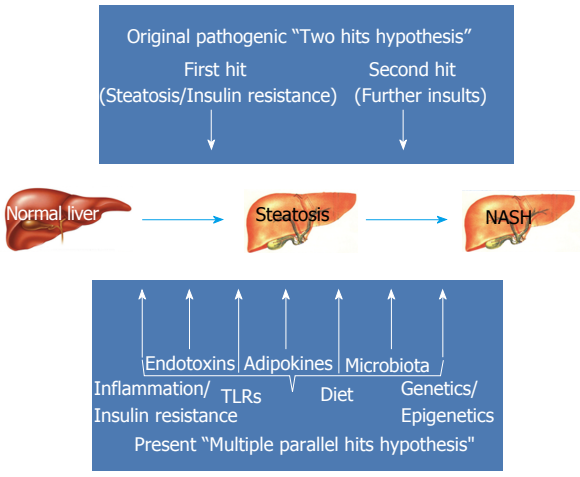


Figure 2 Nonalcoholic fatty liver disease pathogenesis: from two to multiple parallel hits. Schematic representation of the increasing grade of complexity gained in moving from earlier theories[27] to more updated pathogenic paradigms[28]. Top: Former "two hits" hypothesis: steatosis, the first 'hit', sensitizes the liver to the second "hits": oxidative stress, endotoxin, ATP depletion and adduct formation[44]. Bottom: The "multiple hits" hypothesis considers multiple insults acting together on genetically predisposed subjects to induce NAFLD and provides a more accurate explanation of NAFLD pathogenesis. Such hits include IR, hormones secreted from the adipose tissue, nutritional factors, gut microbiota and genetic and epigenetic factors[28]. NAFLD: Nonalcoholic fatty liver disease; TLRs: Toll-like receptors.

the general population, women are protected from developing NAFLD owing to their hormonal profile and/or set of chromosomes. Conversely, women with T2D are exposed to the same risk of NAFLD as men, indicating that T2D abrogates such a gender-related protection[7,14].

PATHOGENESIS OF NAFLD: FROM TWO TO MULTIPLE HITS

In recent years, it has increasingly become evident that what had initially been called the NAFLD "two hit hypothesis", in which IR acted as the "first hit" by inducing lipid accumulation in the hepatocytes and increasing the vulnerability of the liver to further insults, referred to as the "second hit", that, in their turn, promoted hepatic injury, inflammation and fibrosis, is probably over-simplistic[27,44]. Human NAFLD is rather a multi-factorial, non-communicable disease resulting from a complex interaction between multiple environmental and metabolic "hits" and a predisposing genetic background[45] (Figure 2). A "multiple parallel hits hypothesis" seems more appropriate to recapitulate the complexity of NAFLD pathogenesis[28], which results from numerous events originating within liver, adipose tissue, gastrointestinal tract and the muscle[45].

An unhealthy lifestyle, characterized by sedentariness and high-calorie diet, is crucial for NAFLD development and progression. The unbalance between caloric intake and expenditure determines the expansion of fat depots, which become inflamed and insulin resistant, and release increased amounts of free fatty acids into the bloodstream, leading to ectopic fat accumulation in the liver, skeletal muscles and pancreas[46]. The hepatic lipid content mainly derives from the increased lipolysis of triglycerides in the adipose tissue and, to a lesser extent, from dietary fats and sugars, and from de novo lipogenesis[47]. Moreover, the dysfunctional adipose tissue overproduces pro-inflammatory cytokines, such as leptin and resistin, whereas the release of anti-inflammatory adipokines, such as adiponectin, is reduced, so further enhancing NAFLD progression[48]. The role of intra-hepatocytic accumulation of specific toxic lipids, *via* lipotoxicity and inflammation, has emerged as a key player in the pathogenesis of NASH and its systemic complications[49]. Saturated fatty acids have been found elevated in NASH patients and induce inflammation and hepatocyte apoptosis through activation of JNK/endoplasmic reticulum stress and oxidative stress/mitochondrial dysfunction[50]. The accumulation of diacylglycerol and ceramide in the liver impairs hepatic insulin signaling, fuels gluconeogenesis and promotes the development of persisting hyperglycemia and, eventually, T2D[51].

Unhealthy lifestyle also results in dysbiosis, *i.e.* quantitative and qualitative changes of gut microbiota composition[52]. In NAFLD an increased Firmicutes/Bacteroidetes ratio and changes in metagenomic-based functional aspects of gut microbiota have all been described[53]. Gut microbiota may contribute to the development and progression of NAFLD by triggering (both directly or *via* end-products of bacterial metabolism) different signaling pathways, by increasing the efficiency of caloric extraction from the food, and by inducing translocation of bacterial products *via* increased gut permeability[54-57]. Circulating pathogen-associated molecular pattern (PAMPs) and damage-associated molecular patterns (DAMPs) interact with the family of pattern recognition receptors (toll-like receptors, TLRs) within the liver and induce several pro-inflammatory pathways, including over-expression of cytokines/chemokines, production of reactive oxygen species and activation of the inflammasome[58,59]. Of note, activation of Nod-like receptor protein 3 inflammasome, upregulated by TLRs in response to the presence of PAMPs and DAMPs, has been associated with a novel cell death mechanism, referred to as hepatocyte pyroptosis[60].

Several nuclear receptors with their molecular cascades are promising pharmacological targets for the treatment of NAFLD, given their pivotal role in the regulation of energy homeostasis and metabolic pathways. Owing to the development of specific targeted drugs, peroxisome proliferator-activated receptors (PPARs), farnesoid X receptor (FXR) and liver X receptors (LXRs), now deserve full attention. The PPARs superfamily includes PPAR-α, PPAR-β/δ

and PPAR-γ. PPAR-α, mainly expressed in liver and muscle, modulates the rates of fatty acids catabolism, lipogenesis and ketone body synthesis by acting as a nutrient sensor. PPAR-γ, abundantly expressed in the adipose tissue, promotes adipocyte differentiation and storage of triglycerides, and regulates glucose homeostasis. PPAR-β/δ, universally expressed in all organ tissues, regulates glucose and lipoprotein metabolism and exerts an anti-inflammatory role. FXR, mainly expressed in the liver and gut, acts as a sensor of bile acids and mediates the signaling effects exerted by bile acid on glucose and lipid metabolism. LXRs serve as lipid sensors in the liver and participate in regulating the metabolism of cholesterol and fatty acids. The role of nuclear receptors in NAFLD pathophysiology has been comprehensively reviewed elsewhere[15].

The role of skeletal muscle in the pathogenesis of NAFLD is a matter of increasing research[23]. Intramyocellular lipid storage is an early step of ectopic fat accumulation and systemic IR, leads to increased delivery of glucose to the liver, where it becomes substrate for hepatic de novo lipogenesis, and has been associated with NAFLD[61,62]. Accordingly, sarcopenia, a pathological condition featured by generalized loss of skeletal mass and strength, has recently been proposed as an additional key factor in the pathogenesis of NAFLD[63-65]. The myokines, such as irisin, which is produced in response to physical activity and exerts several beneficial metabolic effects, may partly account for these associations[66,67].

NAFLD AS A HETEROGENEOUS DISEASE ENTITY: THE ROLE OF GENETICS

NAFLD is a complex disorder with a variable natural history. Subtle inter-patient genetic variation and environmental factors combine to determine variation in disease progression[68]. Several clinical features are clues to genetics playing a major role in NAFLD. First, up to a quarter of the general population are at risk of progressive disease; however only a minority will experience associated liver-related morbidity[69]. Second, both the development and the progression of disease tend to cluster as distinct traits among "NAFLD families"[7,70]. Third, among non-diabetics, NAFLD will typically affect men more often than women[7] which, further to hormonal variations, may mirror genetic factors. Fourth, although NAFLD is strictly linked with obesity[7] not all obese subjects will develop NAFLD and, more importantly, NAFLD can also be found in non-obese individuals[20]. On these grounds, in the last few years scientific attention has focused on NAFLD genetic features.

Role of PNPLA3, TM6SF2 and MBOAT7

Among all analyzed genes patatin-like phospholipase domain-containing protein 3 (PNPLA3, also called adiponutrin) and trans-membrane 6 super family 2 (TM6SF2) are deemed to play the most significant role. More recently, membrane bound O-acyltransferase domain-containing 7 gene (MBOAT7) has also gained attention. They are all involved in the process of intrahepatic accumulation of triglycerides.

PNPLA3 gene encodes for a 481-aminoacid protein expressed in the liver and adipose tissues, with function of triglyceride hydrolase and acetytil-CoA-indipendent transacylase, suggesting a double role of catabolic lipase activity and anabolic lipogenic activity[71-73]. The nonsynonymous variant rs738409 of *PNPLA3* is a genetic polymorphism characterized by the substitution of isoleucine to methionine at position 148 (I148M). Studies consistently show a strong association between I148M variant and hepatocellular triglycerides accumulation[74-76]. I148M variant is a loss of function allele that determines an altered hepatic lipid metabolism, eventually leading to hepatic droplet lipid accumulation, lower hepatic VLDL secretion and lower adiponectin production[77]. Adiponectin is a protein with anti-inflammatory activity and onco-suppressive properties; lower circulating levels of adiponectin can account for the propensity of I148M variant to the progression from NAFLD to NASH and the increased risk of HCC[77,78].

TM6SF2, also known as KIAA 1926, is a gene at the 19p13.11 locus, with unknown biological function that encodes a protein high expressed in the small intestine, kidney and liver. It is involved in the pathophysiology of NAFLD by modifying hepatic lipid secretion; a decreased hepatic expression of this protein is associated with an increased size and number of hepatocytic lipid droplets[79]. In particular, studies have shown that a variant of *TM6SF2*, rs58542926 c.449 C>T (E167K), is associated with NAFLD evolution, also with dyslipidemia and cardiovascular risk. The minor variant allele (T) is linked with a reduction of the excretion of hepatic VLDL and a lower level of circulating triglyceride and LDL-cholesterol. Conversely, C-allele is associated with a higher hepatic excretion of VLDL, serum triglyceride and cholesterol. Thus, the minor allele exhibits a higher risk of advanced hepatic disease, though protecting from metabolic and cardiovascular disease[80]. Conversely, increased metabolic risk is associated with C-allele[81]. Morris *et al*[82]. based on their large meta-analysis have hypothesized a correlation with T-allele variant and risk developing T2D, but this theory remains to be confirmed[82]. Recently, it has been shown that TM6SF2 (*E167K*) gene variant is associated with an altered lipidomic profile featuring deficiency of polyunsaturated phosphatidylcholine and excess of polyunsaturated free fatty acids in the liver. This lipidomic profile leads to decreased concentrations of circulating VLDL and increased liver fat content[83]. Moreover, the same polymorphism has been found to modulate postprandial lipidmetabolism, nutrient oxidation and glucose homeostasis in response to dietary fat[84]. These findings

are compatible with the above reported notion that carriers of TM6SF2 polymorphisms may be specifically exposed to liver-related as opposed to vascular manifestations of NAFLD.

Finally, it has been recently shown that the rs641738 C>T variant in MBOAT7 gene, which is involved in phosphatidylinositol acyl-chain remodeling, increases the risk of developing NAFLD; inflammation and fibrosis[85,86].

Lean NAFLD

As previously reported in this review, genetic factors play a major role in development and progression of NAFLD. Such a role of genetics is further reinforced when lean NAFLD patients are taken into consideration. It is well-known that NAFLD is often found in obese and dysmetabolic patients. Accordingly, the "Lean NAFLD" phenotype has attracted interest among researchers in as much as it features selectively expanded visceral adiposity though preserving a normal body mass index and, sometimes, even a normal waist circumference. Worryingly, these individuals develop IR and atherogenic dyslipidemia similarly to obese patients and have, therefore, in the past been alluded to as "metabolically obese, normal weight"[87]. Lean NAFLD cases have been described among different ethnic population, mainly among Asians, who tend to develop the metabolic complications of obesity for BMI values inferior to those of Caucasians[88,89]. The mechanism(s) through which lean NAFLD develop(s) is/are poorly characterized and the disease goes often under-recognized. Though the role of genetic factors is likely to be more substantial in "lean NAFLD" than that found in "obese NAFLD"[90], a recent statistically sophisticated study has suggested that both lean and obese NAFLD patients share cardio-metabolic risk commonalities and that the former, despite normal body weight, nonetheless have excess of abdominal adiposity and other MetS features[19]. Accordingly, also lean NAFLD patients may benefit from increased physical activity which is effective in reducing visceral fat[91]. Of note, the findings of a very recent study confirm that NAFLD development and progression derive from a complex interplay between genetic and environmental factor, by clearly demonstrating the presence of a synergy between adiposity and the genetic risk of NAFLD conferred by multiple loci[92].

DIAGNOSTIC STRATEGIES

Diagnosis and staging of NAFLD fully rely on liver biopsy. Histological examination of liver tissue specimens is the gold-standard for quantitating steatosis, diagnosing NASH and staging fibrosis. The last of these three tasks bears major prognostic significance given that fibrosis is associated with long-term liver-related and, probably, cardiovascular outcomes[93-95]. Over the past two decades, different criteria have been suggested for scoring and staging histological lesions, and different definitions of NASH have been used[40,96-99]. Besides discrepancies and subjectivity in interpretation, liver biopsy has additional limitations such as cost, invasiveness and sampling variability which limit the feasibility of this procedure in all the patients with suspected NAFLD and have called for the development of alternative non-invasive diagnostic and staging procedures. Despite some intrinsic and specific drawbacks[100,101], these non-invasive tools, especially if used in combination or sequentially, may help in restricting the number of and assist in a better selection of potential candidates to liver biopsy[102].

Histological classification: what's new?

Steatosis, namely a minimal threshold of 5% of hepatocytes containing fat droplets in biopsy specimen, is a prerequisite for the diagnosis of NAFLD. The histological definition of adult NASH is based on a combination of three elementary lesions, *i.e.*, steatosis, hepatocellular ballooning and lobular inflammation[41,97].

Four main pathologic classifications of NAFLD have been proposed: Matteoni's, Brunt's, Kleiner's classification with the NAFLD Activity Score (NAS) and the recent Fatty Liver Inhibition of Progression (FLIP) algorithm with the Steatosis, Activity, and Fibrosis (SAF) score[40,96-99].

In 2005 the NASH Clinical Research Network designed and validated a histological feature scoring system that addressed the full spectrum of lesions of NAFLD. Currently, NAS is the most widely used histological classification for NAFLD/NASH and its use is recommended for defining and quantifying disease activity in clinical trials[98]. NAS, ranging from 0 to 8, is derived from the sum of steatosis (0-3), ballooning (0-2) and lobular inflammation (0-3). However, at least two out of its three components show a high variability among pathologists; and data increasingly suggest that NAS is poorly correlated with metabolic risk factors[103], is unable to predict fibrosis progression[104] and to prognosticate liver-related and overall mortality in NAFLD patients[93,94,105].

More recently, Bedossa and the FLIP Consortium created a simple histological algorithm (FLIP algorithm) based on a scoring system, the SAF score (Steatosis, Activity, Fibrosis) intended for pathologists to reliably diagnose NASH, assess disease severity and limit interobservervariation[40,99]. The SAF score separately assesses steatosis, activity and fibrosis. Activity, which ranges from 0 to 4, is derived from the combination of the semi-quantitative values of hepatocellular ballooning (0-2) and lobular inflammation (0-2). NASH according to the FLIP algorithm is diagnosed when steatosis, ballooning and lobular inflammation are all scored $\geqslant$ 1. The main discrepancy between NAS and SAF scores is that SAF does not include steatosis in the activity score, owing to the questionable role of steatosis *per se* as a marker of disease progression and prognosis. Moreover, fibrosis stage, the strongest

Table 1 Main features and differences between Nonalcoholic Steatohepatitis Activity score and Steatosis, Activity, and Fibrosis score

	NAS	SAF
Scoring system	Steatosis + Lobular Inflammation + Ballooning: 0-8	Steatosis, Activity (Lobular Inflammation + Ballooning), Fibrosis: S_{0-3}, A_{0-4}, F_{0-4}
Details of scoring	Steatosis 0: < 5% 1: 5%-33% 2: 34%-66% 3: > 67% Lobular inflammation 0:00 1: < 2/20X 2: 2-4/20X 3: > 4/20X Ballooning (number of ballooned hepatocytes) 0: None 1: Few 2: Many Fibrosis stage F0: None F1a: zone 3 perisinusoidal, delicate F1b: perisinusoidal, dense F1c: portal only F2: 1a or 1b + portal F3: bridging F4: cirrhosis	Steatosis 0: < 5% 1: 5%-33% 2: 34%-66% 3: > 67% Lobular inflammation 0:00 1: < 2/20X 2: ⩾ 2/20X Ballooning (size and shape of hepatocytes) 0: normal hepatocytes 1: clusters, reticulated cytoplasm 2: enlarged hepatocytes Fibrosis stage F0: 0 F1: zone 3 perisinusoidal (all), or portal only F2: zone 3 + portal F3: bridging F4: cirrhosis

NAS: NASH activity score; SAF: Steatosis, Activity, and Fibrosis score; NASH: Nonalcoholic steatohepatitis.

predictor of outcomes in chronic liver disease, was not included in the NAS. This novel histological classification has shown excellent applicability and reproducibility among pathologists for the assessment of liver lesions in morbidly obese patients and in NAFLD patients with MetS[40,99]. However, it will have to undergo further validation in clinical practice. Table 1 shows the main features of NAS and SAF scores and highlights the main differences between these scoring systems.

Role of imaging techniques

Imaging methods are the most commonly used non-invasive tools for the diagnosis of steatosis. The main advantages of ultrasonography are its safety, low cost, wide availability and the overall scanning of abdominal organs. Traditionally, ultrasonography has been considered a technique with a low sensitivity in identifying fatty liver infiltration when less than 30% of hepatocytes are steatotic. Of note, a recent study has demonstrated that ultrasonography, especially when implemented with standardized measurements and semi-quantitative scores, is sensitive for an amount of steatosis as low as 10%[106] and may predict metabolic derangements and liver histology changes[106-108]. Further studies are necessary to confirm these novel findings. The main limitations of ultrasonography are its inability in differentiating steatosis from fibrosis, the issues with morbid obese individuals, and its operator and machinery dependency.

The controlled attenuation parameter (CAP) is a new promising screening method that measures ultrasound attenuation in the liver using signals obtained by the Vibration Controlled Transient Elastometry (TE) (Fibroscan®). Interestingly, CAP appears to be more sensitive in detecting lesser degrees of steatosis compared to other widely available imaging methods. Moreover, being coupled with liver stiffness measurements, it has the advantage of simultaneously estimating liver fibrosis[108-110]. However, the underlying disease, BMI and T2D may affect the findings and thus must be taken into consideration in the interpretation of CAP results[111].

Over the last 10 years, several ultrasound elastographic techniques have been implemented, including TE, acoustic radiation force impulse (ARFI) elastography, 2D shear-wave elastography and real-time strain elastography. TE, is the most studied elastographic method for the estimation of liver fibrosis in chronic liver disease; in NAFLD, TE can be used to confidently rule out severe fibrosis and cirrhosis (with a nearly 90% negative predictive value) and for monitoring disease progression[11,112]. Of note, the amount of steatosis has emerged as a significant factor affecting the performance of liver stiffness measurements with TE[112,113]. For this reason, it has been suggested that taking into account CAP values may improve the prediction of liver fibrosis by TE[113].

Magnetic resonance imaging techniques, and particularly magnetic resonance spectroscopy, are able to accurately quantify liver fat content; the advantages include high sensitivity for assessing small amounts of intrahepatic triglycerides and sampling a large liver volume[114]. More recently, magnetic resonance elastography and multiparametric magnetic

resonance offer the possibility of performing a thorough characterization of liver tissue by assessing steatosis, fibrosis and haemosiderosis at the same time[115,116], and have been shown to be able to predict clinical outcomes in patients with various chronic liver disease, notably including NAFLD[117]. Cost and limited availability, however, restrict their use to research purposes.

Performance and limitations of biomarkers

Several serum biomarker panels have been developed for predicting steatosis, diagnosing NASH and estimating fibrosis[118] which are, in theory, the preferred diagnostic tool for large-scale screening and epidemiological studies on NAFLD[4,38]. For example, Bedogni's Fatty Liver Index (FLI) has been largely used to capture steatosis in epidemiological studies[119]. The strength of FLI as a surrogate marker comes from the demonstration that it can predict clinical outcomes related to the MetS, such as risk of T2D, accelerated atherosclerosis and cardiovascular risk, as well as hepatic, cardiovascular and cancer-related mortality[120,121]. While clinically relevant, these associations might be conveyed independently of steatosis *per se* since FLI incorporatesvariables that are part of the MetS phenotype[101]. In fact, recent studies have found only a limited correlation between FLI and the amount of liver fat *per se*, as defined by liver biopsy or magnetic resonance spectroscopy[101,122,123]. As for the FLI, other steatosis biomarkers, such as the NAFLD liver fat score, the Hepatic Steatosis Index and the Lipid Accumulation Product, generally show good performance in identifying steatosis, though they are unable to predict liver fat content and are confounded by hepatic histological changes other than steatosis, mainly necro-inflammation and fibrosis[101,123].

With regard to the possibility of non-invasively differentiating NASH from simple steatosis, several biomarkers (acute-phase proteins, cytokines, markers of oxidative stress and apoptosis, as is the case of cytokeratin-18 fragments) and multiple complex models have been investigated, but the majority of them lack external validation, have only been tested among selected populations and have been inconsistent in their performance of detecting NASH[102,118].

Several simple non-invasive fibrosis scoring systems, notably including NAFLD Fibrosis Score and Fib-4 can, however, reliably exclude advanced fibrosis in a high proportion of NAFLD patients, allowing a more targeted use of liver biopsy[124]. Their use in clinical practice is recommended by updated hepatological guidelines[4,38], and they reliably predict liver-related and cardiovascular complications and death in NAFLD patients[125-127]. However, they were principally developed and validated in patients aged < 65 years of age, hence their cut-offs need to be adapted in the elderly[128]. It has been shown that the combination or the sequential use of different scoring systems or different non-invasive techniques (TE plus biomarker) improve the diagnostic accuracy in detecting severe liver fibrosis, thus further reducing the need for liver biopsy[129,130].

Recently, several miRNAs[131], epigenetic biomarkers[132], lipidomic[133,134], metabolomics[135] and proteomic[136] profiles have been found differently expressed in NAFLD patients and could be used as surrogate markers of progressive liver injury. Probably, a multi-domain, multi-component approach which integrates variables from a diverse set of data sources deriving from phenotypic, genomic, lipidomic, metabolomic and proteomic domains will be the avenue for future investigations in the noninvasive assessment of NAFLD[136,137].

NAFLD: CLINICO-LABORATORY FEATURES

Usually asymptomatic, NAFLD is a phenotypically polymorphic disease which, owing to its systemic nature, has a variable clinical presentation, a multitude of potentially associated disease and a rich spectrum of laboratory features[4,12,138]. Understanding this remarkable phenotypic polymorphism is key in guiding the diagnostic process in the individual patient.

Most patients will seek medical advice on the grounds of otherwise inexplicably deranged liver tests/ultrasonographic findings compatible with fatty changes. Collectively, these laboratory/ultrasonographic abnormalities tend to be often discovered either during routine medical check-up performed by asymptomatic individuals or by patients who have nonspecific and causally unrelated complaints[139,140].

Insulin resistance, hyperuricemia and atherogenic dyslipidemia

The association of NAFLD with IR and its close connection with the MetS was independently reported by three unrelated groups of investigators as early as 1999[141-143]. In particular, Marchesini *et al*[141] by comparing 46 patients with NAFLD to 92 age and sex-matched healthy controls with the homeostasis model assessment method found that NAFLD was associated with IR and hyperinsulinemia even in lean subjects with normal glucose tolerance. Consistently, Cortez-Cortez-Pinto *et al*[142] in 30 patients with NAFLD found that 80% were either obese or dyslipidemic; 50% had arterial hypertension and 33% T2D; moreover, glucose metabolism was altered in 69% and hyperinsulinemia and hyperleptinemia were common. Along the same line, Lonardo, based on his systematic search of the available literature, concluded that fatty liver most commonly affected middle-aged men with obesity, altered glucose metabolism, hyperlipidemia, and hypertension[143].

Over time, these pioneer studies suggesting an intimate connection of NAFLD with IR and the MetS have extensively been confirmed and carried further.

For example Ballestri *et al*[144] have recently shown that HOMA-IR, a widely used index of IR, along with serum uric acid concentrations, is an independent predictor of both steatosis and ballooning; waist circumference predicts both ballooning and hepatic fibrosis[144]. Moreover, the same group of investigators, also found that baseline NAFLD predicts the development of incident T2D and MetS over a median 5-year follow-up[22]. Taken collectively, these findings strongly support the notion that NAFLD should no longer be considered a mere "hepatic manifestation of the MetS" but rather, and more appropriately, both a precursor and a consequence of the MetS[21,31,145-147].

Lonardo *et al*[139] were first in reporting that both fasting insulin (a sensitive marker of IR) and uric acid were independent predictors of NAFLD. These findings have been independently confirmed by several studies[148-152], including two meta-analytic reviews[153,154]. Of interest, although serum uric acid concentrations are associated with indices of IR, both parameters are independently associated with NASH, at least in children and adolescents[155], suggesting that each probably carries an independent contribution to the development of progressive liver disease.

NAFLD is typically characterized by atherogenic dyslipidemia featuring larger and triglyceride over-enriched circulating very-low-density lipoproteins (VLDLs), small dense low-density lipoproteins (LDLs) and low and dysfunctional high-density lipoproteins (HDLs). Of note, when NAFLD progresses to severe fibrosis and cirrhosis, dyslipidemia will apparently improve, probably owing to failing hepatic synthetic capacity[144,156-159].

Liver tests, parameters of iron metabolism and non-organ specific autoantibodies

Liver tests are typically normal or mildly elevated in NAFLD. Gamma-glutamyltransferase (GGT) may be slightly elevated and is increasingly recognized as a marker of metabolic disturbances and cardiovascular risk[160]. Aminotransferases do not identify progressive disease, given that patients with normal liver enzymes are spared neither from NASH nor significant fibrosis[161].

The diagnostic process followed to investigate a suspect liver disease may include the determination of parameters of iron metabolism and non-organ specific autoantibodies (NOSA). Elevated serum ferritin, closely associated with hepatic iron deposition, is common in NAFLD and is strongly correlated with IR, more advanced disease and increased mortality[162-164]. Whether therapeutic strategies aimed at correcting iron metabolism may be beneficial in NAFLD remains controversial.

It is important to highlight that the positivity of NOSA, in the appropriate clinical and epidemiological context, is compatible with NAFLD. Loria *et al*[165], in their pilot study, were first in reporting that NOSA positivity in NAFLD was more prevalent than in the general population and that high-titer ANA was associated with IR. Bringing this line of research further, the clinical research network, in a multicenter study including a total of 864 NAFLD patients found that NOSA are frequently positive in NAFLD in the absence of autoimmune hepatitis and their occurrence is not associated with more advanced histological features[166].

NAFLD AS A MULTISYSTEM DISEASE: CARDIO-METABOLIC AND CANCER RISK

As early as 1995, was it first suggested that NAFLD was a systemic condition with a specific cardio-metabolic involvement[167], a notion which is now universally accepted[12,168].

In particular, our understanding of the close and mutual relationship of NAFLD with the MetS has evolved and the bidirectional relationship among the two conditions is now largely acknowledged (Figure 3)[4,31,146,147]. IR, in its turn, will drive the course of liver disease to progressively fibrosing liver disease culminating with cirrhosis, but also promote the development of T2D[169]. NAFLD almost doubles the risk of incident T2D[22]. However, NAFLD patients usually die of extra-hepatic causes, frequently for cardiovascular diseases, which underpins the importance to early diagnose and aggressively treat CVD risk factors, as extensively discussed elsewhere[170]. A meta-analysis of 27 cross-sectional studies has shown a robust association between NAFLD and several markers of subclinical atherosclerosis burden, such as an increased risk, independent of classical CVD risk factors, of coronary artery calcification, carotid intima-media thickness, impaired flow-mediated vasodilatation and arterial stiffness[171]. Moreover, studies have shown that NAFLD is also associated with increased risk of atrial fibrillation and aortic valve sclerosis[172-175].

While cardiovascular mortality is the leading cause of death in NAFLD patients, malignancies, mainly affecting the gastrointestinal tract (liver, colon, esophagus, stomach, and pancreas) and extra-intestinal sites (kidney in men, and breast in women) rank second[176,177]. The association between NAFLD and colorectal cancer (CRC) has been investigated: some studies have shown a higher prevalence of CRC in NAFLD population than patients without, and this association is increased in presence of NASH[178].

NAFLD AND T2D: A DANGEROUS COMBINATION

T2D accounts for more than 90% of those 415 million people worldwide who live with diabetes; T2D may lead to micro-macrovascular complications that cause profound sufferings and put a heavy burden on health-care systems worldwide[179].

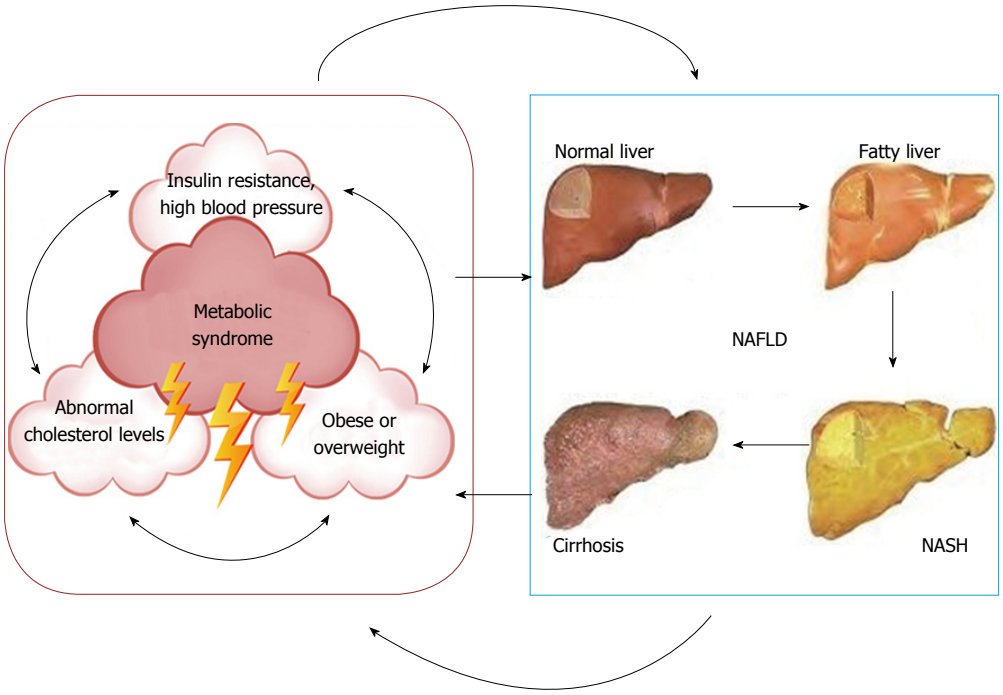


Figure 3 The closed loop nonalcoholic fatty liver disease-metabolic syndrome circuit. Schematic representation illustrating the mutual cause-and-effect relationship of NAFLD with the Metabolic syndrome[4,31,146,147]. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

The common soil and the mutual relationship

The prevalence of NAFLD is increased among those with T2D and T2D rescinds the typical gender dimorphism of NAFLD[7,180,181]. Conversely, NAFLD is associated with a 2-to-5-fold risk of developing T2D after adjustment for several metabolic confounders[22,169,182].

Common features in the epidemiology, risk factors and natural history of NAFLD and T2D can be identified: overweight and obesity[183-185]; a hypercaloric diet coupled with a sedentary behavior leading to weight gain[186,187]; IR and MetS components, including combined dyslipidemia[147,188].

As previously highlighted, NAFLD is not only a consequence, but also a cause of T2D and MetS generating a "vicious circle" and worsens the course of T2D[21,24,31,189]. It is mandatory to interrupt this circle and in this regard, it is encouraging that even transient remission/improvement of NAFLD is independently associated with reduced incidence of T2D[190,191].

The consequences of the association

T2D and NAFLD interact in enhancing the risk of promoting endothelial dysfunction, atherosclerosis, cardiovascular diseases, chronic kidney disease and advanced retinopathy[21,168,192,193]. The coexistence of T2D and NAFLD predicts the development of hepatic fibrosis[194-198]. Treatment of NAFLD, by affecting those pathogenic factors linking fatty liver with atherosclerosis (hence the so called "atherogenic liver") was predicted to be able to reduce cardiovascular risk a few years ago[199]. Consistent with such a prediction, a recent study reported that reduced CIMT progression in the entire cohort of 92 patients was independently associated with decreased liver fat content assessed with magnetic resonance spectroscopy[200].

T2D and NAFLD are also associated with an increased incidence and mortality from several cancer types and, similarly, mortality for malignancies is the second cause of mortality in patients with NAFL[178,201-203]. Similar to NAFLD, T2D enhances the risk of developing HCC and may aggravates HCC outcomes[204-206].

NAFLD in those with T2D will follow a worse prognosis *via* hastened progression to NASH, liver cirrhosis and its complications and increased risk of developing NAFLD-HCC[43,207-210]. Specularly, concurrent NAFLD will often worsen the course of T2D[211].

Diagnostic issues

An early diagnosis and treatment of NAFLD may potentially increase life expectancy of T2D patients[209]. Conversely, in subjects with NAFLD, periodical screening for T2D is encouraged, given that poor metabolic control will lead to progressive liver disease which,in its turn, is associated with enhanced cardiometabolic risk[38,193,212].

NAFLD: A LARGE SPECTRUM OF CLINICAL ASSOCIATIONS

An impressively heterogeneous spectrum of clinical conditions have been associated with NAFLD spanning from carotid atherosclerosis through polycystic ovarian syndrome[23]. Some will be shortly reviewed here; others have recently been covered in detail

elsewhere[4,213,214].

Atherosclerosis and gallstones

A pioneer study by Lonardo *et al*[215] evaluated the triangular association of NAFLD, gallstones and atherosclerosis. A decade later, we now know that the most severe NAFLD forms are strongly associated with excess cardiovascular mortality, probably as a direct/indirect result of hepatic fibrosis[193,212]. Concerning the NAFLD-gallstone duo, a systematic review and meta-analysis conducted in 12 observational studies (9 cross-sectional studies, 2 cohort studies and 1 case-control study) found that gallstones are significantly associated with NAFLD[216]. Further studies should be conducted regarding the underlying mechanism of this association. However, it is now clear that bile acids are no longer "a detergent which facilitates the absorption of fatty foodstuffs" such as it was deemed in the past; but rather, they must be regarded as signaling molecules, which coordinately regulate metabolism and inflammation acting not only in entero-hepatic tissues, but in peripheral organs as well[217].

Psoriasis

This line of research was almost anecdotally inaugurated by a report of three cases from the POLI. ST.E.N.A study[218]. Several systematic studies have subsequently confirmed this novel association and a recent systematic review and meta-analysis supports an association between psoriasis and NAFLD suggesting that screening for NAFLD may be warranted among those with psoriasis[219].

Endocrine derangements

Again, this topic was originally initiated by pioneer clinical observations. First, it was reported that endocrine derangements may eventually be conducive to NAFLD as a result of hormones being master regulators of body fat distribution and cell metabolism[220]. Next, endocrine pathways specifically leading to NASH were identified[221]. Among these, hypothyroidism has been associated to the whole NAFLD spectrum, steatosis to NASH-HCC[156, 221-224].

A large Rotterdam population-based survey, aimed at investigating the association between variations in thyroid function and NAFLD, prospectively, in 9419 adults with a median follow-up time of 10 years; the presence of fibrosis in those with NAFLD was assessed with TE[225]. Data have shown that, compared to euthyroid individuals, hypothyroid ones were exposed to a higher risk of NAFLD and of clinically relevant fibrosis; consistently, on the opposite side of the spectrum, hyperthyroidism was associated with a protection from developing NAFLD[225]. These findings may potentially disclose novel treatment strategies.

NAFLD: PRINCIPLES OF TREATMENT

It is widely accepted that lifestyle changes, namely diet and physical exercise are the mainstay of treatment of NAFLD, a condition no drugs are specifically licensed for[4,38].

Diet

A 3%-5% weight loss will improve steatosis; however, 5%-7% reductions are necessary to decrease hepatic inflammatory changes and 7%-10% to obtain remission of NAFLD/NASH and regression of fibrosis. Calorie restriction *per se* rather than specific diets are deemed to be beneficial[226]. In particular, despite emphasis is usually put in restricting dietary carbohydrates, moderate-carbohydrate diets do not induce a more profound reduction in liver enzymes (a non-validated surrogate index of NAFLD severity) than low/moderate-fat diets[227].

Physical exercise

Exercise effectively reduces visceral and perhaps liver fat. A recent meta-analysis found that aerobic exercise but not resistance training exercise was effective in reducing visceral fat in overweight/obese adults with T2D[228]. The benefits of exercise in reducing intrahepatic triglyceride content will typically occur with minimal or no body weight loss[227,229], suggesting that diet and physical exercise will improve NAFLD and should be associated whenever possible given that they act *via* different pathways. Indeed, diet combined with exercise is associated with improvement in NAFLD activity score[227].

Drugs

Drug treatment (better if within the frame of a clinical trial) should be reserved to those NASH individuals who do not respond to lifestyle changes. Drugs used to treat concurrent metabolic disorders (*e.g.*, statins and antidiabetics) should be differentiated from drugs aimed at treating NASH *per se*.

Statins, glitazones and liraglutide belong to the first group. Our appreciation of the role of statins in hepatology has undergone a dramatic evolution from under-prescription based on fears of hepatotoxicity[230] to their identification as potentially protectors from the full spectrum of liver damage in NASH, notably including liver fibrosis and HCC[231-238].

As regards the oral antidiabetic agents glitazones, a meta-analysis of five eligible randomized controlled trials showed that these drugs improved neither fibrosis nor IR; however, lobular inflammation decreased in NASH patients who received TZD treatment[239]. However, a more recent meta-analytic review was specifically aimed at investigating the role of thiazolidinediones in advanced hepatic fibrosis in those with NASH analyzed 8 RCTs (5 evaluating pioglitazone and 3 evaluating rosiglitazone) enrolling 516 patients with biopsy-proven NASH for a duration of 6 to 24 mo. Among all studies combined, thiazolidinedione therapy was associated with improved advanced fibrosis, fibrosis of any stage

Table 2 Drugs useful in treating nonalcoholic steatohepatitis - A neck-to-neck comparison of findings from two different meta-analytic studies

	Sawangjit *et al*[244]	Singh *et al*[243]
Fibrosis	OCA[1] improves fibrosis. PTX[2], TZDs plus Metformin[2], TZDs plus Losartan[2] might potentially be effective.	PTX[1] and OCA[1] improve fibrosis
Resolution of NASH	TZDs[1] and Vitamin E[1] use is associated with resolution of NASH	Vitamin E[1], TZDs[1], and OCA[1] improve ballooning degeneration
NAS	PTX[1], OCA[1], vitamin E[1], and TZDs[1] improve NAS	
Steatosis		PTX[1], Vitamin E[2], TZD[1] and OCA[2] significantly improve steatosis
Lobular inflammation		PTX[1], TZDs[1] and OCA[2] significantly improve lobular Inflammation

[1]High quality of evidence; [2]Moderate quality of evidence. NASH: Nonalcoholic steatohepatitis; NAS: NASH activity score; OCA: Obeticholic acid; PTX: Pentoxyphylline; TZDs: Thiazolidinediones.

and NASH resolution. Analyses restricted to RCTs enrolling patients without diabetes yielded similar results. All effects were accounted for by pioglitazone use[240]. Weight gain and lower limb edema occurred more frequently with thiazolidinedione therapy[240]. These novel data indicate that pioglitazone improves advanced fibrosis in NASH, even in patients without diabetes.

A phase 2 randomized study conducted on 52 patients (26 per arm) tested the efficacy of liraglutide (1.8 mg/daily *via* subcutaneous route) *vs* placebo. The use of liraglutide was associated with a 39% NASH resolution rate *vs* 9% in the placebo arm (RR = 4.3, 95%CI: 1.0-17.7, P = 0.019). This study was funded by the manufacturers of liraglutide[241].

A few drugs**,** such as vitamin E and obeticholic acid (OCA) have specifically been tested against NASH. Based on a meta-analysis of five RCTs vitamin E significantly reduced liver enzymes (AST, ALT, ALP); steatosis, inflammation and hepatocellular ballooning compared to the control group. Vitamin E treatment in adult NASH was also associated with reduction of fibrosis[242]. Concerns have been raised, however, on the long-term safety of Vitamin E and the lack of data in patients with T2D and cirrhosis. A network meta-analysis found that pentoxyphylline and OCA improve fibrosis, and vitamin E, TZDs, and OCA improve ballooning degeneration in patients with NASH[243]. A subsequent network meta-analysis substantially confirmed these findings by showing that OCA significantly improves fibrosis, while vitamin E and TZDs result in a significant increase in NASH resolution[244]. Table 2 compares the findings from the above two meta-analytic studies[243,244].

Thinking out of the box. Innovative treatment strategies

Further to the above discussed notions on the relationship of the entire NAFLD spectrum with endocrine and metabolic derangements, a line of innovative treatment options can be envisaged. For example, administration of thyroid receptor agonists (GC-1 and KB2115) improves NAFLD in a ob/ob mouse model and a xantine oxidase inhibitor (febuxostat) antagonizes the development of disease in a rodent NASH model[245,246].

Also our understanding of the role of nuclear receptors in the pathogenesis and natural course of NAFLD may lead to novel treatments[15]. The above-mentioned OCA exerts its effects by agonizing FXR. Elafibranor, a dual PPAR-α/δ agonist, has been shown to resolve NASH without fibrosis worsening and to improve the cardiometabolic profile in a recent phase 2 randomized trial of patients with NASH[247]. Oltipraz is a synthetic inhibitor of LXR-α whose antisteatosic properties have previously been shown in a mouse model. A recent phase 2 randomized controlled trial found that, in patients with NAFLD, a 24-wk course of oltipraz significantly reduced intrahepatic fat content assessed with magnetic resonance spectroscopy in a dose-dependent manner; however IR, liver enzymes, lipids and cytokines were unaltered[248].

CONCLUSION

NAFLD is an emerging multifaceted systemic disease with a heavy epidemiological burden. Further to hepatic risks, NAFLD also affects cardiovascular, metabolic, renal and endocrine systems as well as cancer development with a significant hepatic and extra-hepatic impact on morbidity and mortality[4]. The bi-directional relationship between NAFLD and MetS-T2D is an important emerging topic underpinning the mutual influence and the need to interrupt the *vicious circle* associating liver and dysmetabolism. However, we now know that IR is a necessary though not sufficient requisite of the development of NAFLD and that gender, genetic background and microbiota also play a major role[4,14]. It is anticipated that pathogenic complexity and multi-factoriality will have to be translated into more finely tailored diagnostic and treatment strategies. This implies that *"ad hoc"* studies are necessary to more precisely identify the individual target populations to be addressed. Presently, lifestyle changes effected *via* dietary restrictions and physical exercising remain the only recommended treatment of NAFLD; in all overweight/obese patients, weight loss is mandatory[4]. However, given that only a small minority of such individuals will succeed in maintaining their ideal body weight, and that no currently marketed

drugs are specifically licensed for NASH, data of ongoing studies are eagerly awaited to offer these numerous and heterogeneous patients effective and safe tailored drug treatment options.

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REVIEW

New therapeutic perspectives in irritable bowel syndrome: Targeting low-grade inflammation, immuno-neuroendocrine axis, motility, secretion and beyond

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Abstract

Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, distention, and changes in bowel habits. Although there are several drugs for IBS, effective and approved treatments for one or more of the symptoms for various IBS subtypes are needed. Improved understanding of pathophysiological mechanisms such as the role of impaired bile acid metabolism, neurohormonal regulation, immune

dysfunction, the epithelial barrier and the secretory properties of the gut has led to advancements in the treatment of IBS. With regards to therapies for restoring intestinal permeability, multiple studies with prebiotics and probiotics are ongoing, even if to date their efficacy has been limited. In parallel, much progress has been made in targeting low-grade inflammation, especially through the introduction of drugs such as mesalazine and rifaximin, even if a better knowledge of the mechanisms underlying the low-grade inflammation in IBS may allow the design of clinical trials that test the efficacy and safety of such drugs. This literature review aims to summarize the findings related to new and investigational therapeutic agents for IBS, most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

Key words: Therapy; Low grade inflammation; Motility; Secretion; Irritable bowel syndrome; Immunoendocrine axis

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Core tip: Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, distention, and changes in bowel habits. Despite there are several drugs for IBS, effective and approved treatments for one or more of the symptoms for various IBS subtypes are needed. The understanding of pathophysiological mechanisms such as the role of impaired bile acid metabolism, neurohormonal regulation, immune dysfunction, the epithelial barrier and secretory properties of the gut has led to advancements in the treatment of IBS. This literature review aims to summarize the findings relating the new and investigational therapeutic agents for IBS, most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

Sinagra E, Morreale GC, Mohammadian G, Fusco G, Guarnotta V, Tomasello G, Cappello F, Rossi F, Amvrosiadis G, Raimondo D. New therapeutic perspectives in irritable bowel syndrome: Targeting low-grade inflammation, immuno-neuroendocrine axis, motility, secretion and beyond. *World J Gastroenterol* 2017; 23(36): 6593-6627 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6593.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6593

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic, recurring, and remitting functional disorder of the gastrointestinal (GI) tract characterized by abdominal pain, distention, and changes in bowel habits that do not have a known structural or anatomical explanation[1].

IBS is a global problem, with anywhere from 5% to 15% of the general population showing symptoms that would satisfy a definition of IBS[2-4]. IBS considerably affects quality of life and imposes a profound burden on patients, physicians and the health-care system[5]. For example, the IBIS-C study recently assessed the socio-economic burden of moderate-to-severe IBS with constipation in six European countries (France, Germany, Italy, Spain, Sweden and the United Kingdom), showing that IBS represents a main cause of absenteeism in the workplace[6].

Regarding the sex-related prevalence of IBS, in Western countries, the prevalence of IBS in women outnumbers that in men by 2:1[7,8], and within the patient population who have consultations with primary care physicians, women outnumber men by 3:1[7,9]. Finally, in tertiary care settings, the number of women with IBS is 4 to 5 times higher than the number of men[7-10].

According to Rome Ⅲ, IBS is defined based on the presence of: recurrent abdominal pain or discomfort at least 3 d/mo in the past 3 mo associated with two or more of the following: (1) improvement with defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool.

These criteria should be fulfilled for the past 3 mo with symptom onset at least 6 mo before diagnosis[11]. Recently, the Rome Ⅳ criteria implemented the knowledge accumulated since Rome Ⅲ was published almost ten years ago.

According to Rome Ⅳ, IBS is defined on the basis of the presence of: Recurrent abdominal pain, on average, at least 1 d per week in the last 3 mo, associated with 2 or more of the following criteria: (1) related to defecation; (2) associated with a change in frequency of stool; and (3) associated with a change in form (appearance) of stool. These criteria should be fulfilled for the last 3 mo with symptom onset at least 6 mo before diagnosis[12].

In contrast to the Rome Ⅲ criteria, the term discomfort has been deleted from the last definition and from subsequent diagnostic criteria because not all languages have the term "discomfort". This word has different meanings in different languages, which can result in ambiguity with patients[12]. Furthermore, the last definition implies a change in the frequency of abdominal pain, highlighting that patients should have symptoms of abdominal pain at least 1 d per week during the past 3 mo[12]. Finally, the sentence "improvement with defecation" was substituted in the current definition by "related to defecation", as a large subset of IBS patients do not have an improvement in abdominal pain with defecation but instead complain of worsening[12].

According to the Rome Ⅳ criteria, IBS is subtyped according to the predominant bowel habit as follows:

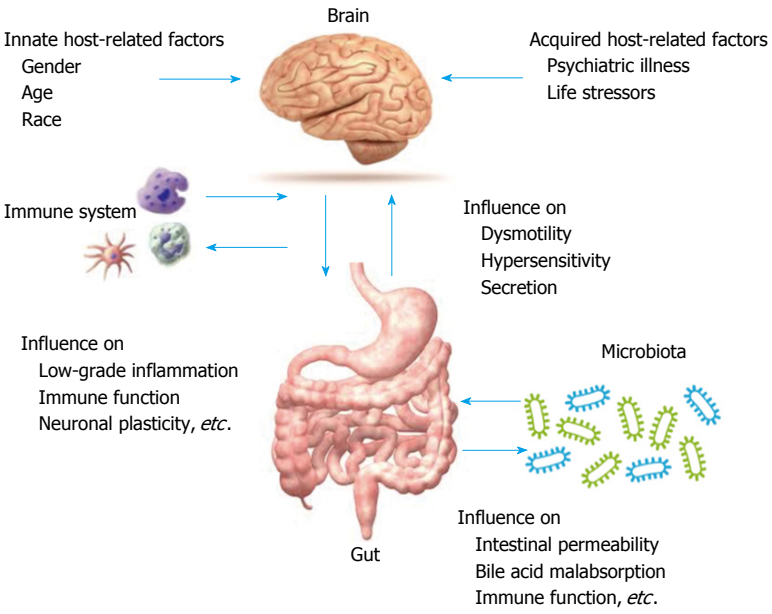


Figure 1 Complex interplay between low-grade inflammation, immuno-neuroendocrine axis, and microbiota. Brain (influenced by multiple innate and acquired factors) and gut interact bidirectionally to shape the clinical phenotype of irritable bowel syndrome (IBS). This bi-directional pathway acts not only on gastrointestinal motility, visceral sensitivity and secretion; however, the influence of both the immune system and microbiota modulates several functions that could create the definitive clinical phenotype of IBS.

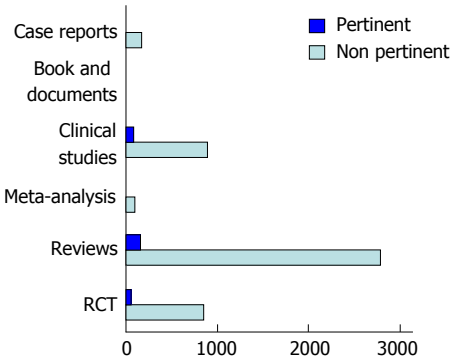


Figure 2 Literature findings on the relationship between irritable bowel syndrome and inflammation (*n* = 317). RCT: Randomized controlled trials.

IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), mixed type (IBS-M), and unclassified (IBS-U)[12]. The definition of bowel habit type is based on the patient's description of the stool form by referring to the Bristol Stool Scale[13]. Furthermore, IBS patients can be grouped into sporadic (nonspecific) and post-infectious (PI-IBS)/inflammatory bowel disease (IBD)-associated (IBD-IBS)[14,15].

Although there are several drugs for IBS in the pipeline, there is a continuous need for effective and approved treatments for one or more of the symptoms of IBS subtypes[16-18]. The understanding of pathophysiological mechanisms such as the role of altered bile acid metabolism, neurohormonal regulation, immune dysfunction, the epithelial barrier and secretory properties of the gut has led to progress in the treatment options of IBS (Figure 1)[18,19].

This literature review aims to summarize the findings relating the new and investigational therapeutic agents for IBS most recently developed in preclinical as well as Phase 1 and Phase 2 clinical studies.

MATERIALS AND METHODS

We carried out a bibliographic search in MEDLINE for the period January 1966 to December 2016 and focused on identifying publications describing the new therapeutic pharmacological approaches in IBS. Information was also obtained from abstracts and the latest results found in the Clinicaltrial.gov database. The keywords used were: irritable bowel syndrome, inflammation, immunoendocrine axis, intestinal permeability, IBS-C, IBS-D, therapy. The inclusion criteria to select articles were based on design (systematic reviews, meta-analysis, clinical trials, and experimental studies on animals) and population (adult patients > 18 years of age). We excluded articles not relevant for this topic.

According to the abovementioned criteria, 5127 studies were found and 4810 studies were excluded because they were not relevant for this topic (Figure 2).

LOW-GRADE INFLAMMATION IN IRRITABLE BOWEL SYNDROME

Recently, the scientific community has focused its attention on the pivotal role of low-grade mucosal inflammation in IBS, considering evidence showing that some patients with IBS have an increased number of inflammatory cells in the colonic and ileal mucosa, with regard to control patients[20].

In fact, the intestinal mucosa harbours a florid immune system that can be regarded as "physiologically inflamed"[20,21]. Thus, low-grade inflammation, which likely plays a multifactorial role in IBS pathophysiology, can only be evaluated using quantitative assessments[20-22].

The available data[23,24] on low-grade inflammation in IBS patients is often expressed as average numbers and are mainly focused on IBS-D. Thus, it is unclear whether this event occurs only in selected subsets of IBS patients[25].

Therefore, IBS could be considered a micro-organic disease, where there is an increased number of mucosal immunocytes (*i.e.,* mast cells, eosinophils, and T cells) in adult and paediatric patients. Several precipitating factors have been claimed, including food allergy, abnormal microbiota, bile acid malabsorption, and increased intestinal permeability[26]. The magnitude of the inflammatory response is several-fold less than that seen in acute inflammation in inflammatory bowel disease. The above-reported evidence provides a rationale to evaluate the efficacy of intestinal anti-inflammatory therapies in patients with IBS that we will touch upon in the next section.

ANTI-INFLAMMATORY THERAPIES IN IRRITABLE BOWEL SYNDROME

Corticosteroids

In the study performed by Dunlop *et al*[24], twenty-nine patients with post-infectious irritable bowel syndrome underwent a randomized, double-blind, placebo-controlled trial of 3 wk of oral prednisolone, 30 mg/d. Mucosal enterochromaffin cells, T lymphocytes and mast cells were evaluated in rectal biopsies before and after treatment, and bowel symptoms were reported in a daily diary. In this study, enterochromaffin cell counts did not change significantly after either prednisolone or placebo. Although lamina propria T-lymphocyte counts decreased significantly after prednisolone, but not after placebo, this was not linked with any significant treatment-related improvement in abdominal pain, diarrhoea, frequency or urgency[24].

Antibiotics

Rifaximin is a rifamycin derivative that acts by inhibiting bacterial ribonucleic acid (RNA) synthesis. It is virtually unabsorbed after oral administration, so it is used mainly to treat local dysfunctions within the gastrointestinal tract[27].

The Food and Drug Administration (FDA) initially approved rifaximin to treat traveller's diarrhoea caused by Escherichia coli and to prevent the recurrence of hepatic encephalopathy.

Successively, the FDA approved rifaximin in IBS-D "naive" patients at a dose of 550 mg three times a day for 14 d as well as in patients experiencing a recurrence of symptoms.

Rifaximin improves IBS symptoms through a variety of mechanisms directed at the gastrointestinal tract. In fact, much evidence from animal experiments shows that rifaximin either improves or maintains microbiota diversity and bacterial composition in IBS, reduces intestinal cytokine inflammation, provides gut-barrier protection preventing attachment and internalization of coliforms and pathogens with reduced epithelial cell inflammation and pathogen-induced inflammatory response, and reduces visceral hyperalgesia[28].

In a combined analysis of two separate Phase 3 trials (TARGET 1 and 2), a 14-d course of rifaximin 550 mg three times daily in IBS-D patients significantly increased the percentage of relief of global IBS symptoms and improved IBS-related distention and abdominal pain, discomfort, and loose or watery stools compared with placebo for up to 10 wk post-treatment[29,30].

Successively, TARGET 3 was performed to test the safety and efficacy of a repeated treatment with rifaximin in patients experiencing a recurrence of IBS symptoms. In this study, the percentage of responders during the 18-wk follow-up (in terms of pain and stool consistency improvements) to randomized repeat treatment was significantly greater with rifaximin *vs* placebo[31]. The safety profile of rifaximin in patients with IBS-D was generally similar to that observed with placebo[30].

In fact, constipation was only reported in 1 (0.3%) patient in the rifaximin group and 3 (1.0%) patients in the placebo group. Only one patient in each treatment group suspended the drug. One case of Clostridium difficile infection occurred (in a patient who had been off of rifaximin for several weeks but was receiving a concomitant systemic antibiotic)[30,31].

In conclusion, these trials show that a 2-wk course of rifaximin could improve IBS-D-related symptoms, and in the case of persistence of symptoms, retreatment may ameliorate abdominal pain and stool consistency with possible improvements in bloating and stool urgency in some patients. While patients were retreated within an 18-wk period of follow-up in the study, it is still unclear as to when and how often treatment should be given. In addition, the identification of those patients who might likely respond to rifaximin remains to be investigated.

Recently, Ghoshal *et al*[32] evaluated symptom resolution among IBS patients with or without small intestinal bacterial overgrowth (SIBO) on norfloxacin treatment and its efficacy in obtaining negative SIBO test results as compared with placebo. In this study, 80 IBS patients (Rome Ⅲ) were evaluated for SIBO by gut aspirate culture. Patients with a colony count ≥ 10 CFU/mL and those without SIBO were separately randomized to 800 mg/d norfloxacin for 10 d or placebo. The global symptom score (blind), Rome Ⅲ

criteria, aspirate culture, and glucose hydrogen breath test were assessed before and 1 mo after treatment, and patients were followed up for 6 mo. Although norfloxacin was more effective at decreasing the symptom score at 1 mo among patients with compared with those without SIBO but not placebo, the scores were comparable at 6 mo. Symptoms more often resolved to turn Rome Ⅲ negative in SIBO patients treated with norfloxacin compared with placebo at 1 mo. Patients without SIBO and a colony count of 10 CFU/mL responded more than those with a colony count less than 10 CFU/mL[32].

Mast cell stabilizers

Since mast cell activation was thought to be involved in visceral hypersensitivity, a study was undertaken by Klooker *et al*[33] to evaluate the effect of ketotifen, a mast cell stabilizer, on rectal sensitivity and symptoms in patients with IBS. In this case-control study, 60 patients with IBS underwent a barostat study to assess rectal sensitivity before and after 8 wk of treatment. After the initial barostat, patients were randomised to receive ketotifen or placebo. Ketotifen increased the threshold for discomfort in patients with IBS and visceral hypersensitivity but not placebo. This effect was not observed in normosensitive patients with IBS. Ketotifen significantly reduced abdominal pain and other IBS symptoms and improved quality of life. However, whether this effect was secondary to the mast cell stabilising properties of ketotifen or H1 receptor antagonism remains a topic of future research[33].

Successively, Lobo *et al*[34] showed a clinical Benefit of Disodium Cromoglycate (DSCG) in IBS in a double-blind, placebo-controlled clinical assay with prolonged (6 mo) oral administration of DSCG (DSCG), since it induces mast cell-mediated recovery of the healthy-like innate immunity gene expression profile in the jejunal mucosa[34].

Finally, since histamine sensitizes the nociceptor transient reporter potential channel V1 (TRPV1) and has been observed to play role in visceral hypersensitivity in animals, Wouters *et al*[35,36] investigated the role of ebastine, an antagonist of histamine receptor H1 (HRH1), in reducing symptoms of patients in a randomized placebo-controlled trial. After a 2-wk run-in period, subjects were enrolled randomly to groups given either the HRH1 antagonist ebastine or placebo for 12 wk. Rectal biopsy specimens were collected, barostat studies were performed, and symptoms were recorded (using the validated gastrointestinal symptom rating scale) before and after the 12-wk period. Patients were followed up for a further 2 wk. The primary end point of the study was the evaluation of ebastine efficacy on the symptom score evoked by rectal distension. Compared with the placebo group, patients treated with ebastine had reduced visceral hypersensitivity, increased symptom relief, and reduced abdominal pain scores[35,36].

Mesalazine

The therapeutic potential of aminosalicylates, whose benefits in chronic inflammatory bowel diseases are well known, has been focused on as a potential cure for IBS[37,38].

The largest studies on mesalazine in IBS have been conducted by Barbara and Lam. Barbara *et al*[39] conducted a phase 3, multicentre, tertiary setting, randomised, double-blind placebo-controlled trial in patients with Rome Ⅲ-confirmed IBS. Patients were randomly assigned to either 800 mg mesalazine or placebo three times daily for 12 wk and were followed for an additional 12 wk. The primary efficacy endpoint was satisfactory relief of abdominal pain/discomfort for at least half of the weeks of the treatment period. The secondary endpoint was satisfactory relief of overall IBS symptoms. The responder patients were 68.6% in the mesalazine group *vs* 67.4% in the placebo group. However, with the 75% rule or > 75% rule, there was a higher percentage of responders in the mesalazine group than placebo of 11.6% and 5.9%, respectively, although these differences were not significant. For the key secondary endpoint, in the mesalazine group, overall symptom improvement was observed and a significant difference of 15.1% *vs* placebo with the > 75% rule was reached. The authors concluded that mesalazine treatment was not superior to placebo on the study primary endpoint, but a subgroup of patients with IBS had a sustained therapy response and benefits from mesalazine therapy[39].

On the other hand, Lam *et al*[40] conducted a double-blind, randomised placebo-controlled trial of 2 g mesalazine twice daily compared with placebo for 3 mo in Rome Ⅲ criteria patients with IBS-D. The authors compared the mesalazine and placebo effects on stool frequency as the primary endpoint and secondarily assessed the effect of mesalazine on abdominal pain, stool consistency, urgency and satisfactory relief of IBS symptoms. In total, 136 IBS-D patients (82 female, 54 male) were enrolled; 10 patients withdrew from each group. The intention to treat analysis showed that the mean daily stool frequency during weeks 11 and 12 was 2.8 (SD 1.2) in the mesalazine group and 2.7 (SD 1.9) in the placebo group, with a group difference of 0.1. The authors concluded that mesalazine did not ameliorate abdominal pain, stool consistency or percentage with satisfactory relief compared with placebo during the last 2 weeks' follow-up. However a post hoc analysis in 13 post-infectious patients with IBS tended to show benefit, even though this finding needs to be confirmed in larger studies[40].

A point of weakness of these studies is that the use of endpoints for response may be easily met by patients in the placebo arm, resulting in placebo response rates of almost 70% for satisfactory relief

Table 1 Summary of the literature findings about anti-inflammatory therapies in irritable bowel syndrome

Drug	Ref.	No. of patients	Study design	Outcome
Corticosteroids (prednisolone)	Dunlop *et al*[24]	29 patients with post-infectious irritable bowel syndrome	Randomized, double-blind, placebo-controlled trial of 3 wk of oral prednisolone, 30 mg/d	Not associated with any significant treatment-related improvement in abdominal pain, diarrhoea, frequency or urgency
Antibiotics (Rifaximin)	Pimentel *et al*[29] Target 1 e 2	623 IBS patients in TARGET 1 and 637 IBS in TARGET 2	Phase 3 trials, 14 d with rifaximin 550 mg 3 times daily	Significantly increased the percentage of relief of global IBS symptoms and improved IBS-related bloating and abdominal pain, discomfort, and loose or watery stools, with regard to placebo for up to 10 wk post-treatment
Antibiotics (norfloxacin)	Ghoshal *et al*[32]	80 IBS patients evaluate for SIBO	Randomized, double-blind, placebo-controlled trial; patients were randomized to 800 mg/d norfloxacin for 10 d or placebo	Although norfloxacin was more effective at reducing the symptom score at 1 mo among patients with compared with those without SIBO but not placebo, the scores were comparable at 6 mo. Symptoms more often resolved to turn Rome Ⅲ negative in SIBO patients treated with norfloxacin compared with placebo at 1 mo
Mast cell stabilizers (Ketotifen)	Klooker *et al*[33]	60 IBS patiens	Case Control study; abarostat study to assess rectal sensitivity before and after 8 wk of treatment and, after the initial barostat, patients were randomised to receive ketotifen or placebo	Ketotifen but not placebo increased the threshold for discomfort in patients with IBS with visceral hypersensitivity, but this effect was not observed in normosensitive patients with IBS. Ketotifen significantly decreased abdominal pain and other IBS symptoms and improved quality of life
Mast cells stabilizers (DSCG)	Lobo *et al*[34]		Randomized, double-blind, placebo-controlled trial; with prolonged (6 mo) oral administration of DSCG	Induces Mast Cell-Mediated Recovery of Healthy-Like Innate Immunity Genes Expression Profile in the Jejunal Mucosa
Mast cells stabilizers (ebastin)	Wouters *et al*[35]	65 IBS patients	Double-blind placebo-controlled trial, after 2-wk run-in period, subjects were assigned randomly to groups ebastine (20 mg/d; n = 28) or placebo (n = 27) for 12 wk	Compared with subjects given placebo, those given ebastine had reduced visceral hypersensitivity, increased symptom relief, and reduced abdominal pain scores
Mesalazine	Barbara *et al*[39]	185 patients with IBS	A phase 3, multicentre, tertiary setting, randomised, double-blind, placebo-controlled trial in patients with Rome Ⅲ confirmed IBS. Patients were randomly assigned to either mesalazine, 800 mg, or placebo, three times daily for 12 wk, and were followed for additional 12 wk	Mesalazine treatment was not superior than placebo on the study primary endpoint, but a subgroup of patients with IBS showed a sustained therapy response and benefits from a mesalazine therapy
	Lam *et al*[40]	136 patients with IBS-D	A double-blind, randomised placebo-controlled trial of 2 g mesalazine twice daily compared with placebo for 3 mo	The authors concluded that mesalazine did not improve abdominal pain, stool consistency or percentage with satisfactory relief compared with placebo during the last 2 weeks' follow-up, however a post hoc analysis in 13 post-infectious patients with IBS appeared to show benefit but this needs confirmation in a larger group[40]

IBS : Irritable bowel syndrome.

of abdominal pain or discomfort and > 60% for satisfactory relief of overall IBS symptoms in the trial performed by Barbara *et al*[39] and in > 40% for satisfactory relief of IBS symptoms in the trial performed by Min *et al*[41].

It may have therefore have been preferable to use a once daily dosing schedule in both trials in order to reduce the placebo response rates, thus increasing the likelihood of detecting a statistically significant difference between mesalazine and placebo.

Based on this evidence, it is necessary that further studies prove the efficacy of mesalazine for IBS. Studies aimed at evaluating the role of aminosalicylates and other potential anti-inflammatory treatment options, including probiotics, non-absorbable antibiotics, histamine receptor antagonists and protease inhibitors on IBS symptoms or pathophysiology are now warranted[39].

Table 1 sums up the literature findings about anti-inflammatory therapies in irritable bowel syndrome.

INTESTINAL PERMEABILITY IN IRRITABLE BOWEL SYNDROME

An increase in intestinal permeability can be seen in

many conditions, such as infectious gastroenteritis and irritable bowel disease[42]. The intestinal barrier has long been a focus of gastroenterological research[43] and its role in IBS has been discussed in many studies. Most studies show an increase in intestinal permeability of patients with IBS-D and post-infectious IBS (PI-IBS)[43-46].

Among the first to describe intestinal permeability in patients with PI-IBS were Spiller *et al*[47], who detected an increased lactulose/mannitol ratio in the urine of IBS patients compared to healthy controls.

Marshall *et al*[48] also described an increase in permeability of patients with IBS after an outbreak of bacterial gastroenteritis but could not show a difference in permeability between PI-IBS and non PI-IBS.

There are genetic risk factors for developing PI-IBS and CDH1, which codes for E-cadherin, a tight junction (TJ) protein that is involved in the epithelial barrier function of the gut[49], hence suggesting the pathophysiological mechanism through which some patients experience increased permeability.

The mechanism of increased permeability in patients with IBS is suggested to involve tight junction dysfunction or involvement of the adherence proteins[44]. Among factors that could influence permeability is stress. Male soldiers were evaluated in a prospective study during and after combat training with an increase in physiological and psychological stress. Their training induced an increase in gastrointestinal symptoms and alteration in the permeability of the gut barrier[50]. Since stress has been suggested to be one of the pathophysiological factors involved in developing IBS, this mechanism could explain the reason for the gastrointestinal symptoms[51].

Another factor that has been evaluated is the intraluminal content of patients with IBS, where faecal supernatants from patients have increased the colonic permeability in mice[52,53].

Both intracellular [zonula occludens (ZO)-1, ZO-2, and ZO-3, and cingulin] and surface-membrane proteins [occludin, claudins, and junctional adhesion molecules (JAM)] are the main components of TJ[26,54]. Adherens junctions are mainly made up of e-cadherin, catenin, and actin filaments[26,55].

Inflammation has also been described to be a factor in increasing intestinal permeability, not only in inflammatory bowel disease[43] but also in IBS, where the increase in mast cells and the mediators increased the effects on the intercellular junctions[44].

Finally, other factors, such as hormonal and neuro-hormonal pathways, nutritional factors, ethanol consumption and several drugs (nonsteroidal anti-inflammatory drugs, methotrexate, tacrolimus, protonic pump inhibitors), could affect the intestinal barrier, a factor that needs to be further evaluated[26]. The knowledge of affection of the intestinal permeability in IBS patients will help in the development of new therapies in order to restore the gut barrier, a topic we will touch upon in the next section.

Table 2 sums up the literature findings about therapies restoring intestinal permeability in irritable bowel syndrome.

THERAPIES RESTORING INTESTINAL PERMEABILITY IN IRRITABLE BOWEL SYNDROME

Probiotics

The human intestinal microbiota represents one of the densest, biodiverse, and rapidly evolving bacterial ecosystems. The intestinal microbiome, that is, its collective genome, is an adaptive entity that varies with diet, lifestyle and environment, providing a further metabolic flexibility to the human super organism and functional traits that humans have not evolved on their own[56]. Therefore, the potential of manipulating the gut microbiota in these disorders is assessed[57].

The mechanisms through which probiotics alter the intestinal microbial flora could be direct, changing the bacterial macroenvironment of the lumen, or indirect, through the stimulation of the immune system and the improvement of mucosal function, for example, by modulating the invasion and adherence of the epithelial cells of the gut by pathogenic bacteria, thus normalizing gut permeability[58-60]. The use of probiotics in patients with IBS seems to be effective in achieving improvement in the global IBS symptoms[61,62], but how it affects the intestinal permeability is less evaluated in humans[46]. Most studies have shown that altering the intraluminal content affects the barrier functions of the gut, and studies on rodent models of IBS have shown different data[63].

Despite the growing interest of the scientific community in research in the field of probiotics, the interpretation of the scientific literature on the value of these preparations' results is difficult due to the wide variability in the species, strains and doses employed in the preparations as well as the low methodological quality of the available trials, often due to the poor design and the small sample size.

Several meta-analysis have been published on this topic[64-66], all concluding that probiotics might be efficacious in IBS, but the actual benefit and the most effective species and strains are uncertain.

In the meta-analysis performed by Ford *et al*[61], including forty-three randomized controlled trials, probiotics showed beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores[61]. Probiotics appeared to be successful in chronic idiopathic constipation (CIC), but there were only two randomized controlled trials, and again, since trials for probiotics are few in number, no specific conclusions could be obtained[61].

Table 2 Summary of the literature findings about therapies restoring intestinal permeability in irritable bowel syndrome

Drug	Ref.	No. of patients	Study design	Outcome
Probiotics	Ford *et al*[61]	Forty-three RCTs were eligible for inclusion	Metanalysis	Probiotics had beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores. Data for prebiotics and synbiotics in IBS were sparse. Probiotics appeared to have beneficial effects in CIC (mean increase in number of stools per week = 1.49; 95%CI: 1.02-1.96), but there were only two RCTs. Synbiotics also appeared beneficial (RR of failure to respond to therapy = 0.78; 95%CI: 0.67-0.92). Again, trials for prebiotics were few in number, and no definite conclusions could be drawn
	Mazurak *et al*[67]	Fifty-six papers	Metanalysis	The heterogeneity of the studies of probiotics in IBS questions the value of meta-analyses and the use of different bacterial strains and different mixtures of these strains, as well as different dosages, are the main contributors to this heterogeneity
Glutamine	Akobeng *et al*[73]	Two randomized trial	Cochrane analysis	Not significant difference in the permeability and no effect in the clinical remission
Larazotide acetate	Leffler *et al*[78]	342 adults with celiac disease who had been on a gluten free diet (GFD) for 12 mo or longer and maintained their current GFD during the study	Randomized, double-blind, placebo-controlled study assessed larazotide acetate 0.5, 1, or 2 mg 3 times daily	Reduce signs and symptoms in celiac disease patients on a GFD better than a GFD alone

In the last updated meta-analysis performed by Mazurak *et al*[67], including fifty-six papers (twenty-seven studies using multi-species bacterial preparations and twenty-nine using single-strain probiotics), they analysed the efficacy of probiotics regarding patients included, treatment duration, probiotic dosage, and outcome measures. According to the authors, the heterogeneity of the studies of probiotics in IBS impairs the value of meta-analyses. The use of different bacterial strains and different mixtures of these strains, as well as different dosages, may be the main factors contributing to this heterogeneity[67]. Currently, there is limited evidence for the efficacy of a small number of single-strain probiotics in IBS (mostly bifidobacteria), and this evidence leads to the performance of trials with inclusion and exclusion criteria closely following the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) guidelines for clinical trials in IBS[68,69], including the definition of minimal severity for inclusion, global primary endpoints, and adequate secondary end-points (pain, bloating, and a clinically meaningful responder definition). Such trials should include at least 8 wk of therapy, an adequate follow-up period and restriction to one of the different IBS subtypes[67].

Glutamine

Glutamine is one of the compounds that has been investigated as a treatment of conditions with leaky gut. It has been shown to regulate the protein turnover in enterocytes of pig[70], reduce intestinal permeability in intestinal cell cultures and maintain transepithelial resistance[71]. Glutamine has also been shown to maintain the integrity of the intestinal barrier in critically ill patients by reducing the incidence of infections[72]. Glutamine treatment in patients with Crohn's disease was recently reviewed in a Cochrane analysis[73]. In this review, only two randomized controls were included, and neither showed a significant difference in the permeability and neither had any effect on clinical remission.

Glutamine treatment in patients with IBS is less examined. Glutamine synthetase expression is lower in the small bowel and colonic mucosa of patients with IBS-D with increased intestinal permeability[74]. Therefore, one recent pilot study on IBS-D showed that with a higher glutamine concentration, Claudin-1 expression increases, thus improving the permeability[75]. However, further studies are needed for using glutamine as a supplement treatment for IBS.

Larazotide acetate

Larazotide acetate (LA) is a tight-junction regulator peptide preventing the opening of intestinal epithelial TJ[76]. The safety, tolerance and pharmacokinetics of LA were studied in a randomized double-blind placebo-controlled study conducted on celiac disease subjects challenged with gluten[76,77].

Recently, in a multicentre, randomized, double-blind placebo-controlled study, LA at doses of 0.5, 1, or 2 mg 3 times daily was evaluated to relieve ongoing symptoms in 342 adults with celiac disease who had been on a gluten-free diet (GFD) for 12 mo or longer and maintained their current GFD during the study. A 0.5 mg dose of Larazotide acetate appeared to reduce signs and symptoms in celiac disease patients on a

GFD better than a GFD alone. Although the results were mixed, this study resulted in the successful use of a novel therapeutic agent targeting tight junction regulation in those patients with CeD who are symptomatic despite a GFD[78].

Therefore, the modulation of the tight junction could represent a paradigm shift in the treatment of immune mediated and inflammatory diseases (Celiac Disease, IBD, IBS, *etc*).

THERAPEUTIC TARGETS IN CONSTIPATION-PREDOMINANT IRRITABLE BOWEL SYNDROME

Constipation-predominant irritable bowel syndrome (IBS-C) is a frequent disorder and represents one of the main causes of ambulatory visits. Abdominal pain and discomfort characterize IBS-C, making it different from chronic idiopathic constipation[79].

It is now well known that treatment focusing only on bowel transit does not provide complete relief to patients with IBS-C. A global evaluation of the pathophysiology of IBS-C has led to the use of sensory end points like complete spontaneous bowel movements and the FDA combined end point (abdominal pain and complete spontaneous bowel movements) in clinical trials[79].

For example, new information on the mechanisms underlying pain sensation in chronic visceral hypersensitivity as well as insights into the mechanism of action of new drugs targeting abdominal pain in IBS have recently been obtained by preclinical experiments in rodent models[80]. A number of drugs that we will touch upon in the next section are actually in development.

Linaclotide

Linaclotide (MD-1100 acetate) is a novel orally active 14-amino acid peptide of the guanylin family of cyclic guanosine monophosphate (cGMP)-regulating guanylate cyclase-C (GC-C) agonists. It has been approved by the FDA and by the EMA for the treatment of moderate to severe IBS-C in adults. Its action is focused on the increase of fluid secretion, favouring gastrointestinal transit, and has GC-C-mediated analgesic effects[81].

It is recommended at a dose of 290 μg orally once a day before meals. Linaclotide is converted to an active metabolite (MM-419447) that has the same pharmacodynamics and pharmacokinetics as the parent drug.

In 2007, Andresen *et al*[82] investigated the effect of 5 d of linaclotide on transit and bowel function in 36 women with IBS-C according to Rome Ⅱ criteria randomized in a 1:1:1 fashion for placebo, linaclotide 100 μg, and linaclotide 1000 μg.

Patients with slow colonic transit or slower transit than the mean for healthy controls were studied for 5 d at baseline and 5 d during the treatment. Patients collected all the information regarding gastric, small bowel, and colonic transit by scintigraphy and bowel function using stool diaries, which included Bristol Stool Form Scale (BSFS) scores for stool consistency, ease of stool passage scores, and completeness of evacuation.

Linaclotide did not show any effect on gastric emptying or colonic filling. It did show a significant effect on ascending colon emptying t½ times ($P = 0.015$) and on overall total colonic transit times at 48 h ($P = 0.02$) at the 1000 μg dose ($P = 0.004$), but not at the 100 μg dose, as well as on increased stool frequency, decreased stool consistency, improved ease of passage, and acceleration of time to first bowel movement ($P < 0.001$)[82].

In 2010, Johnston *et al*[83] investigated the efficacy and safety of 12 wk of linaclotide at a daily dose range of 75-600 μg in a phase Ⅱb randomized double-blind parallel-group multicentre placebo-controlled trial conducted on 420 patients with IBS-C (female patients = 92%). Patients had to meet Rome Ⅱ criteria, with fewer than three spontaneous bowel movements (SBMs) per week, and straining, lumpy/hard stools, or sensation of incomplete evacuation more than 25% of the time for at least 12 wk in the 12 mo preceding study entry. The primary endpoint was a change in the number of complete spontaneous bowel movements (CSBMs). Secondary endpoints were the effect on individual symptoms, quality of life (QOL), the number of patients who were CSBM responders (at least three CSBMs/wk and an increase of one CSBM from baseline for 75% of the study duration), and global relief responders (symptoms being somewhat, considerably, or completely relieved for 100% of the study duration or completely relieved for 50% of the study duration).

For the 75, 150, 300 and 600 μg linaclotide doses, the mean change in CSBMs per week was 2.90, 2.49, 3.61 and 2.68, respectively ($P < 0.01$), and the percentage of patients who were CSBM responders was 25%, 19.5%, 32% and 24%, respectively. Patients treated with linaclotide showed an adequate relief response (33%-51% *vs* 22%) and a global relief response (44%-55% *vs* 29%) compared to placebo. All doses of linaclotide significantly improved bowel habits, including frequency of short bowel movements (SBMs) ($P \leqslant 0.001$) and CSBMs ($P \leqslant 0.01$), severity of straining ($P \leqslant 0.001$), stool consistency ($P \leqslant 0.001$), and abdominal pain scores ($P \leqslant 0.05$), than placebo. Abdominal discomfort, bloating, and global IBS-C measures were also improved for all doses except for the 75 μg (abdominal discomfort) and 150 μg (bloating) doses. The linaclotide effect was observed at the first week and lasted throughout the 12 wk of treatment.

The approval of linaclotide for IBS-C was based on two randomized double-blind placebo-controlled phase Ⅲ trials similar in study design, end points, and patient demographics[84-86].

Primary end points included both the FDA-recommended combined primary end point and a more rigorous combined primary end point that required

even more CSBM responses for 9 of 12 wk. Secondary end points included patient-reported abdominal pain, discomfort, and bloating; straining severity; and weekly SBM and CSBM frequency and stool consistency. The first phase Ⅲ trial included 804 adults with IBS-C who were randomized 1:1 to receive linaclotide 290 lg or placebo daily for 26 wk, with change-from-baseline end points measured at 12 and 26 wk[84]. Attrition rates were 18.5% at 12 wk and 25.6% at 26 wk. At 26 wk, the majority of patients withdrew from the study due to adverse events in the linaclotide arm (10.2%) and perceived lack of efficacy in the placebo arm (8.2%). Over 12 wk, the FDA combined primary end point was achieved by 33.7% of patients receiving linaclotide compared with 13.9% of patients receiving placebo ($P < 0.0001$).

Linaclotide was also superior to placebo in the more rigorous investigator-defined combined primary end point that was reached by 12.7% of linaclotide-treated patients *vs* 3.0% of placebo-treated patients ($P < 0.0001$). At 26 wk, 32.4% of patients receiving linaclotide and 13.2% of patients receiving placebo ($P < 0.0001$) reached the FDA combined primary end point.

Improvements in all secondary end points occurred in the linaclotide group at weeks 12 and 26. The second phase Ⅲ trial of linaclotide was composed by a 12-wk treatment phase followed by a 4-wk randomized withdrawal phase[86]. A total of 803 adults with IBS-C were randomized to receive linaclotide 290 lg or placebo once/d for 12 wk. Approximately 78% of patients completed the entire 16-wk study, and most of the patients who suspended the study did so due to adverse events in the linaclotide arm (7.9%).

In the 12-wk active treatment phase, linaclotide demonstrated statistically significant improvements in all primary and secondary efficacy end points compared with placebo. Approximately one-third (33.6%) of patients receiving linaclotide fulfilled both components of the FDA end point compared with 21% of patients receiving placebo ($P < 0.0001$). Statistically significant improvements were observed also in abdominal pain, discomfort, and bloating in linaclotide-treated patients, with a mean reduction of about 2 points from baseline (on an 11-point scale) compared with reductions of 1.1 with placebo ($P < 0.0001$ for each measure).

In the linaclotide arm, an improvement in severity of straining, constipation, and stool consistency was observed compared with the placebo arm (all $P < 0.0001$).

Linaclotide caused diarrhoea, abdominal pain, flatulence, headache, viral gastroenteritis, and abdominal distension as adverse events. Diarrhoea, the most common, occurred in less the 20% of patients, probably due to increased fluid secretion and accelerated colonic transit[84,86].

In Johnston *et al*[83] phase Ⅱb dose-ranging trial, diarrhoea of mild to moderate severity was the primary dose-dependent adverse effect observed. It occurred in 11.4%, 12.2%, 16.5% and 18.0% of patients in the 75, 150, 300 and 600 μg linaclotide dose groups, respectively, compared with 1.2% in the placebo group. Dehydration or electrolyte disturbances were not found, although one instance of faecal impaction occurred[83]. In the studies by Rao *et al*[86] and Chey *et al*[84], 4.5%-5.7% of the linaclotide-treated patients and 0.2%-0.3% of the placebo group discontinued the study due to diarrhoea.

In a phase Ⅲ clinical trial in IBS-C, patients experienced adverse events more in the linaclotide 290-lg group (65.4%) than in the placebo (56.6%, $P < 0.05$) group[87]. In another IBS-C phase Ⅲ trial, adverse effects in the linaclotide group were reported at a similar rate to placebo (56.2% *vs* 53.0%, $P = 0.39$)[88]. Adverse events were reported by 60.5% of patients receiving linaclotide 145 lg, 55.7% of patients receiving linaclotide 290 lg, and 52.1% of patients receiving placebo[89].

In phase Ⅲ clinical trials in patients with IBSC, diarrhoea was the most frequently reported adverse event, occurring in 19.5%-19.7% of patients in the linaclotide groups compared with 2.5%-3.5% of patients receiving placebo ($P < 0.0001$).

In randomized trials, linaclotide at 145 μg/d was best tolerated with improvement in CSBM/Wand symptoms in patients with CIC. Patients with IBS-C best responded to the 290-μg daily dose[84,85]. Linaclotide appeared to be very well tolerated.

Linaclotide is approved for the treatment of IBS-C in both male and female adults at a dosage of 290 lg once/d and for the treatment of CIC at a dosage of 145 lg once/d. The medication should be taken 30 min prior to breakfast. Renal or hepatic impairment is unlikely to affect the metabolism or clearance of linaclotide or its metabolite due to its low systemic exposure.

In conclusion, linaclotide can represent a targeted approach that addresses the complexity of symptoms associated with the syndrome. Linaclotide has been reported to safely improve IBS-C abdominal pain severity, bowel movement quality, and bowel movement frequency as well as key symptoms of abdominal fullness, bloating, and discomfort, with associated improvements in QOL. Based on the United States FDA and the EMA, linaclotide fulfils the recommended endpoints with a number needed to treat (NNT) ranging from 4.39 to 7.9. It is effective and can be associated with diarrhoea as the most common adverse effect leading to suspension of the medication in approximately 5% of patients. According to recent clinical evidence, linaclotide should be considered for patients with IBS-C due to its effect on abdominal pain and bowel symptom improvement.

Plecanatide

Plecanatide is a 16-amino acid GC-C agonist currently used in phase Ⅲ clinical trials for CIC and phase Ⅱ

trials for IBS-C[90]. Plecanatide mimics the endogenous agonist of the GC-C receptor in the intestinal tract. Like that of uroguanylin, plecanatide's actions are pH-dependent, with the most favorable efficacy in the acidic environment of the duodenum. Similar to linaclotide, plecanatide luminally activates the GC-C receptor on gastrointestinal mucosal epithelial cells, leading to intracellular secretory and extracellular anti-nociceptive effects *via* a cGMP-mediated second messenger pathway[91]. A phase Ⅲ randomized double-blind trial in 951 patients with CIC treated with 0.3, 1 or 3 mg plecanatide or placebo once/d for 12 wk was conducted[92]. The primary end points were weekly (more than three CSBMs/wk and an increase of more than one CSBM/wk from baseline) or an overall study response (weekly response for 9 of 12 wk, including 3 of the last 4 wk to ensure durability of response). The percentage of overall responders was significantly higher in the plecanatide 3 mg group compared with placebo (19% *vs* 10.7%, $P = 0.009$). Weekly responder rates were also significantly higher in plecanatide 3 mg than placebo for weeks 1-12. Patients treated with 3 mg showed an improvement in stool frequency, consistency, straining, and quality of life compared with placebo. Data for other plecanatide doses were not shown.

Plecanatide potentially has low risk of adverse cardiovascular effects, as its systemic absorption is very low. According to the phase Ⅰ study for evaluation of the safety and tolerability of plecanatide in humans[93], no measurable systemic absorption was observed at any doses of oral plecanatide. Plecanatide was safe and well tolerated up to the highest dose. Diarrhoea was the most prevalent side effect, but its frequency did not statistically significantly differ between placebo and plecanatide, and appeared not to be dose-related in the plecanatide-treated subjects. Other gastrointestinal events were nausea, abdominal discomfort and pain, and vomiting. In a Phase Ⅱ dose escalation trial involving a total of 84 chronic constipation patients recruited with modified Rome Ⅲ criteria, 14 d of plecanatide therapy improved stool frequency, stool consistency, straining and overall relief of chronic constipation symptoms. To confirm the safety and efficacy of plecanatide, two Phase Ⅲ trials (NCT01982240andSP304203-00) have been planned. In the United States and Canada, the Phase Ⅲ trial NCT01982240 was initiated in November 2013 with adult chronic constipation patients and was expected to be completed in February 2015[94].

Prucalopride

Prucalopride is authorized in several countries (not in the United States) for women with CIC unresponsive to laxatives[95]. As a very highly selective 5-HT4 agonist, prucalopride has no measurable affinity for other receptors. In safety evaluation tests, prucalopride showed no h ERG (humanether-à-go-go-related gene) channel inhibitory activity. It is not arrhythmogenic, and it promotes colonic motility[96].

At dosages of 2 mg and 4 mg per day, this drug produced a low incidence of QT interval prolongation. Even up to 20 mg per day (10-fold higher than the recommended dosage), prucalopride displayed no clinically relevant effects on cardiovascular parameters in healthy volunteers. Prucalopride improved stool frequency and consistency, and it dose-dependently enhanced colonic transit in healthy controls or chronic constipation patients with no negative impact on gastric emptying or small bowel transit[97]. The patients' quality of life was significantly improved by prucalopride treatment.

In three pivotal trials, prucalopride showed a good efficacy in increasing CSBMs per week and in improving perceived disease severity and quality of life in patients with CC. A study conducted on 620 patients with CC treated with 2 or 4 mg of prucalopride for 12 wk showed that it increased one or more CSBMs per week compared to the control group[98-100]. In another trial conducted on 713 patients with CC, 2 or 4 mg of prucalopride increased the frequency to three or more CSBMs per week and improved evacuation completeness, perceived disease severity, and quality of life[101]. In another study conducted on patients 65 years or older with CIC, prucalopride at a dose of 1 mg for 4 wk did not cause any changes in an electrocardiogram or corrected QT (QTc) interval, showing its safety for the treatment of CIC in the elderly[97,102]. A study conducted on Asian subjects with CIC reported similar efficacy and safety as that observed in Western populations[103]. In a pooled analysis of the study with Asian subjects and the three pivotal trials, increased stool frequency of approximately three or more CSBMs per week was observed in Asian (34% *vs* 11%, $P < 0.001$) and non-Asian (24.6% *vs* 10.6%, $P < 0.001$) women. Prucalopride was shown to be safe and well tolerated[104], improving CIC abdominal symptoms such as abdominal discomfort, bloating, straining, and painful bowel movements[105]. Another study conducted on a small number of patients showed the efficacy of prucalopride not only in the treatment of slow transit constipation but also of obstructed defecation and IBS-C[106].

In a recent analysis, Camilleri *et al*[106] evaluated the efficacy of prucalopride using the data from six phase 3 and 4 multicentre double-blind randomized placebo-controlled parallel-group trials performed across three continents.

Over the 12-wk treatment period, prucalopride-treated patients consistently achieved a mean of 3 SCBMs/wk compared to placebo with the treatment response observed in the individual trials[98-100,102]. On the other hand, the SPD555-401 trial was the only trial that failed to demonstrate a statistically significant effect of

prucalopride on this primary endpoint after both 12 and 24 wk of treatment, without any plausible explanation of this lack of efficacy[107,108]. In the current study, no differences were found between men and women, although over time, a difference in the response rate has been reported. This could be related to differences in demographics (other than gender) and disease characteristics at baseline or to intrinsic differences in responsiveness to prucalopride between men and women. Furthermore, prucalopride was significantly more effective than placebo, as demonstrated by many secondary endpoints, including improvements in PAC-SYM (Patient Assessment of Constipation Symptoms) and PAC-QOL (Patient Assessment of Constipation Quality of Life) scores and rescue medication use. An exploratory efficacy analysis showed that prucalopride treatment was effective even in patients with very severe CIC and those with no SBMs at baseline.

In the current integrated analyses, the NNT with prucalopride used to achieve the primary efficacy endpoint in one patient was 8.8 (95%CI: 7.1-11.6). In a meta-analysis of data from three trials of linaclotide in patients with CIC, the NNT for the primary endpoint of these trials (3 SCBMs/wk and an increase of 1 SCBM/wk, for 75 % of weeks) was 7 (95%CI: 5-8)[109]. Prucalopride has a favorable safety and tolerability profile[110]. Notably, no cardiovascular safety signals were observed. Indeed, the mean QT interval corrected according to Bazett's formula (QTcB) and the mean QT interval corrected according to Fridericia's formula (QTcF) were both/470 ms. A potential limitation of this integrated analysis is moderate heterogeneity (I^2 = 56%) due to a deviation of the results of one of the six trials compared to the others.

Prucalopride was well absorbed from the gastrointestinal tract, with an absolute oral bioavailability of more than 90%. Its main elimination route was *via* theurine (60%-70% excreted unchanged in the urine). Because prucalopride has a low level of metabolism by liver, its pharmacokinetics is unlikely to be altered by hepatic impairment, and no CYP3A4 drug interactions are anticipated. In Europe, 2 mg of prucalopride has been approved for the treatment of chronic constipation in women who have no adequate response to laxatives[111].

Headache (in 25%-30%), nausea (12%-25%), abdominal pain (16%-23%), and diarrhoea (12%-19%) were observed as adverse events.

Recently, a randomized trial compared prucalopride with Macrogol/PEG 3350 plus electrolytes in patients with CIC. Prucalopride showed a non-inferiority for the primary outcome, even though PEG showed a superiority in improving gastrointestinal transit, stool frequency, and number of spontaneous bowel movements[112]. Although no studies have yet evaluated the efficacy of prucalopride in IBS-C, it is expected that it may also be efficacious for the disease symptoms. However, the worsening of abdominal pain may limit its use in clinical practice.

YKP10811

YKP10811 is a novel substituted benzamide derivative, small molecule with high binding affinity to the 5-HT4 receptor[113]. In cellular functional assays conducted with the 5-HT4 receptor, YKP10811 showed weak agonist activity that was dose dependent and reproducible. These results indicated that YKP10811 acts as a partial agonist of the 5-HT4 receptor. YKP10811 did not show any significant off-target binding to any other receptors, enzymes, or serotonin-receptor subtypes at 1 mmol/L, except for binding to the 5-HT2A receptor and the 5-HT2B receptor. Thus, YKP10811 has 120-fold and 6-fold lower affinity, respectively, for 5-HT2A and 5-HT2B receptors than for 5-HT4. In cellular functional assays, YKP10811 showed antagonist activity at the 5-HT2B receptor with a median inhibitory concentration. In rats, YKP10811 accelerated colonic transit by 37% at a dose as low as 1 mg/kg. In dogs, 0.3 mg/kg YKP10811 accelerated colonic transit by 45.5% at 2 h after dosing. The accelerated colonic transit in dogs was associated with significantly increased colon contractions and defecation. YKP10811 significantly reduced visceral hypersensitivity in multiple pain models in rats. In a phase Ⅰ double-blind randomized 9-d placebo-controlled multiple-ascending dose study in healthy volunteers at doses of 5, 15, 30 and 45 mg once daily, YKP10811 was well tolerated with minimal side effects. In a single-center randomized parallel-group double-blind placebo-controlled study[114] in patients with functional constipation, YKP10811 enhanced gastrointestinal and colonic transit and improved bowel function during an 8-d treatment trial. The effect of YKP10811 on colonic transit was mirrored by improvements in softer stool consistency and faster time to first bowel movement, suggesting that YKP10811 has encouraging effects on these clinical end points. In addition to pharmacodynamic effects in patients with functional constipation, improvements in bowel functions are validated and measurable end points recommended for the treatment of functional constipation[115]. These findings suggest that YKP10811 may be a potential new medication for the treatment of functional constipation. YKP10811 had a robust effect on accelerating, by 30% to 40%, colonic emptying when compared with placebo. Ascending colon emptying has been reported to have the greatest contribution to overall colonic transit[116] because the ascending and transverse colon constitute the "reservoir" or storage regions of the human colon[117]. Among the other 5-HT4-receptor agonists previously studied with the same method, 4 mg prucalopride and 30 and 50 mg velusetrag[118] also accelerated AC emptying. Emptying of the proximal colon correlates linearly with faecal weight[119], which largely reflects

stool water content, and as expected based on prior studies, the overall colonic transit was correlated linearly with stool consistency, with less significant association with the number of bowel movements per day. The results also showed the dual action (agonist/antagonist) of YKP10811 seen in *in vitro* studies. YKP10811 facilitated the electrical field stimulation-induced neurogenic twitch of guinea pig ileum at lower concentrations. This type of dual action (agonist/antagonist) of YKP10811 under the same assay conditions was also shown in the peristaltic reflex test, with an EC50 of 0.5 mmol/L and an IC50 of 21 mmol/L. There is a significant gap in concentration ranges (> 40-fold difference) for stimulatory *vs* inhibitory effects of YKP10811 *in vitro* (unpublished data; SK Life Science, Inc). Two participants, 1 receiving placebo and 1 receiving 20 mg YKP10811, had prolonged QTc (> 470 ms). Both participants discontinued the study on the advice of the investigators, even though the QTc prolongation was minimal (functions in patients with functional constipation). Thus, YKP10811 is likely to be of benefit to patients with functional constipation without rectal evacuation disorders. The safety and efficacy of this novel agent should be studied in larger multicentre clinical trials. With further studies, the current data suggest that YKP10811 would expand the therapeutic options beyond the recently approved secretagogue medications for the treatment of functional constipation, lubiprostone and linaclotide[120,121].

YKP10811 was reported to be safe and tolerable in healthy volunteers. Except for a Phase Ⅱ clinical trial in C-IBS patients (NCT02082457)[122], there were only two registered Phase Ⅱ trials that evaluated the efficacy and safety of YKP10811 in comparison with placebo in subjects with CIC (NCT015 23184, NCT01989234)[123,124]. Collectively, 420 eligible subjects were enrolled to be treated with different doses of YKP10811 or placebo once daily for 8 d and 12 wk in two trials. The results have not been completed for reporting yet. This drug is pending to pass Phases Ⅱ and Ⅲ of clinical trials, expected in 2016.

Renzapride

Renzapride (a novel benzamide substitute) is a full agonist for the 5HT4 receptor and an antagonist to 5HT2b and 5HT3 receptors. It can accelerate the gastrointestinal tract transit and motility stimulating the 5HT4 and 5HT2b receptors[125], and it appears to be a promising therapeutic agent for constipation, which is predominant in IBS patients. It is safe and has only a few adverse effects[126,127]. Several clinical trials have been performed to evaluate its potential efficacy in IBS patients, confirming that renzapride does not cause cardiac arrhythmias in clinical doses, unlike cisapride[126,127]. It is excreted renally and is not metabolized by cytochrome P450 enzymes. Thus, no drug interactions *via* affecting cytochrome P450 enzymes have been reported[125,127]. Renzapride stimulates colonic transit and reduces transit time and pain in IBS patients due to its prokinetic property, providing a benefit in those patients with constipation[128]. In addition, a dose-dependent efficacy of this drug has been demonstrated[126]. In a phase Ⅱ study of 46 women with IBS-C, renzapride at a dose of 4 mg q.d. favoured colonic transit and increased ascending colon emptying compared to placebo[129]. A large multicentre European trial confirmed the effects of 4 mg renzapride q.d. in the improvement of frequency of bowel movements and stool consistency in IBS-C[130]. Much pharmacodynamic data support renzapride's prokinetic effects. As for the prior European study, statistically significant differences in the frequency of bowel movements and stool consistency in favour of renzapride 4 mg q.d. were relatively small. In addition, renzapride did not improve the feelings of completeness of bowel movements or the amount of straining. Several systematic reviews have shown the efficacy of 5HT receptor modulators in IBS patients. In 2009, Ford *et al*[131] conducted a meta-analysis by reviewing placebo-controlled clinical trials up to 2008 on the efficacy of known 5-HT3 antagonists and 5-HT4 agonists in IBS. They observed that renzapride and cisapride were not more effective than placebo in IBS patients.

Other investigators also evaluated the efficacy of combined 5HT3 antagonists/5HT4 agonists (cisapride and renzapride) in IBS patients[132] and observed that 1 and 2 mg of renzapride was ineffective in relieving IBS symptoms, supporting the results obtained by Ford. However, these authors showed that 4 mg of renzapride was significantly more effective than placebo.

Recently, a meta-analysis[133] from randomized placebo-controlled clinical trials, including 2528 C-IBS, non-C-IBS, and non-D-IBS patients according to the Rome criteria, was performed. The study confirmed that renzapride had no significant effects in relieving symptoms in IBS patients compared to placebo. To reach a convincing conclusion on the effectiveness of renzapride, a clinical trial compared with placebo was performed. Renzapride at a dose of 4 mg was compared to placebo for 5 wk or less and more than 5 wk. Although the differences were not statistically significant, the results were clinically important and significant for both treatment durations. Therefore, these results could be considered for renzapride 4 mg, while more trials are necessary to determine the effectiveness of this novel drug more precisely. As regards adverse effects, no statistically significant differences between renzapride and placebo were found, except for diarrhoea occurrence, which was higher in patients treated with renzapride. In addition, renzapride caused more withdrawals due to adverse effects and/

or low efficacy in patients. One of the limitations of this meta-analysis was the evaluation of trials with different patient inclusion criteria (age, sex, lifestyle and compliance). In addition, the trials evaluated had different durations of treatment and endpoints. The treatment durations ranged from 2 wk[134] to 12 wk[135]. To avoid heterogeneity, patients were divided into two groups according to treatment duration and time of reporting the results (5 wk or less and more than 5 wk), although there were few data in each group. The safety data from these phase Ⅲ studies indicated that renzapride was generally well tolerated, even though ischaemic colitis was reported in the long-term study in 3 patients. However, evaluating the total of patients treated with renzapride during the study, the overall rate of ischaemic colitis appeared comparable with that reported for other 5-HT3 receptor antagonists[136]. In conclusion, renzapride is not only superior to placebo in relieving IBS symptoms (abdominal pain and discomfort), but it also causes increased diarrhoea occurrence compared with placebo and appears to be associated with many drop-outs. Therefore, this drug might be a cost burden to patients, without any advantages in efficacy. Indeed, during the trial, no improvements in frequency of bowel movements, straining, or completeness of evacuation were observed in patients treated with renzapride. Taken together, these data suggest that renzapride is unlikely to provide clinically meaningful improvement in IBS symptoms.

Velusetrag

Velusetrag is an orally administrated available 5-HT4 agonist developed by Theravance. The binding affinity of this drug for the 5-HT4 receptor is more than 500-fold that of other 5-HT receptor subtypes[137]. The major metabolite detected in plasma after oral velusetrag is THRX-830449, which is a full agonist and is approximately equipotent to velusetrag. Metabolism occurs through the CYP3A4 system. In healthy subjects[138], at steady a state, the THRX-830449 to velusetrag AUC ratio is approximately 0.5 following once-daily dosing of velusetrag (15 mg).

Increased smooth muscle contractility of the antrum, fundus, duodenum and jejunum was observed in velusetrag-treated dogs[139]. Velusetrag increased guinea-pig colonic transit and produced dose-dependent relaxation of the rat esophagus[140]. Relief of constipation using velusetrag was also confirmed in chronic constipation patients[141].

Velusetrag was approximately 6- or 86-fold more potent than cisapride or mosapride after intravenous dosing and 9- or 18-fold more potent than tegaserod or cisapride, respectively, after intraduodenal administration[141].

Its low risk for cardiovascular events has been confirmed in an *in vitro* investigation demonstrating no effect on hERG channel conductance[140]. In a preclinical study that compared *in vivo* activity of velusetrag *vs* tegaserod in guinea pig, the subcutaneous administration of velusetrag increased colonic transit more than tegaserod did. Velusetrag was more potent than tegaserod when orally administered in a dog GI smooth muscle contractility model[142]. Velusetrag exhibited an acceptable oral bioavailability in rats and dogs[140], while the systemic effect of the drug was increased by an increase in the administered dose in healthy volunteers[143-145]. Both single (up to 70 mg) and multiple (up to 50 mg, for 2 wk) dosing of velusetrag in healthy subjects showed a dose-dependent effect on GI motility[145].

There have been two Phase Ⅱ clinical trials to evaluate the clinical efficacy of this drug. In one of these two studies[138], 60 healthy volunteers were randomly assigned, in double-blind fashion, to placebo or 5, 15, 30 or 50 mg velusetrag, with transit measurements after single and 6-d dosing.

The GI transit was evaluated in a randomized double-blind placebo-controlled study conducted on 60 healthy subjects randomly assigned to receive velusetrag at a dose of 5, 15, 30 or 50 mg or placebo either as a single dose or for 6 d[137]. Velusetrag at single dose (30 and 50 mg) favoured colonic transit, evaluated by colonic filling at 6 h and geometric center at 24 h, while this effect was not observed in patients treated with placebo. Similarly, velusetrag at multiple doses (15-50 mg doses) favoured gastric emptying compared with placebo (P = 0.002). In this study by Manini *et al*[115], an improvement of stool frequency and consistency by velusetrag in a subset of 11 patients with chronic constipation was also reported. Pharmacokinetic evaluations demonstrated a similar profile in healthy and CIC subjects[143]. Velusetrag was well tolerated in the Phase I study when administered in single and repeated doses in healthy subjects. In the Phase Ⅰ clinical trial, the most commonly reported adverse event was diarrhoea, which is expected because of velusetrag's mechanism of action[141,143]. In a Phase Ⅱ randomized, double-blind, placebo-controlled trial, the efficacy and safety of velusetrag were compared with placebo in 401 subjects with CC. SBM frequency, CSBM and other associated symptoms with CIC were significantly improved compared with placebo in patients who received velusetrag for 4 wk. The most effective dose was 15 mg once daily. Most of the adverse events, such as diarrhoea, headache, nausea and vomiting, were mild to moderate. These adverse events were common in the first days of treatment with the dose of 50 mg once daily. The number of withdrawals due to adverse events was 18 *vs* 1 for the velusetrag- and placebo-treated subjects, respectively. The number of withdrawals were 4, 3 and 11 in the 15-, 30- and 50-mg treated groups, respectively. However, the medicine was well tolerated with no cardiac complications[146]. Another Phase Ⅱ study of

velusetrag in 401 patients with chronic constipation treated for 4 wk showed that there were significant treatment effects on the average daily number of bowel movements compared with placebo[147].

The most common adverse effects of velusetrag were those frequently associated with 5-HT4 agonists, including diarrhoea, headache and nausea. These dose-dependent adverse effects were mild to moderate and usually occurred within the initial days of dosing. Clinically relevant doses of velusetrag in animals or humans did not generate severe side effects on blood pressure, heart rate or electrocardiogram. In isolated porcine or canine coronary arteries, velusetrag showed no contractile activity[148]. In the randomized, double-blind, placebo-controlled study in 60 healthy subjects, there was no significant treatment effect on heart rate recorded by ECG after treatment for the prior 5-6 d. In this study, there were also no serious adverse events, and predictable GI effects such as diarrhoea and altered bowel movement were the main adverse events recorded[140]. These results suggest that velusetrag appears to be well tolerated. Further careful clinical studies will be required to further evaluate the safety and tolerability of this drug.

Naronapride

Naronapride (ATI-7505)[149] is a benzamide 5-HT4 receptor agonist that activates 5-HT4 receptors but has almost no actions on the other 5-HT subtypes. The design of ATI-7505 was based on the prototypical agent, cisapride. However, unlike cisapride, which is a mixture of (3R, 4S) and (3S, 4R) isomers of substituted piperidine-based scaffolds, ATI-7505 is the pure (3S, 4R) isomer. ATI-7505, with its (R)-quinuclidinyl moiety, is metabolized by ubiquitous carboxyl esterases to a single metabolite, ATI-7500.

This potent and selective 5-HT4 receptor agonist showed different pharmacodynamic and pharmacokinetic properties from previous nonselective 5-HT4 agonists. Hydrolytic esterase metabolism, unlike oxidative CYP450 metabolism, is a large-capacity metabolic system that can easily handle therapeutic amounts of xenobiotics. This large-capacity system implies that other drugs metabolized by esterases are not expected to induce drug-drug interactions of ATI-7505 with other drugs. There is also no interaction with drugs metabolized by a different enzymatic system, such as CYP450[150]. Naronapride is not metabolized by CYP450 enzymes, and thus, less drug-drug interaction occurs.

A thorough QT study showed that naronapride had no obvious effect on cardiac repolarization at either therapeutic or supratherapeutic doses. The structure of naronapride is similar to that of cisapride, but it is more selective than cisapride and thus interacts minimally with hERG channels and 5-HT3 receptors[150]. ATI-7500, the main metabolite of naronapride, is 100-fold less active than the parent drug. Unlike prucalopride and velusetrag, neither naronapride nor ATI-7500 can pass the blood-brain barrier, therefore reducing the incidence of side effects. This new benzamide exhibited GI prokinetic effects, stimulated colonic transit and reduced stool consistency in healthy male and female subjects[151]. One Phase Ⅱ randomized double-blind placebo-controlled dose definition study evaluated several doses of orally administered naronapride (20, 40, 80 and 120 mg twice a day) in 210 patients with CC. This study evaluated the clinical effects of 9 days' treatment with three doses of ATI-7505 at 3, 10 and 20 mg on GI and colonic transit using a validated scintigraphic method. There were borderline effects on gastric emptying at half-time. However, ATI-7505 stimulated colonic transit at 24 h and ascending colonic emptying. There was looser stool consistency as measured by the Bristol stool form scale with the 10- and 20-mg t.i.d. doses. This finding suggests that ATI-7505 appears to have prokinetic properties in both stomach and colon in healthy subjects and, particularly, in the colon. Further clinical trials of larger numbers of patients with functional gastrointestinal disorders, such as patients with CIC, are required to evaluate clinical efficacy.

The inhibition of the delayed rectifier K+ current in response to ATI-7505 in patch-clamped HEK293 (human embryo kidney) cells transfected with the human IKr channel is very weak, suggesting that there would be an adequate safety window between activity in the GI tract and potential cardiac toxicity. In addition, the primary metabolite ATI-7500 is 100-fold less active than the parent drug at the 5-HT4 receptor and, as with ATI-7505, has no detectable HERG channel inhibitory activity at concentrations up to 100 μmol/L. Preliminary data on intensive cardiac safety monitoring suggest that ATI-7505 is safe as regards the cardiac profile[149].

The most common drug-related adverse events were headache, diarrhoea, nausea and vomiting. Headache and abdominal pain were reported more frequently by the maximum dose of naronapride[152].

Chenodeoxycholic acid

Chenodeoxycholic acid (CDCA) is a bile acid that can induce colonic electrolyte secretion by acting on the membrane-bound bile acid GPBA receptor (TGR5) on enterocytes, subsequently leading to the stimulation of cAMP generation and electrogenic chloride secretion. Supplementation with specific bile acid analogues or by using drugs that inhibit ileal bile acid reabsorption may benefit constipation patients.

Oral chenodeoxycholic acid at doses of 750-1000 mg/d can increase bowel movements, decrease stool consistency, and reduce the time to defecation in IBS-C[153].

They were previously used for the dissolution of

gallstones, and they are known to favour diarrhoea at high doses in healthy controls and constipation patients[154]. The effects of CDCA on gastrointestinal and colonic function have been evaluated in healthy volunteers and patients with irritable bowel syndrome with constipation. In a randomized controlled trial, 500 mg and 1000 mg CDCA given to 60 healthy volunteers for 4 d led to dose-dependent acceleration of colonic transit. In addition, significant increases in stool frequency, decreases in stool consistency, and improvements in ease of stool passage were reported with CDCA[155]. In a double-blind placebo-controlled study, Rao *et al*[153] demonstrated that sodium chenodeoxycholate[156] stimulated colonic transit and improved bowel function in 36 women with irritable bowel syndrome with constipation. Increased stool frequency, greater ease of stool passage and looser stool consistency were observed in patients treated with sodium chenodeoxycholate 500 mg or 1000 mg for 4 d as compared with controls. Unfortunately, over 40% of sodium chenodeoxycholate-treated patients had light abdominal cramping or pain. Whether these side effects could be mitigated at a lower dose remains to be determined.

Elobixibat

Elobixibat is an orally administrated available potent inhibitor of ileal bile acid transporter with minimal systemic exposure[157]. Elobixibat (A3309) reduces bile acid enterohepatic recirculation and upregulates bile acid synthesis as measured by serum C4 levels. It also depletes liver cholesterol and reduces serum LDL[158], thus increasing the delivery of bile acids to the proximal colon, which in turn increased fluid secretion, colonic motility and stool frequency, and it improved stool consistency and relieved constipation-related symptoms in chronic idiopathic constipation patients[159,160].

In a phase Ⅰ trial, elobixibat stimulated colonic transit in a dose-dependent way. In a randomized phase Ⅱ trial, elobixibat at doses of 15 and 20 mg/d showed an improvement of stool consistency and of stool passage, increased the number of SBMs and reduced straining in female patients with CIC[161]. In a dose-finding randomized trial, elobixibat increased C4, reduced LDL cholesterol, increased colonic transit from 3 to 1.9 d and increased the number of SBM and CSBM/wk in patients with CIC compared to placebo. The treatment with elobixibat also resulted in an improvement of bloating severity, but no effects on abdominal pain or discomfort were reported[157]. The well-tolerated doses were 5-10 mg, with a discontinuation rate during the phase IIb trial of 13%, rising to 23% for the 15 mg group. Fifty-four percent of patients developed adverse events, such as abdominal cramps, relieved by defecation, and diarrhoea. However, the side effects were not different from those of the placebo group[162]. In a large randomized trial conducted on patients with CIC, the 10- and 15-mg doses increased SBMs and reduced the time to SBM (12 h with the 10-mg dose, 7 h with the 15-mg dose and 24 h with the placebo). In patients treated with elobixibat, an increased spontaneous laxation within 24 h was observed compared with placebo (75 % on 15 mg/d and 45 % on placebo).

The side effects of elobixibat are mainly gastrointestinal tract-related. Although higher dosages of elobixibat caused abdominal pain and diarrhoea more frequently, no severe adverse effects occurred in the Phase Ⅰ and Phase Ⅱ clinical trials. The Phase Ⅲ clinical trials are ongoing to determine the best tolerated dose and to examine the effects of long-term administration.

Complete spontaneous bowel movements per week increased in a dose-dependent way. An improvement of stool consistency and bloating was observed at the 10- and 15-mg doses. Side effects such as abdominal pain and diarrhoea were also dose-dependent, notably for the 15-mg dose[157].

Elobixibat is a promising anti-constipation drug. However, there are no studies in cancer or in OIC (opioid induced constipation) patients. Due to its prokinetic activity, elobixibat is not recommended in patients with mechanical bowel obstruction.

According to the results of Phase Ⅱ trials in chronic idiopathic constipation patients, elobixibat was safe and generally well to lerated, even at a dose up to 20 mg per day.

As illustrated by elobixibat, the advantages of IBAT inhibitors may be especially attractive, which may boost research on other IBAT inhibitors, such as SC-435, S-8921 and S-0960[163-165].

Lubiprostone

Lubiprostone, a first-in-class drug for the treatment of chronic idiopathic constipation and irritable bowel syndrome in adult women with constipation is believed to be a highly selective locally acting activator of ClC-2 channels[166]. Lubiprostone can tautomerize between the inactive form Ⅰ and the active form Ⅱ[167]. Lubiprostone acts mainly by activating specific type-2 chloride channels (ClC-2) on the apical membrane of the enterocytes[166] that are involved in ion and fluid transport across the epithelial membrane. Once channels are opened, chloride enters the enterocyte in the basal membrane through the action of Na-K-2Cl active cotransporters. This mechanism results in an electrochemical gradient favouring chloride secretion. It leads to an overall concentration-dependent raise in intestinal fluid secretion without any impairment on serum sodium and potassium levels. These mechanisms explain how lubiprostone increases the number of colonic spontaneous bowel movements per week. However, lubiprostone efficacy on the abdominal pain score is only partially known

and needs further investigation. Lubiprostone also activates a prostaglandin receptor (EP4), which in turn activates cystic fibrosis conductance regulators (CFTR)[168]. The activation of EP4 receptors favours colonic smooth muscle and gastric longitudinal muscle *via* vagal nerve endings[169]. Lubiprostone changes mucin, which improves the gut microbiome, creating an anti-inflammatory environment[170]. Unlike linaclotide, lubiprostone does not increase pain thresholds[171].

Lubiprostone pharmacokinetics is not impaired by renal failure. However, great adverse events with a standard dose of lubiprostone can result in cases of mild-to-moderate hepatic impairment (Child-Pugh class A and B), which increases the lubiprostone metabolite M3. Thus, in cases of liver impairment, a reduction of lubiprostone starting doses is required. Lubiprostone metabolism does not involve cytochromes. The catabolism is mediated by carbonyl reductase in the stomach and jejunum[172]. Lubiprostone is unlikely to have major drug interactions.

In healthy subjects, a reduction in gastric emptying, an increase in gastric fasting volume, a reduction in maximum tolerated gastric volumes and a stimulation of small bowel and colon transit was observed with lubiprostone at a dose of 24 μg twice daily[173]. However, the effects on gastric motility may mask the nausea side effect.

Two 12-wk double-blind randomized multicentre placebo-controlled phase Ⅲ clinical trials[174] and one 36-wk open-label extension study[175] contributed to the FDA's approval of lubiprostone for the treatment of IBSC in women. A total of 1171 patients were randomized 2:1 to receive either 8 μg lubiprostone or placebo twice/d. The primary end point of each study was the evaluation of response rate, measured by patient-reported improvements from baseline in IBS-C symptoms. As secondary end points, monthly responder rates, changes from baseline in SBM frequency, stool consistency, straining, distention, abdominal pain/discomfort (each measured on a 5-point Likert scale) and change in health-related quality of life were evaluated.

The discontinuation rate in both studies was 24%, firstly due to withdrawal of consent and secondly to adverse events and perceived lack of efficacy. Lubiprostone was superior in the primary end point compared to placebo (17.9% *vs* 10.1%, $P = 0.001$). Patients treated with lubiprostone reported more improvements in all secondary end points than placebo. Lubiprostone was associated with a more significant improvement in abdominal pain/discomfort than placebo from baseline to month 2 (0.43 *vs* 0.35, $P = 0.039$) and month 3 (0.45 *vs* 0.36, $P = 0.028$).

Lubiprostone significantly changed the mean SBM frequency from baseline to month 1 compared with placebo, even though the numerical data were not included.

Two 4-wk phase Ⅲ randomized double-blind placebo-controlled multicentre clinical trials were conducted on a total of 479 patients to evaluate the short-term efficacy and safety of lubiprostone in patients with CIC with identical study designs and primary end points. After a 2-wk baseline period, eligible subjects received 24 lg lubiprostone or placebo twice/d.

The number of patient-reported SBMs, defined as any BM occurring 24 h or longer after the use of an alternative drug used to relieve constipation (rescue medication), during the first week of treatment was the primary end point of each study[176,177]. Lubiprostone was associated with a statistically higher frequency of SBMs during the first week of treatment than placebo.

Improvements in other secondary end points, such as stool consistency, straining, and constipation severity were also observed in patients treated with lubiprostone compared with placebo in all 4 wk in both studies. However, significant improvement in abdominal distention and discomfort compared with placebo was not observed in either study.

As regards side effects, a similar percentage of patients reporting at least one treatment-related adverse event for IBS-C was observed in the lubiprostone (50%) and placebo (51%) groups. The most common side effects were gastrointestinal (19% with lubiprostone *vs* 14% with placebo). Serious adverse events were similar between the two groups (1%). Nausea was the most frequent treatment-related event[11,12], although it may be reduced by administering lubiprostone with meals.

A unique adverse effect occurred with the initial dose. In rare cases, acute transit dyspnea and ischemic colitis were observed[176,178].

Caution in the use of lubiprostone should be used for infants of breastfeeding mothers due to the risk of diarrhoea[179]. Limited data are available on the lubiprostone effects in paediatric patients, and further, larger studies are required. In an open-label 4-wk clinical trial conducted on paediatric patients with CIC (mean age 10.2 years)[179], lubiprostone was efficacious and well tolerated at daily doses of 12-48 lg. The recommended dose of lubiprostone for the treatment of CIC in both adult men and women is 24 lg twice/d, while for the treatment of IBSC in adult women, it is 8 lg twice/d. Due to its minimal systemic absorption and its metabolism through a cytochrome P450-independent pathway, lubiprostone-drug interactions are unlikely, even though *in vitro* studies have suggested that methadone may decrease the efficacy of lubiprostone by reducing chloride channel type 2 activation[179].

In patients with moderate or severe hepatic impairment (Child-Pugh class B or C), a dose reduction might be suggested, while in patients with renal failure, no dosage adjustment is recommended.

A small percentage (8%-13%) of patients over 65 years were included in clinical trials with lubiprostone. The safety profile was similar in elderly and younger patients, even though, due to the limited the number of patients over 65 years, no differences in clinical response were observed[179], and further studies are needed.

Like linaclotide, lubiprostone is contraindicated in mechanical bowel obstruction. To confirm the indications in the treatment of IBS-C in adults, more and larger trials are required. Due to the chronic nature of IBS-C and CIC, post-marketing studies are necessary to confirm the long-term efficacy and safety of lubiprostone. All randomized clinical trials were of limited duration (12-26 wk). However, in open-label extension studies, a safety over 52 wk was demonstrated.

Despite the efficacy, the side effects (*e.g.,* nausea, abdominal pain) and the high cost may limit the use of lubiprostone.

Tenapanor

Tenapanor, also known as AZD1722 or RDX5791, is a first-in-class orally available inhibitor of NHE3 that is minimally absorbed in the gastrointestinal tract-this constitutes a significant therapeutic benefit, as it may act on the drug target[180,181]. Consequently, tenapanor increases intestinal Na^+ contents, which leads to an increase in intestinal fluid volume and accelerates the whole GI transit, as shown in rats. Moreover, tenapanor inhibits the absorption of phosphorus, which is independent of typical phosphorus transporters in the intestines, namely, sodium-dependent phosphate transport protein 2B (NaPi2b) and Na (+)-dependent phosphate transporter (PiT1). Tenapanor is stable at room temperature and is formulated into tablets ranging from 1 to 50 mg. Absorption, distribution, metabolism and excretion (ADME) studies have revealed that tenapanor is minimally absorbed and metabolized. For example, experiments in rats showed 92.2% ± 1.6% recovery of tenapanor in faeces upon oral administration[182]. In humans, the inactive metabolites of tenapanor were found in plasma, but they were only approximately 9% of the parent compound. In pharmacokinetic studies, tenapanor was observed at relatively low concentrations in plasma (average < 3 ng/mL) of rats and dogs, but only sporadically (29/76 and 0/92, respectively).

Oral administration of tenapanor (at doses of 0.1 and 3 mg/kg) produced a dose-dependent increase in faecal water content and stool consistency in rats. The effect of tenapanor at a dose of 50 mg/kg twice daily on stool form was assessed in cynomolgus monkeys. The animals were observed for 4 d before treatment. Soft or watery stools were observed in monkeys on tenapanor treatment, and stool consistency was normalized on day 6 of the experiment. Under physiological conditions, tenapanor given orally at doses of 3, 10, 30 and 50 mg/kg did not affect visceral sensitivity or the changes in intestinal volumes induced by colorectal distension in comparison with the control and tegaserod-treated (5 mg/kg administered per os) groups[183]. However, tenapanor (30 and 50 mg/kg) had a dose-dependent antinociceptive effect in the acute restraint stress-induced intestinal hypersensitivity to colorectal distension. The antinociceptive potential of tenapanor was comparable with that of the tegaserod-treated group.

The safety and tolerance of tenapanor were assessed in a randomized, double-blind, placebo-controlled study[184,185]. Eighty healthy volunteers were included in the study (male and female). Tenapanor was given orally at the doses ranging from 10 to 900 mg (as a single administration) and for 7 consecutive days at doses ranging from 3 to 100 mg to assess the safety of tenapanor administration. Tenapanor was also beneficial for the percent of days with a spontaneous bowel movement. Finally, no serious side effects were observed, and there were very few adverse events[186]. Phase Ⅱa In a Ⅱa double-blind randomized placebo-controlled study on 181 patients with IBS-C[187], tenapanor was given orally at doses of 10, 30 and 100 mg once daily for 4 consecutive weeks with 2 wk follow-up. The primary end point (change in complete spontaneous bowel movements from baseline to week 4) was not met in this study, and the incidence of diarrhoea was comparable with that of the placebo group. However, an improvement in bloating and abdominal pain was noted in IBS-C patients. In Phase Ⅱb In a Ⅱb randomized double-blind placebo-controlled multicentre study, 371 IBS-C patients were divided into four groups: placebo and tenapanor (5, 20 and 50 mg) treated twice daily for 12 wk with 4 wk follow-up. The primary efficacy end point was met in 60.7% of the tenapanor-treated group (at a dose of 50 mg) *vs* 33.7% of the placebo-treated group. The overall responder was met in 50.0% of the tenapanor-treated group (50 mg) *vs* 23.6% for placebo (after 12 wk). After 12 wk, adequate relief in IBS-C symptoms was observed in 63.1% of the tenapanor-treated group (50 mg twice daily) *vs* 39.3% in placebo. The effectiveness of tenapanor therapy was maintained during entire time of the clinical study. The treatment satisfaction patient scale questionnaire showed that tenapanor-treated (50 mg) IBS-C patients were quite or very satisfied (65% *vs* 38% for the placebo-treated group). The drug was well tolerated in all groups, and no serious adverse effects were noted. The most common adverse effect was diarrhoea in the tenapanor-treated group (50 mg twice daily), reported in 11.2% of IBS-C patients *vs* 0% in placebo. Safety and tolerability In the preclinical studies in rats, tenapanor did not influence gastric emptying[187].

Piromelatine

Melatonin is engaged in the regulation of gastrointestinal motility and sensation. When administered

orally in pharmacological doses, it has shown beneficial effects on abdominal pain in IBS patients without any effects on sleep disturbances[188]. It was also shown that oral melatonin significantly stimulated colonic transit time in healthy subjects, and it may be a promising option for future research on the agents modulating bowel motility[189]. Melatonin synthesized in the enteroendocrine cells of the intestinal mucosa reaches the liver *via* the portal vein[190]. Melatonin is a potent accelerator of duodenal mucosal bicarbonate secretion, which neutralizes the acid content of the stomach in the duodenum, and it seems to be engaged in the acid-induced stimulation of the secretion[191]. Melatonin protects the gastrointestinal mucosa due to an antioxidant action, a decrease in secretion of hydrochloric acid, stimulation of the immune system, promotion of epithelial regeneration, and increased microcirculation[192,193].

Recently, it was shown that patients with IBS had significantly lower 6-SMLT (6-sulphatoxymelatonin)/creatinine level compared with healthy controls[194]. The lack of statistical difference in 6-SMLT/creatinine levels between the constipation and diarrhoea groups is difficult to explain. In some patients, the symptoms could be recurrent, or there could be some subjects with mixed (IBS-M) or unsubtyped (IBS-U) IBS. This study's results agree with those obtained by Bultman[195] and Lu *et al*[196] who performed the study on female patients with IBS and found decreased salivary melatonin and urine 6-SMLT level compared to non-IBS volunteers. Low melatonin levels were observed in women with eating disorders. Low melatonin concentrations have been associated with increased depressive symptoms, such as sadness, bodily discomfort, inner tension, difficulties in attention concentration and pain.

Serotonin, an endogenous amine and the precursor of melatonin, synthesized and released from enteroendocrine cells of the gastrointestinal mucosa is thought to play an important role in the pathogenesis of IBS[197]. Antagonists of the serotonin 5-HT3 receptor are beneficial in patients with IBS-D, whereas the partial agonist of the serotonin 5-HT4 receptor (tegaserod) alleviates symptoms of IBS with constipation, especially in females. The role of melatonin as the regulator of circadian and seasonal rhythmicity has been established[198,199]. Patients with functional disorders of the gastrointestinal tract also had sleep disorders, and some of them suffered from increased neural excitability and anxiety[199,200]. There were speculations concerning a possible role of melatonin in functional dyspepsia (FD), particularly ulcer-like dyspepsia. In two types of FD, one with epigastric pain and another with postprandial distress syndrome, the melatonin level is varied, and different dyspeptic symptoms may be related to differences in melatonin secretion. Sleep disturbances are common in patients with IBS and are among the most important extraintestinal symptoms, markedly affecting quality of life and psychosocial well-being[201]. In a double-blind placebo-controlled study, Camilleri *et al*[202] showed that melatonin improves abdominal pain in IBS patients with sleep disturbances. Currently, conventional treatment for irritable bowel syndrome is quite unsatisfactory. Despite multiple therapeutic interventions, no long-term effect has been achieved. On the other hand, up to 80% of patients with IBS treated with hypnotherapy showed an improvement of their symptoms[203]. These observations emphasize the possible role of melatonin in the pathogenesis of irritable bowel syndrome and in its therapy[204,205].

Daikenchuto

Daikenchuto (TU-100), a traditional Japanese drug (Kampo medicine), is indicated in the treatment of adhesive bowel obstruction[206,207]. TU-100 is a mixture of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder. In many trials, the TU-100 prokinetic effect has been demonstrated to be useful in treating GI hypomotility[208]. Studies conducted on postoperative patients after gastrointestinal surgery showed that TU-100 prevented postoperative ileus, but little is known about the TU-100 effects in patients who did not undergo major gastrointestinal surgery[209]. Iturrino *et al*[210] performed a randomized controlled trial to evaluate the effects of oral TU-100, 2.5 g t.d.s. or 5 g t.d.s. compared to placebo t.d.s. on gastrointestinal and colonic transit, rectal compliance and sensation thresholds, anal sphincter pressures and bowel function in women with functional constipation. In this study, there were no significant effects on gastrointestinal and colonic transit, rectal compliance, anal sphincter pressures, recto-anal pressure difference, or rectal sensation thresholds. The highest dose was associated with lower rectal sensation thresholds for first sensation and gas. There were no treatment effects on psychosensory symptoms, stool frequency, stool consistency or quality of life[211].

On the other hand, Manabe *et al*[211] reported that TU-100 provided a clinically significant promotility effect in small bowel and ascending colon transit in healthy subjects. TU-100 is quite safe and well tolerated and is a potential treatment for IBS-C and functional constipation[210].

Recently, however, Acosta *et al*[212] did not report any significant effects of TU-100 on rectal sensation ratings, sensation thresholds, rectal fasting or postprandial tone, rectal compliance, bowel function, abdominal pain or bloating scores, or IBS quality of life. Further randomized controlled trials in patients with IBS-C or functional constipation using both clinical and validated biomarkers are required.

DA-6886

DA-6886, a gastrointestinal prokinetic benzamide

derivative, is a novel 5-HT4 receptor agonist. Experimental studies showed that it may represent a highly potent and selective 5-HT4 receptor agonist to stimulate colonic transit in mice, having a favorable safety profile in patients with IBS-C and chronic constipation[213]. Currently, a phase Ⅰ dose block-randomized double-blind placebo-controlled single/multiple dosing dose escalation clinical trial with an open-labelled food effect is being conducted to evaluate the safety and pharmacokinetics of single-dose DA-6866 in healthy male subjects[214].

Table 3 sums up the literature findings about irritable bowel syndrome-C therapies.

THERAPEUTIC TARGET IN DIARRHOEA-PREDOMINANT IRRITABLE BOWEL SYNDROME

To date, the treatment options for IBS-D are limited and frequently unsuccessful. However, the incidence of IBS-D is currently increasing, thus causing a heavy economic burden both for patients and health care systems worldwide. As for IBS-C, a complete understanding of IBS-C pathophysiology has favoured the use of sensory end points such as complete spontaneous bowel movements and the FDA combined end point (abdominal pain and complete spontaneous bowel movements) in clinical trials[79].

Furthermore, also in the setting of IBS-D, preclinical studies in rodents have recently improved the understanding of the mechanisms underlying the alterations in gastrointestinal motility, sensitivity and secretion. A number of drugs that we will touch upon in the next section are actually in development.

Ramosetron

Ramosetron is a potent and selective 5-HT3 receptor antagonist. 5-HT3 receptors can be widely found in the central and peripheral nervous system[215]. Intraluminal stimuli favour the release of 5-HT from enterochromaffin cells located in the mucosa[215]. When secreted, 5-HT can activate 5-HT3 receptors located on intrinsic primary afferent neurons with submucosal terminals. Thus, the peristaltic reflex and intestinal secretion can occur[215,216]. 5-HT also activates 5-HT3 and 5-HT4 receptors located on primary afferent neurons of both splanchnic and vagal fibres, which are involved in sensory and motor responses[217].

In experimental studies, corticotrophin-releasing hormone (CRH) exogenously administered or released from the central nervous system by stress peripherally activates the release of 5-HT, which in turn promotes defecation through the 5-HT3 receptor. Ramosetron decreased defecation by CRH in a dose-dependent way[218-219].

The first 5-HT3 receptor antagonist to be introduced was Alosetron, which has been demonstrated to be effective in the treatment of female patients with IBS-D[220]. However, due to serious gastrointestinal events (ischemic colitis and severe constipation), it is still only available in the United States and is indicated for women with severe D-IBS refractory to conventional therapy.

Ramosetron was first tested by Lee *et al*[221] in a multicentre randomized open-label trial on 343 men with IBS-D. Patients were randomized to a 4-wk treatment of ramosetron 5 mg once daily or mebeverine 135 mg three times daily. An improvement in abdominal pain/discomfort and bowel habits in the ramosetron and mebeverine groups was observed during the treatment period. A significant reduction in abdominal pain/discomfort and urgency, stool form score, and stool frequency severity scores in both treatment arms was reported compared with the baseline.

Adverse events were observed in 7% and 4% of patients treated with ramosetron and mebeverine, respectively, even though no statistical significant differences were reported. Additionally, all the side effects were mild or moderate[221].

Successively, Fukudo *et al*[222] performed a randomized double-blind placebo-controlled trial to determine whether ramosetron reduces diarrhoea in 296 male outpatients with IBS-D. Patients were treated with 5 mg of oral ramosetron (n = 147) or placebo (n = 149) once daily for 12 wk after a 1-wk baseline period. The primary end point was increased stool consistency in the first month. Secondary end points were the relief of overall IBS symptoms and the improvement of IBS-related quality of life. In the first month, patients on ramosetron treatment (74, 50.3%) showed an improvement of stool consistency compared to placebo (29, 19.6%) (P < 0.001). In patients treated with ramosetron, the monthly relief of overall IBS symptoms and IBS-related quality of life was demonstrated compared with placebo. Safety was evaluated in all 296 patients, with side effects occurring in 46.9% and 51.7% of ramosetron and placebo patients, respectively. All constipation and hard stools experienced in the ramosetron group that were related to the pharmacologic actions of ramosetron were classified as mild and resolved early without using rescue drugs[222].

In another randomized double-blind placebo-controlled trial performed by Fukudo *et al*[223] on 576 female outpatients with IBS-D, patients were given either 2.5 μg ramosetron or placebo once daily for 12 wk. Patients treated with ramosetron reported global improvement, increased stool consistency, a significant decrease in abdominal pain and discomfort and significant improvement in QOL compared with placebo. Of the patients tested with ramosetron, 11.0% complained of constipation[223]. Successively, in a phase Ⅲ open-label uncontrolled long-term safety

Table 3 Summary of the literature findings about irritable bowel syndrome-C therapies

Drug	Ref.	No. of patients	Study design	Outcome
Linaclotide	Andresen *et al*[82]	36 women with IBS-C	Phase Ⅱa randomized, double-blind, placebo-controlled trial. Patients were randomized in a 1:1:1 fashion to placebo, linaclotide 100 μg, and linaclotide 1000 μg and was evaluated the effect of 5 d	No treatment effects were seen for gastric emptying or colonic filling with linaclotide. Significant treatment effects were found for ascending colon emptying t ½ times (P = 0.015) and overall total colonic transit times at 48 h (P = 0.02), for the 1000 μg dose (P = 0.004) but not the 100 μg dose, as well as overall treatment effects on increased stool frequency, decreased stool consistency, improved ease of passage, and acceleration of time to first bowel movement (P < 0.001)[82]
	Johnston *et al*[83]	420 patients with IBS-C	Phase Ⅱb randomized, double-blind, parallel-group, multicenter, placebo-controlled trial evaluate 12 wk of linaclotide at a daily dose range of 75-600 μg	Compared with placebo, all doses of linaclotide significantly improved bowel habits, including frequency of SBMs and CSBMs, severity of straining, stool consistency, as well as abdominal pain scores. Abdominal discomfort, bloating, and global IBS-C measures were also improved, for all doses except for the 75 μg (abdominal discomfort) and 150 μg dose (bloating). Effects were present for the first week, and sustained throughout the 12 wk of treatment
	Chey *et al*[84]	804 adults with IBS-C	Phase Ⅲ trials randomized, double-blind, placebo-controlled to receive linaclotide 290 lg or placebo daily for 26 wk, with change-from-baseline end points measuredat 12 and 26 wk	Over 12 wk, the FDA combined primary end point was achieved by 33.7% of patients receiving linaclotide compared with 13.9% of patients receiving placebo (P < 0.0001)
	Videlock *et al*[109]	7 trials of linaclotide in patients with IBS-C or CC	A meta-analysis from MEDLINE, EMBASE, and the Cochrane central register of controlled trials were searched for randomized, placebo-controlled trials	The NNT for the primary endpoint of these trials (3 SCBMs/wk and an increase of C1 SCBM/wk, for 75% of weeks) was 7 (95%CI: 5-8)
	Rao *et al*[86]	803 adults with IBS-C	Phase Ⅲ trials randomized, double-blind, placebo-controlled to receive linaclotide 290 lg or placebo once/d for 12 wk	Linaclotide demonstrated statistically significant improvements in all primary and secondary efficacy end points compared with placebo Severity of straining, constipation, and stool consistency also improved in the linaclotide group compared with the placebo group
Plecanatide	Miner *et al*[92]	951 patients with CIC	Phase Ⅲ, randomized, double-blind trial, received plecanatide 0.3, 1 or 3 mg, or placebo once/d for 12 wk	The proportion of overall responders was significantly greater with plecanatide 3 mg compared with placebo (19% *vs* 10.7%, P = 0.009); weekly responder rates were also significantly greater for plecanatide 3 mg than placebo for weeks 1-12. Improvements in stool frequency, consistency, straining, and quality of life were also noted with the 3-mg dose *vs* placebo. Data for other plecanatide doses were not reported
Prucalopride	Quigley *et al*[88]	620 patients with CC	A double-blind, placebo-controlled study. Patients receiving 2 or 4 mg of prucalopride for 12 wk	Increased one or more CSBMs per week compared to patients in the control group
	Camilleri *et al*[98]	713 patients with CC	A double-blind, placebo-controlled study. Patients receiving 2 or 4 mg of prucalopride for 12 wk	Increased frequency of three or more CSBMs per week, and improved evacuation completeness, perceived disease severity, and quality of life
	Müller-Lissner *et al*[97]	Elderly patients aged 65 years and older with CC	A double-blind, placebo-controlled study Patients receiving 2 or 4 mg of prucalopride for 12 wk	No changes in electrocardiogram or corrected QT (QTc) interval were reported, indicating its safety for the treatment of CC in the elderly
	Ke *et al*[102]	4 Randomized, Placebo-controlled Studies	A Pooled Analysis	Safe and well-tolerated It was also effective in improving the abdominal symptoms of CC such as abdominal discomfort, bloating, straining, and painful bowel movements
YKP10811	Shin *et al*[114]	55 patients	A single-center, randomized, parallel-group, double-blinded, placebo-controlled study were assigned randomly to groups given YKP10811 10 mg (n = 15), 20 mg (n = 16), 30 mg (n = 15), or placebo (n = 11) daily for 8 d	Enhanced gastrointestinal and colonic transit and improved bowel function during an 8-d treatment trial. In general, the 10-mg and 20-mg doses were the most effective in accelerating colonic transit. No serious adverse events were observed
Renzapride	Camilleri *et al*[101]	46 women with IBS-C	In a phase Ⅱ study	Renzapride 4 mg q.d. accelerated colonic transit and increased ascending colon emptying *vs* placebo
	George *et al*[130]	510 patients	Multicentre, randomized, placebo-controlled, double-blind study men and women were randomized to placebo or renzapride (1, 2 or 4 mg/d) for 12 wk	4 mg renzapride q.d. in terms of improving frequency of bowel movements and stool consistency

	Ford *et al*[131]	29 RCTs were eligible for inclusion	Meta-analysis of placebo-controlled clinical	Renzapride and cisapride were not more effective than placebo in IBS patients
	Mozaffari *et al*[133]	2528 C-IBS and non C-, non D-IBS patients	Meta-analysis from randomized placebo-controlled clinical trials	Renzapride has no significant advantage over placebo in relieving symptoms in IBS patients
Velusetrag	Manini *et al*[115]	60 healthy volunteers	Phase Ⅱ clinical trials, pt were randomly assigned, in double-blind fashion, to placebo, 5, 15, 30 or 50 mg velusetrag, with transit measurements after single and 6-d dosing	Single doses of velusetrag (30 and 50 mg), but not placebo, accelerated colonic transit, as measured by colonic filling at 6 h and geometric center at 24 h
	Goldberg *et al*[140]	401 subjects with CC	In a Phase Ⅱ randomized, double-blind, placebo-controlled trial	Short bowel movement (SBM) frequency, complete SBM and other associated symptoms with CC were significantly improved in comparison with placebo in patients who received velusetrag for 4 wk
Naronapride	Dennis *et al*[149]	210 patients with CC.	Phase Ⅱ, randomized, double-blind, placebo-controlled, dose definition study (orally 20, 40, 80 and 120 mg twice a day) to evaluate the clinical effects of 9 days′	There were borderline effects on gastric emptying at half-time; however, ATI-7505 accelerated colonic transit at 24 h and ascending colonic emptying
Chenodeoxycolic acid	Odunsi-Shiyanbade *et al*[155]	60 healthy volunteers	Randomized controlled trial, CDCA 500 mg and 1000 mg given for 4 d	Significant increases in stool frequency, decreases in stool consistency, and improvements in ease of stool passage were reported with CDCA
	Rao *et al*[153]	36 female patients	Double-blind placebo-controlled study	Accelerated colonic transit and improved bowel function
Elobixibat	Simrén *et al*[162]	30 patients	Dose-finding randomized trial five dose-levels (range: 0.1-10 mg/d) or to placebo	Increased C4, reduced LDL cholesterol and increased colonic transit from 3 to 1.9 d, and increased the number of SBM and CSBM/W in patients with CIC
	Chey *et al*[157]	190 patients	Were randomized to 5, 10, or 15 mg A3309 or placebo once daily. 8-wk, multicenter, randomized, double-blind, placebo-controlled, parallel group, phase Ⅱb study,	A3309 increased stool frequency and improved constipation-related symptoms in CIC; effects were maintained over 8 wk of treatment
Lubiprostone	Drossman *et al*[173] Chey *et al*[174]	1171 patients in total	Two double-blind, randomized, multicenter, placebo-controlled phase Ⅲ clinical trials′, a 12-wk randomized 2:1 to receive either lubiprostone 8 lg or matching placebo twice/d with food; a 36-wk open-label extension study	Lubiprostone was superior to placebo in the primary end point of overall responders, greater improvements in all secondary outcome measures compared with placebo
	Johanson *et al*[175] Barish *et al*[82]	479 patients in total	Two 4-wk phase Ⅲ, randomized, double-blind, placebo-controlled, multicenter clinical trials	Patients treated with lubiprostone had a statistical higher frequency of SBMs during the first week of treatment compared with placebo
	Hyman *et al*[176]	127 pediatric patients with CIC (mean age 10.2 yr)	An open-label 4-wk clinical trial	Demonstrated that lubiprostone was efficacious and well tolerated at total daily doses of 12-48 lg
AZD1722	Rosenbaum *et al*[185]	181 patients with IBS-C	Phase Ⅱa In a Ⅱa double-blind, randomized placebo-controlled study, Tenapanor was given orally at the doses of 10, 30 and 100 mg once daily for 4 consecutive weeks with 2 wk follow-up	The primary end point [change in complete spontaneous bowel movements (CSBM) from baseline to week 4] was not met in this study and the incidence of diarrhea was comparable with placebo group. However, improvement in bloating and abdominal pain was noted in IBS-C patients
	Rosenbaum *et al*[186]	371 IBS-C patients	Phase Ⅱb In a Ⅱb randomized, double-blind, placebo-controlled, multicenter study	The overall responder was met in 50.0% of tenapanor-treated group (50 mg) *vs* 23.6% for placebo (after 12 wk). After 12 wk, adequate relief in IBS-C symptoms was observed in 63.1% of tenapanor-treated group (50 mg twice daily) *vs* 39.3% in placebo
	Rosenbaum *et al*[187]	356 patients	A double-blind, placebocontrolled, randomized phase 2b trial 12-wk dose-ranging study evaluating tenapanor 5 mg, 20 mg or 50 mg b.i.d. *vs* placebo (1/2)	Tenapanor 50 mg b.i.d. significantly improved CSBM responder rate (primary endpoint) compared with placebo in patients with IBS-C. Tenapanor 50 mg b.i.d. also improved key secondary endpoints compared with placebo, including overall responder rate, abdominal pain responder rate and stool frequency. In addition, improvements were observed in several exploratory endpoints addressing a range of symptoms in patients with IBS-C. Tenapanor was generally well tolerated and had minimal systemic availability. Tenapanor shows promise as a future treatment option for patients with IBS-C

Neu p11 (piromalatine)	Camilleri *et al*[202]	40 IBS patients	In double blind placebo controlled study were randomly assigned to receive either melatonin 3 mg (n = 20) or matching placebo (n = 20) at bedtime for two weeks	Melatonin 3 mg at bedtime for two weeks significantly attenuated abdominal pain and reduced rectal pain sensitivity without improvements in sleep disturbance or psychological distress. The findings suggest that the beneficial effects of melatonin On abdominal pain in IBS patients with sleep disturbances are independent of its action on sleep disturbances or psychological profiles

IBS : Irritable bowel syndrome.

trial, the long-term safety and efficacy of 2.5 and 5 μg of ramosetron treatment in women with IBS-D was reported. However, the authors of the study concluded that "clinicians should be aware that one-fifth of women with IBS-D receiving ramosetron may suffer from constipation during treatment"[224].

Finally, a recent systematic review and meta-analysis performed to explore of the safety and efficacy of ramosetron both in male and female patients with IBS-D concluded that ramosetron is efficacious in both male and female patients with diarrhoea-predominant IBS, even if large-scale studies are needed to assess its effects on different ethnicities[225].

LX-1031

LX-1031 is a tryptophan hydroxylase (TPH) inhibitor that reduces peripheral serotonin production. It is indicated for conditions characterized by excess 5-HT expression such as IBS-D and, possibly, carcinoid diarrhoea. The blocking of excess 5-HT effects is well established[226]. Previously, pharmacological attempts aimed at inhibiting 5-HT synthesis, such as para-chlorophenylalanine, but the central adverse effects due to the inhibition of brain 5-HT synthesis with consequent affective disorders blocked their use[227].

LX-1031 does not cross the blood-brain barrier and, thus, does not pose risk of depression[228].

In a phase Ⅱ multicentre randomized double-blind placebo-controlled proof-of-concept study[229] performed on 155 patients, the subjects were assigned randomly in a double-blind fashion to 1 of 2 doses of LX1031 (250 mg 4 times/d or 1000 mg 4 times/d) or placebo taken daily during the 28-d treatment period[229]. Patients treated with LX1031 at the dose of 1000 mg significantly improved the primary efficacy end point, namely, the relief of IBS pain and discomfort, compared with placebo at week 1. No significant improvements were observed at weeks 2, 3 or 4. Adverse effects reported were generally mild, self-limited, and evenly distributed across the placebo and both LX1031 treatment arms[229]. The relationship between clinical improvement and reduction in serotonin synthesis shown in this study supports LX1031's proposed mechanism of action in IBS and thus supports serotonin synthesis inhibition in the GI tract as a novel therapeutic strategy for the treatment of IBS-D[229].

ASP-7147

Activation of the Bombesin-2 receptor may be involved in the regulation of gastrointestinal motility and intestinal secretion. ASP7147 is a novel small molecule Bombesin-2 receptor antagonist that reduces motility and intestinal secretions. Indeed, ASP 7147-mediated inhibition of this receptor may improve symptoms in patients with IBS-D. In the RCT performed by Lembo *et al*[230] on 64 patients during a 4-wk study, ASP7147 showed promise as a safe and effective new therapy for both men and women with IBS-D, demonstrating improvement in multiple symptoms of IBS-D. The persistence of treatment effect suggests the possibility of retained efficacy with less-frequent dosing in follow-on trials[230].

JNJ-27018966

JNJ-27018966 is a dual μ-opioid agonist and δ-opioid receptor antagonist that has been shown to have benefits in patients with IBS-D[231]. A randomized controlled double-blind study compared JNJ-27018966, at doses of 25, 100 and 200 mg twice daily, to placebo in 807 patients with IBS-D. Diarrhoea and pain were significantly improved in patients treated with JNJ-27018966 at the doses of 25 and 200 mg twice-daily compared to placebo (12, 13.8 and 5.7%, respectively, $P < 0.05$ for both comparisons to placebo)[232].

ROSE-010

ROSE-010 is a glucagon-like peptide 1 analogue that decreases gastric emptying and motility[1]. Hellström *et al*[233] conducted a randomized crossover placebo-controlled trial on 160 patients with IBS and associated abdominal pain treated with ROSE-010 100 μg once daily, 300 μg once daily or placebo. ROSE-010 was associated with a twofold greater response to abdominal pain compared to placebo ($P < 0.05$ for all comparisons) and significantly higher patient-reported satisfaction ($P < 0.05$). The most frequent treatment-related side effect was nausea, which was experienced by 19, 37 and 0% of ROSE-010 100 μg, ROSE-010 300 μg and placebo, respectively[233-234].

AST-120

AST-120, also known as kremezin, is an orally administered intestinal sorbent that has been reported to slow

chronic kidney disease (CKD) progression and to delay the initiation of dialysis by reducing the levels of renal toxins or their precursors in the gastrointestinal (GI) tract[235,236].

It has been shown that AST 120 exerts its properties in IBS by acting on intestinal permeability, reduction of visceral sensitivity and alteration of gut motility[236]. In a randomized double-blind controlled study conducted on 115 non-constipation-related IBS patients, AST-120 at a dose of 2 g three times daily significantly improved the percentage of patients with at least a 50% decrease in the number of days with abdominal pain compared to placebo (26.8% *vs* 10.2%, respectively). Additionally, AST-120 significantly improved bloating and stool consistency compared to placebo. The safety profile of AST-120 was similar to that of placebo[237].

Ibodutant

Antagonists of NK2 receptors have been suggested to modulate gastrointestinal chemical-induced impaired motility and stress-induced impaired bowel habits in humans, as recent phase 2 clinical trials have reported[238]. In a randomized double-blind controlled trial conducted on 559 IBS-D patients, ibodutant, a neurokinin-2 receptor antagonist, significantly improved abdominal pain, overall symptoms and quality of life compared to placebo. Ibodutant at doses of 1, 3 or 10 mg once daily showed superiority over placebo, with the 10 mg once daily dose being the most effective and women showing a better response than men[239,240]. Considering the limited number of effective available therapeutic options for IBS-D, ibodutant may become an important and safe treatment option, depending on whether ongoing phase 3 studies will confirm the efficacy observed in phase 2 studies[238].

Asimadoline

Asimadoline, a kappa-opioid receptor agonist, acts peripherally, inducing analgesic and antidiarrheal effects[241]. Action in the central nervous system is not required for asimadoline efficacy in the treatment of IBS. Asimadoline reduces sensation in response to colonic distension at subnoxious pressures in healthy subjects and in IBS patients without impairment of colonic compliance. Asimadoline decreased the appetite and enhanced the postprandial gastric volume (in healthy women). However, there were no significant effects on gastrointestinal transit, colonic compliance, fasting or postprandial colonic tone. In a clinical trial conducted on 40 patients with functional dyspepsia (according to Rome Ⅱ criteria), asimadoline did not significantly impair appetite or symptoms over 8 wk. However, asimadoline at a dose of 0.5 mg significantly reduced the appetite in patients, with higher postprandial fullness scores and daily postprandial fullness severity (over 8 wk). Patients treated with asimadoline at a dose of 1.0 mg showed borderline significant effects. In a clinical trial conducted on patients with IBS, the average pain 2 h post-on-demand treatment with asimadoline was not significantly decreased. At the post hoc analyses, asimadoline was demonstrated to be effective in mixed IBS[242]. Successively, in a randomized controlled double-blind trial conducted on 596 patients with IBS-D, asimadoline at doses of 0.15, 0.5 and 1 mg twice daily was compared to placebo. Patients treated with asimadoline at a dose of 0.5 mg twice daily had a twofold significant improvement in the total number of months with adequate relief of IBS pain, pain scores, urgency and frequency[243,244].

Colesevelam

Bile acids have several physiologic functions and are actively reabsorbed (up to 95%) in the terminal ileum[245,246]. Disruption of the enterohepatic circulation of bile acids due to ileal disease (inflammatory bowel diseases) or idiopathic bile acid malabsorption is responsible for chronic diarrhoea[246]. Faecal concentrations of bile acids in IBS-D or functional diarrhoea are unknown. While earlier studies suggested up-regulation of the ileal active transporter[247] as a result of chronic loss of bile acids (which may reduce the bile acids reaching the colon), other data suggest increased delivery to the colon may occur if the ileal reabsorptive capacity for bile acids is exceeded[246].

Odunsi-Shiyanbade *et al*[155] showed in 12 IBS-D patients that colesevelam modestly affected overall colonic transit (in patients treated with colesevelam, the emptying of the ascending colon was approximately 4 h longer compared to placebo). Furthermore, colesevelam favoured stool passage and somewhat firmer stool consistency. No effects on mucosal permeability or safety were found[246]. Successively, Camilleri *et al*[248] performed a 10-d single-center unblinded single-dose trial on the effects of colesevelam in 12 IBS-D patients. They demonstrated that colesevelam accelerates the delivery of BAs to stool, while improving stool consistency. It also stimulates hepatic BA synthesis, avoiding steatorrhea in patients with IBS-D. The overall effects are due to luminal BA sequestration by colesevelam[248]. All the abovementioned studies suggest that there is an opportunity to diagnose and specifically treat the cause of symptoms in IBS-D.

Solifenacin

Solifenacin is a muscarinic type 3 receptor antagonist recommended in the treatment of overactive bladder (OAB) in adults[249]. Since 1967, M3 receptor antagonists such as mepenzolate bromide have been used in Japan as modulating agents of gastrointestinal motility. However, no clinical trials had been designed to evaluate the efficacy for IBS defined under the modern Rome criteria. Given the high rate of comorbidity between IBS

and OAB[250], and considering that solifenacin acts on bowel dysfunction similarly to darifenacin, a selective M3 receptor antagonist with equivalent potencies, solifenacin was evaluated on symptomatic relief in 20 IBS-D patients in an open-label trial[249]. After a 2-wk observation period, solifenacin was administered for 6 wk. Later, solifenacin was suspended, and ramosetron, a serotonin 3 receptor antagonist, was given for 4 wk. Solifenacin was not inferior to ramosetron in the treatment of IBS with diarrhoea[249].

The results of this study suggested the potential therapeutic application of solifenacin in the treatment of IBS-D. However, the possible placebo effect could not be excluded. Therefore, further placebo-controlled parallel group studies are required to confirm the efficacy of solifenacin[249].

Tiropramide

Tiropramide, a derivative of tyrosine, has a spasmolytic effect on the intestine, decreasing Ca^{2+} release into intestinal smooth muscle[251-253]. In a double-blind placebo-controlled randomized trial performed by Lee *et al*[251], tiropamide was associated with an improvement of total symptom scores for 4 wk compared with 3 wk in the placebo group. In addition, only patients treated with tiropramide improved abdominal pain at week 4[254].

Lee *et al*[251] successively performed a multicentre, randomized, non-inferiority trial involving 287 patients with IBS randomly assigned to either tiropramide 100 mg or octylonium 20 mg t.i.d. (means 3 times a day) for 4 wk[1]. The visual analogue scale (VAS) scores of abdominal pain at week 4 were significantly reduced in both tiropramide and octylonium groups, even though the change from baseline was similar in the 2 groups[251]. In both groups, abdominal pain and discomfort assessed using VAS scores, diaries and IBS-QoL were improved, and no differences in the changes from baseline were observed. Side effects were similar in both groups. No severe side effects involving either drug were observed[251].

Despite the useful results of the abovementioned study, further studies are required to elucidate tiropramide's pharmacodynamic and pharmacokinetic properties and its mechanism of action on the intestine[251].

Eluxadoline

Eluxadoline is a μ- and κ-opioid receptor agonist and δ-opioid receptor antagonist. Its action is directed to the enteric nervous system, with slight side effects in the central nervous system. Its use was approved by the United States Food and Drug Administration on May 2015[255-257].

Patients with IBS-D receiving eluxadoline (100 mg twice daily) in a phase Ⅱ dose-ranging study had greater efficacy compared with patients receiving placebo after 12 wk[258]. Eluxadoline improved the number of daily bowel movements and decreased the episodes of urgency and incontinence experienced by patients during the 3-mo treatment period[258]. Eluxadoline had an overall favorable safety profile, with nausea, abdominal pain, vomiting, and constipation the most commonly reported AEs[258].

Subsequently, in two large Phase 3 trials (IBS-3001 and IBS-3002), the efficacy of eluxadoline in patients with IBS-D was shown[259].

Finally, Cash *et al*[260] reported pooled safety and tolerability data from Phase 2 and 3 clinical studies for the approved doses of eluxadoline: 75 and 100 mg. The authors demonstrated that constipation and nausea were the most common adverse events[260]. Consistent with the known adverse effects of opioid agonists, clinically apparent sphincter of Oddi spasm events were observed in eluxadoline-treated patients without a gallbladder. The majority of these cases were observed in patients on the higher dose of eluxadoline, thus suggesting a possible association[260].

Table 4 sums up the literature findings about irritable bowel syndrome-D therapies.

CONCLUSION

IBS currently remains a field of intense therapeutic research, in which most of the aforementioned studies focus on stool-pattern-specific subcategories of patients with this condition. Multiple further drugs are also under evaluation. Among these, alpha galactosidase (AG) was shown to reduce meteorism associated with black bean ingestion, even though it is unknown whether it may have a benefit on IBS[261]. However, in a subsequent study performed by Hillilä *et al*[262], no evidence to support the use of AG routinely in IBS patients was found.

With regards to therapies restoring intestinal permeability, multiple studies with prebiotics and probiotics[263] are ongoing, even if to date their efficacy has been limited. In parallel, much progress has been made in targeting low-grade inflammation, especially through the introduction of drugs such as mesalazine and rifaximin, even if a better knowledge of the mechanisms underlying the low-grade inflammation in IBS may support the design of clinical trials aimed at evaluating the efficacy and safety of such drugs.

On the other hand, the non-pharmacological treatment of IBS is often viewed as attractive. Faecal microbiota transfer, dietary interventions, holistic and integrative medicine approaches currently represent possible future therapeutic alternatives in this setting.

In conclusion, long-term studies and comparative studies with pharmacotherapy, as well as elucidation of the underlying mechanisms of action, are still needed to find the correct algorithm to manage IBS patients.

Table 4 Summary of the literature findings about irritable bowel syndrome-D therapies

Drug	Ref.	No. of pt	Study design	Outcomes
Ramosetron	Lee *et al*[213]	343 male pt	A multicenter, randomized, open-label trial male patients with IBS-D; pt were randomized to either a 4-wk treatment of ramosetron 5 mg once daily ,or a 4-wk treatment of mebeverine 135 mg three times daily	Global IBS symptoms, abdominal pain/discomfort and abnormal bowel habits in the ramosetron and mebeverine groups significantly increased during the treatment period. The severity scores of abdominal pain/discomfort and urgency, the stool form score, and the stool frequency in both treatment arms were significantly reduced, compared with the baselines
	Fukudo *et al*[222]	296 male pt	A randomized, double-blind, placebo-controlled trial in male patients with IBS-D Patients were given 5 mg oral ramosetron (n = 147) or placebo (n = 149) once daily for 12 wk after a 1-wk baseline period	Improving stool consistency in the first month. The ramosetron group had significantly higher monthly rates of relief of overall IBS symptoms and IBS-related quality of life than the placebo group. Adverse events occurring in 46.9% and 51.7% of ramosetron and placebo patients, respectively
	Fukudo *et al*[223]	576 female pt	A randomized, double-blind, placebo-controlled trial. The subjects received either 2.5 μg ramosetron or placebo once daily for 12 wk.	Global improvement, an increased stool consistency a significant reductions in abdominal pain and discomfort and greater improvement in QOL compared with placebo
Lx1031	Brown *et al*[229]	155 patients	A phase-Ⅱ multicenter, randomized, double-blind, placebo-controlled, the subjects were assigned randomly in a double-blind fashion to 1 of 2 doses of LX1031 (250 mg 4 times/d or 1000 mg 4 times/d) or placebo, taken daily during the 28-d treatment period	Improved significantly in patients given 1000 mg LX1031 compared with those given placebo, at week 1, together with nonsignificant improvements at weeks 2, 3 and 4. Adverse Effects reported were generally mild, self-limited, and evenly distributed across the placebo and both LX1031 treatment arms
ASP-7147	Lembo *et al*[230]	64 patients	RCT performed on during a 4-wk	Demonstrating improvement in multiple symptoms of IBS-D. The persistence of treatment effect suggests the possibility of retained efficacy with less frequent dosing in follow-on trials
JNJ-27018966	[232]	807 patients	A randomized, controlled, double-blind study, 25, 100, and 200 mg twice daily to placebo	The composite of diarrhea and pain was significantly improved in the JNJ-27018966 25 and 200 mg twice-daily groups compared to placebo
ROSE-010	Hellström *et al*[233]		A randomized placebo-controlled trial. Patients were randomized to ROSE-010 100 μg once daily, 300 μg once daily or placebo	Treatment with ROSE-010 resulted in a two fold greater response to abdominal pain compared to placebo and significantly greater patient-reported satisfaction with ROSE-010. The most common treatment-related adverse effect was nausea
AST-120	Tack *et al*[95]	160 patients 115 non-constipation-related IBS patients	A randomized, double-blind, controlled study	AST-120 2 g three times daily significantly improved the proportion of patients with at least a 50% reduction in the number of days with abdominal pain compared to placebo. AST-120 resulted in significantly improved bloating and numerically improved stool consistency compared to placebo. The safety profile AST-120 was similar to placebo
Ibodutant	Trinkley *et al*[231]	559 IBS-D patients	A randomized, double-blind, controlled trial	Improved abdominal pain, satisfactory relief of overall symptoms, and quality of life compared to placebo. All three doses of ibodutant (1, 3, 10 mg once daily) were superior to placebo, but 10 mg once daily was most effective and females responded better than males
Asimadoline	Trinkley *et al*[231]	596 IBS-D patients	A randomized, controlled, double-blind trial compared asimadoline 0.15, 0.5 and 1 mg twice daily to placebo	Asimadoline 0.5 mg twice daily significantly improved by two fold the total number of months with adequate relief of IBS pain, pain scores, urgency and frequency
Colesevelam	Odunsi-Shiyanbade *et al*[155]	12 IBS-D patients	Single center trial	Colesevelam modestly affected overall colonic transit (emptying of the ascending colon took an average 4 h longer in patients given colesevelam compared to placebo). Furthermore, colesevelam was associated with greater ease of stool passage and somewhat firmer stool consistency. No effects on mucosal permeability or safety were identified
	Camilleri *et al*[240]	12 IBS-D patients	A 10-d single-center, unblinded, single-dose trial	Colesevelam increases delivery of BAs to stool while improving stool consistency, and increases hepatic BA synthesis, avoiding steatorrhea in patients with IBS-D

Solinefacin	Fukushima *et al*[249]	20 IBS-D patients	An open-label trial. After a 2-wk observation period, all participants received solifenacin for 6 wk. Subsequently, the administration of solifenacin was discontinued and ramosetron, a serotonin 3 receptor antagonist, was administered for 4 wk	The efficacy of solifenacin in the treatment of IBS with diarrhea was not inferior to that of ramosetron
Tiropamide	Lee *et al*[251]	287 patients	A multicenter, randomized, non-inferiority Patients randomly allocated to either tiropramide 100 mg or octylonium 20 mg t.i.d (means 3 times a day) for 4 wk	Tiropamide led to symptom improvement in terms of total symptom scores for 4 wk, compared with 3 wk in the placebo group; in addition, at week 4 abdominal pain was only improved in the tiropramide group. The incidence of adverse events was similar in the 2 groups, and no severe adverse events involving either drug were observed

IBS : Irritable bowel syndrome.

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MINIREVIEWS

Glucocorticosteroid therapy in inflammatory bowel diseases: From clinical practice to molecular biology

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Abstract

Inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's disease, are chronic pathologies associated with a deregulated immune response in the intestinal mucosa, and they are triggered by environmental factors in genetically susceptible individuals. Exogenous glucocorticoids (GCs) are widely used as anti-inflammatory therapy in IBDs. In the past, patients with moderate or severe states of inflammation received GCs as a first line therapy with an important effectiveness in terms of reduction of the disease activity and the induction of remission. However, this treatment often results in detrimental side effects. This downside drove the development of second generation GCs and more precise (non-systemic) drug-delivery methods. Recent clinical trials show that most of these new treatments have similar effectiveness to first generation GCs with fewer adverse effects. The remaining challenge in successful treatment of IBDs

concerns the refractoriness and dependency that some patients encounter during GCs treatment. A deeper understanding of the molecular mechanisms underlying GC response is key to personalizing drug choice for IBDs patients to optimize their response to treatment. In this review, we examine the clinical characteristics of treatment with GCs, followed by an in depth analysis of the proposed molecular mechanisms involved in its resistance and dependence associated with IBDs. This thorough analysis of current clinical and biomedical literature may help guide physicians in determining a course of treatment for IBDs patients and identifies important areas needing further study.

Key words: Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Glucocorticoid dependence; Glucocorticoid resistance

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Core tip: Glucocorticoids (GCs) are widely used in patients with Inflammatory Bowel Diseases who have moderate or severe disease activity; however, some of them do not respond to treatment or become dependent. Knowledge of both the clinical approach of GCs treatment as well as the molecular basis underlying their effects will help physicians prescribe drugs that will lead to better outcomes for patients.

Dubois-Camacho K, Ottum PA, Franco-Muñoz D, De la Fuente M, Torres-Riquelme A, Díaz-Jiménez D, Olivares-Morales M, Astudillo G, Quera R, Hermoso MA. Glucocorticosteroid therapy in inflammatory bowel diseases: From clinical practice to molecular biology. *World J Gastroenterol* 2017; 23(36): 6628-6638 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6628.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6628

INTRODUCTION

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are multifactorial disorders comprised of both environmental and genetic factors. Even though they are different disease entities, UC and CD are grouped under the generic term of IBDs given their similar activity/remission stages, chronicity, immunological pathophysiology and uncertain etiology[1-3]. The prevalence of UC is 505 per 100000 people in Europe and 249 per 100000 people in North America, and CD is 322 per 100000 people in Europe and 319 per 100000 people in North America[4]. An increased incidence has been reported in some South American and mid-eastern Asian countries such as Chile[5], Brazil[6], Israel[7], and Malaysia[8]. This tendency might be related to the industrialization phenomenon and the influence of environmental risk factors[4].

IBDS: A MULTIFACTORIAL COMPLEX DISORDER

Various genetic and environmental factors have been associated with development of IBDs. Polymorphisms in a vast number of genes which impair functions such as lymphocyte activation, autophagy, pathogen sensing, stress response, antigen presentation, and chemotaxis, among others have been described[9]. The presence of such genetic variants may contribute to an imbalance in the immune response and increased predisposition to IBDs[10,11]. Moreover, environmental factors, such as diet, smoking, appendectomy, breast-feeding behavior in childhood, vitamin D deficiency and infections, play key roles in inflammatory manifestations, and have been described as risk factors for IBDs[12,13]. Furthermore, intestinal microbiome composition differs in IBDs patients compared to healthy subjects: IBD patients have a more predominant *Proteobacteria* population and decreased *Firmicutes* and *Bacteroidetes* populations[14,15]. Environmental factors might be involved in creating such imbalance in microbiome composition, and this may give rise to a constant inflammatory antigenic stimulus in the gut that causes chronic inflammation in genetically susceptible individuals[14].

These multifactorial components involved in the origin and progression of IBDs, has prevented the development of a specific treatment applicable to all patients. Current treatment options seek to diminish inflammation in order to control symptoms and keep the patient in a state of remission or symptom improvement. For example, aminosalicylates (5-asa derivatives) are used to treat mild disease, while immunosuppressants such as azathioprine/6-mercaptopurine and methotrexate, and biological therapy such as anti-TNF antibodies or anti-integrin are prescribed for moderate to severe disease[16]. Finally, glucocorticoids (GCs) are used to achieve, but not to maintain, remission in patients with moderate to severe activity[17].

GC THERAPY IN IBDs: ADMINISTRATION AND EFFECTIVENESS

The first study that demonstrated the usefulness of GCs (cortisone) in controlling severe UC attacks was published in 1955[18]. Since the 70s, the first generation of GCs (prednisone, methylprednisolone, hydrocortisone) has been used to induce clinical remission in IBDs patients. However, significant adverse effects have led to the development of second generation GCs (budesonide, budesonide MMX, Beclomethasone dipropionate)[19-21]. A characteristic of GC treatments and their use is summarized in Table 1.

Table 1 Glucocorticoids of first and second generation and his medical uses

Glucocorticoids	Component	Indications	Ref.
1st generation	Prednisone	Moderate to Severe cases	[3,25,27]
	Methyl-Prednisolone	of IBDs	[26]
	Hydrocortisone	Short duration of treatment	[29]
2nd generation	Budesonide	Moderate CD cases	[32-34]
	Budesonide MMX	Mild to moderate UC cases	[36,37]
	Beclomethasone dipropionate	Topical administration	[40,41]
	Erythrocyte - Mediated Delivery of Dexamethasone	In research for long term treatments	[42]

IBDs: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

First generation GCs: more effective than 5-asa and immunomodulatory treatment with significant secondary effects

Several studies have compared the efficacy and safety of traditional GCs to other treatments by measuring the rates of remission and adverse side effects. Two independent placebo-controlled double-blind randomized trials showed that 47% of active CD patients treated with prednisone (1 mg/kg, ranging from 40-60 mg/d) achieved clinical remission, defined as CDAI score < 150 (for a review on clinical and endoscopy scoring see references[22-24]), compared to 26% of patients receiving placebo after 4 mo of treatment. Neither sulfasalazine (0.5-1 gr/15 kg) nor azathioprine (2-2.5 mg/kg per day) achieved significant effectiveness[25]. Later, a placebo-controlled study showed that high doses of methylprednisolone (12-48 mg/d), sulfasalazine (3 gr/d), or both in active CD patients, were significantly effective compared to the placebo, and patients treated with methylprednisolone (alone or in combination with sulfasalazine) showed faster improvement compared to sulfasalazine[26].

Despite the effectiveness of GCs demonstrated in patients with severe CD, a prospective multicenter study comprised of patients with active CD on prednisone treatment (1 mg/kg per day) concluded that while 92% achieved clinical remission, only 29% of them reached endoscopic remission[27]. The presence of stenosis or fistula secondary to CD which occurs in patients who do not reach endoscopic remission, indicates a poor response to GC therapy and requires surgical intervention[27]. This suggests that GCs are efficient in improving the inflammatory response but other strategies are needed to increase mucosal healing.

Regarding UC, systemic GCs are the first choice therapy in cases of severe colitis[3]. In acute and critical UC, 76% of the patients treated with prednisolone (20 mg/d), plus a nightly rectal drip of hydrocortisone succinate sodium (100 mg in solution), presented clinical remission *vs* 52% of patients who were treated with sulfasalazine (8 gr/d the first week, 4 gr/d the second week)[28] demonstrating the clinical efficacy of this treatment. Likewise, two consecutive trials in UC patients with left side colitis determined that oral prednisone (1 mg/kg per day) was effective in severe stages of the disease compared with calcium lactate (1-3 gr; placebo), and prednisone treatment led to clinical remission more quickly and more often than those treated with salazopyrin (4 gr/d; a sulfasalazine derivate) and topic hydrocortisone treatments (100 mg/150 mL of sterile water administered as an enema)[29].

Despite the effectiveness of first generation GCs, they are characterized by several serious side effects that limit their long-term use. Among these, some of the most important are: metabolic (altered distribution of fat, Cushing's face or steroid-induced diabetes), eye-related (cataracts and glaucoma), dermatological (bruising and urticarial), gastrointestinal (gastric ulcer and gastrointestinal bleeding), and musculoskeletal side effects (osteopenia to osteoporosis). Additionally, adverse effects on the central nervous system have been described (insomnia, anxiety, hyperactivity, psychotic processes), along with hypertension, hypothalamic-pituitary-adrenal axis suppression and increased susceptibility to infections due to immunosuppression[19]. However, its use in IBDs patients has not be associated with atherosclerosis progression[30]. Finally, there is evidence that GC use increases the likelihood of sepsis of gastrointestinal origin; therefore it should be prescribed with caution in these patients[31].

Second generation GCs: high anti-inflammatory activity and fewer side effects.

The previously described serious side-effects related to first-generation GCs drove the development of second-generation steroids, which maximize the amount of corticosteroid locally available for distal ileum and proximal colon and minimize systemic bioavailability[3,26]. These new formulations of GCs with limited absorption include budesonide, budesonide MMX (multimatrix delivery system MMX) and beclomethasone dipropionate[3,19].

Budesonide, similar effectiveness with fewer adverse effects: Budesonide undergoes hepatic inactivation before reaching the systemic circulation (first-pass metabolism) which reduces its bioavailability[3]. Various placebo-controlled clinical studies have provided evidence about the efficacy of budesonide in IBDs patients. A multicenter randomized, placebo-controlled, double-blind clinical

trial was conducted in CD patients who received three different budesonide doses (3, 9 and 15 mg/d)[32]. After 8 wk of treatment, 51% of those receiving 9 mg budesonide progressed to remission in CD patients with ileum and proximal colon disease activity. Thus, this dose was effective and safe but depended on disease location. A randomized, double-blind, double-dummy controlled trial conducted in patients with mild to moderately active CD demonstrated that budesonide (9 mg/d for 2 mo) was as effective as prednisone (40 mg/d for the first 2 wk, 30 mg/d the third week, and gradually tapered to 5 mg/wk at the end of the study lasting 2 mo) in patients with terminal ileum, cecum, and/or ascending colon inflammation[33].

Subsequently, a multicenter double-blind, randomized trial was conducted in CD patients with moderate activity in the distal ileum and ascending colon. More patients that received budesonide (4.5 mg twice daily, or 9 mg once daily) achieved clinical remission compared to placebo 2 wk after beginning treatment, and this difference was statistically significant[34]. In addition, a pooled analysis of trials in CD patients found significantly fewer GC-related adverse events compared to patients treated with conventional corticosteroids (RR = 0.64, 95%CI: 0.54-0.76)[35].

An improved oral formulation of budesonide uses the colonic delivery technology Multi-Matrix System (MMX) to extend drug release in the colon (Budesonide MMX). Due to this characteristic, it is indicated for the treatment of UC patients that do not respond to standard doses of salicylates. A placebo-controlled study demonstrated that a higher proportion of UC patients with mild to moderate activity treated with budesonide MMX (9 mg), reached clinical remission and symptom resolution compared to placebo. In contrast, a lower dose of budesonide MMX (6 mg) and mesalamine, another salicylate treatment (2.4 gr/d), did not significantly improve either parameter[36]. Furthermore, the higher dose of Budesonide MMX was efficient in inducing remission in patients with mild to moderate UC disease who did not achieve remission with 5-asa treatments such as mesalamine or sulfasalazine[36,37]. Currently, physicians prescribe Budesonide MMX in UC patients that do not respond to traditional maintenance therapy before treating with azathioprine/6-mercaptopurin.

In 2015, data from 5 clinical studies, including double-blind, randomized, and open label studies, showed that rates of adverse effects were similar between budesonide MMX 9 mg and 6 mg (54.5% and 60.6% respectively) and placebo (50.5%) in patients with mild to moderate UC[38]. Furthermore, the open label studies showed less frequency of adverse effects with budesonide MMX (3 mg or 9 mg) compared to placebo. The most common adverse effects in these studies were headache, nausea and urinary tract infection demonstrating that second generation GCs not only have fewer side effects, but those effects are less severe compared to first generation GCs side effects.

Beclomethasone dipropionate, a second generation option with low systemic activity: Beclomethasone dipropionate (BDP) is a second-generation corticosteroid with topical effects and minimal systemic activity. It is administered as a pro-drug and is partially metabolized in the lower gastrointestinal tract[39]. The effectiveness and tolerability of BDP *vs* budesonide have been evaluated in CD patients whose clinical characteristics did not include complications such as stenosis or fistulas. A study showed that BDP (10 mg/d) was less effective than budesonide (9 mg/d) in CD patients treated for 2 mo. They concluded that this was due to the pharmacokinetic properties of budesonide[40].

A study was conducted to evaluate the efficacy and safety of BDP enemas *vs* prednisone enemas in active distal UC after 1 mo of treatment. They determined that clinical and endoscopic remission occurred in 29% of patients who received BDP and in 25% of patients who had standard topical GC treatment, and both groups experienced few adverse effects[41]. This evidence leads to the conclusion that BDP has similar efficacy to traditional GCs.

GC delivery technology, the next step to avoid side effects

Novel drug delivery systems capable of slowly releasing drugs into the bloodstream at a low concentration such as the erythrocyte-mediated drug delivery have been proposed. The membrane characteristics of autologous erythrocytes render them ideal drug carriers because their permeability allows for the diffusion of small molecules. This has been demonstrated using dexamethasone 21-phosphate (Dex 21-P), a biologically inactive compound[42]. Once encapsulated, an enzyme resident in the red blood cell dephosphorylates Dex 21-P to form the corresponding active metabolite, dexamethasone (Dex), which is then released into the bloodstream by passive diffusion through the cytoplasmic membrane of erythrocytes[42].

A study recruited 40 patients with mild to moderate UC, who had not responded to mesalamine and randomly assigned them to one of the following three treatment groups: Dex 21-P encapsulated into erythrocytes (DEE) delivered *via* two infusions 14 d apart, oral prednisolone infusions (0.5 mg/kg for 2 wk by 6 mg/wk tapering), and placebo. The group of DEE and oral prednisolone achieved a higher rate of clinical and endoscopic remission (75%) after 2 mo compared to placebo[42]. DEE-treated patients showed no adverse effects associated with GCs, in contrast to 80% of prednisone group, in whom acne, hirsutism and weight gain were reported. This delivery tool is an attractive choice for patients with IBDs to avoid most of GCs' adverse effects.

Resistance and dependence, an unresolved issue for GC treatment

More than half of IBDs patients on GC therapy respond to treatment, approximately 28% have a partial response, and 19% are non-responders (GC-resistant). However, about 20% of IBDs patients on long-term GC therapy become dependent[43,44]. Therefore, clarifying this GC resistance/dependency is crucial to making appropriate decisions regarding patient treatment. GC resistance or refractoriness is the inability of GCs to exert their effects on target tissues, thus limiting the efficacy of the therapy. In IBDs, GC resistance is defined as the persistence of an active manifestation of the pathology, despite having received standard treatment of 0.75 mg/kg per day for 4 or more weeks[44].

On the other hand, GC dependency is the need for GCs to maintain remission. Patients are considered GC-dependent if they fail to taper to steroid doses below 10 mg within 4 mo (starting dose 0.75-1 mg/kg oral prednisone-equivalent) or if they relapse within 3 mo after the discontinuation of GC treatment[44]. Usually, long-term treatment with GCs is associated with its resistance or dependency and a more aggressive clinical phenotype in IBD patients[45].

MOLECULAR BASIS OF GLUCOCORTICOID TREATMENT

Exogenous GCs are highly lipophilic compounds, making them widely bioavailable. Similar to endogenous cortisol, GCs are primarily transported in the bloodstream bound to corticosteroid-binding globulin and, to a lesser extent, to albumin[46]. GCs have the ability to passively diffuse through cell membranes and interact with the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors[47].

Molecular characteristics of GR and its impact on Immune system

GR is a cytosolic protein with a molecular weight of 94 kDa encoded by the *NR3C1* gene (5q31.3). It contains nine exons that mainly code for two transcripts formed by alternative splicing: the α and β isoforms[48]. In addition to GRβ, three less-well-characterized isoforms have been reported: GRγ, GR-A and GR-P[49]. Moreover, GRα and GRβ can also undergo alternative translation initiation in exon 2, which generates eight additional GR isoforms with truncated N-terminals giving them distinct properties[49].

The amino terminal region of GRα contains a ligand-independent transactivation domain (AF-1), a highly conserved central DNA binding domain (DBD), and a hinge segment. The carboxyl-terminal region contains the ligand-binding domain, which includes an AF-2 region that interacts with co-regulators in a ligand-dependent manner[50]. The GRβ is a shorter protein which differs from GRα in its C-terminal domain and antagonizes the activity of GRα[51]. After GC binding, the receptors undergo conformational changes and expose the DBD, interact with chromatin, and regulate gene expression[50].

The GR-GC complex can inhibit proinflammatory proteins such as nuclear factor κB and AP-1 through protein-protein interactions. These molecular mechanisms down-regulate the expression of proinflammatory cytokines and chemokines, such as IL-1α, IL-1β and IL-8. In addition, GCs up-regulate the expression of other cytokines that suppress the production of inflammatory mediators: such as transforming growth factor-β3 (TGF-β) and IL-10, increasing its anti-inflammatory function. They also inhibit T and B lymphocyte proliferation, and promote a tolerant macrophage profile (M2) (Figure 1)[46,52,53].

Molecular basis of GC resistance, a clue for personalized treatment

The molecular mechanisms of GC resistance in IBDs have been associated with changes in the GR isoform levels, polymorphisms in *NR3C1* or genes involved in GC bioavailability, and impaired cytokine production[46,48,54,55]. The most important genes associated to GC resistance are summarized in Table 2. Changes in levels of GRα and β isoforms have been associated with GC resistance. The α-isoform is the most well-studied protein, but only increased levels of the β-isoform have been associated to GC resistance in clinical trials in UC, asthma, nasal polyposis, cancer and chronic lymphocytic leukemia[53]. The GRβ protein is located primarily in the nucleus and is found mostly in T lymphocytes, macrophages, neutrophils, eosinophils, and peripheral blood mononuclear cells; however, it has also been reported in brain, lung, and heart tissue[56,57]. It has been proposed that GRβ may act as a dominant negative to GRα, since GRβ does not bind to GCs, but rather it interacts with glucocorticoid element response (GRE) causing GC resistance. This mechanism alters GRα signaling independent of GRα-GRβ heterodimer formation[58]. An *in vitro* study showed that overexpression of GRβ in a colonocyte cell model induces a vast deregulation of gene expression without Dex treatment, and a proportion of these genes have been shown to be altered in IBDs patients[59]. Thus, GRβ may directly change transcriptional gene activity of proinflammatory molecules. This effect is independent of GRα function and may promote the GC-resistance.

Furthermore, GRβ can bind to RU-486, a GRα antagonist, in such a way as to change the cellular location of this GRα, and alter its ability to regulate gene expression[60]. In addition, it has previously been shown that the expression of GRβ can be transcriptionally activated in regulatory T lymphocytes and neutrophils by proinflammatory cytokines[61]. Hence, GRβ expression may affect the immune response of these cells promoting inflammation and non-response to GC[55,57,62].

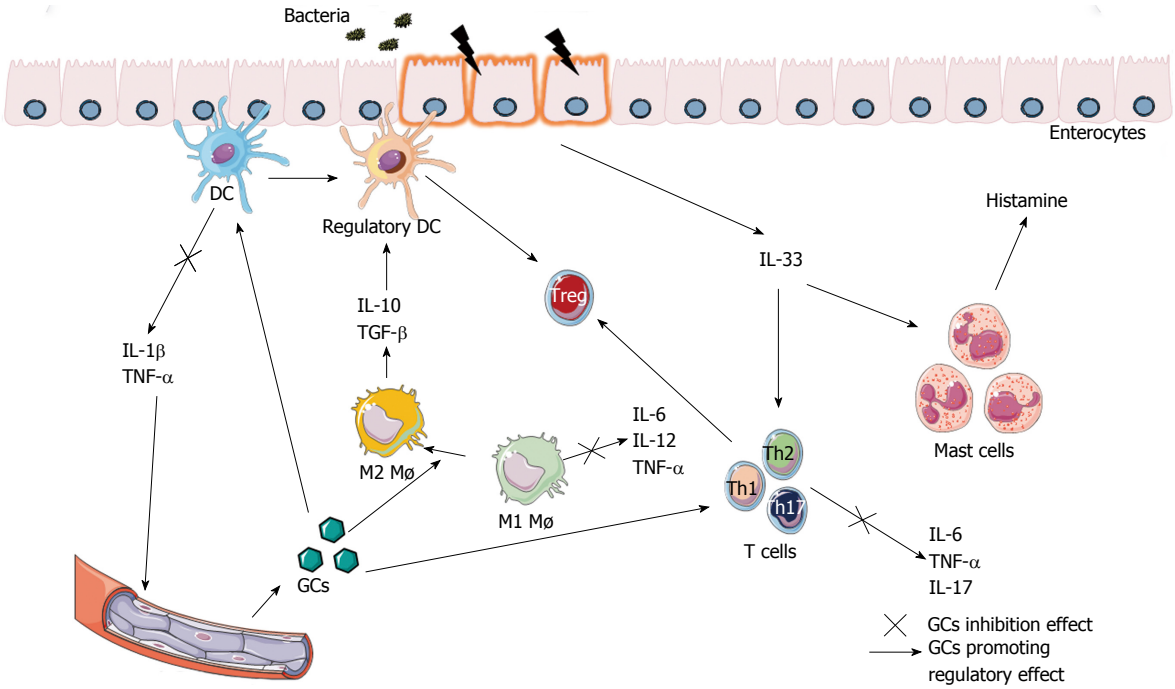


Figure 1 Immunologic dynamics and the responses triggered by glucocorticoids in inflammatory bowel diseases. During activation of the immune response in inflammatory bowel diseases (IBDs), the invasion of bacteria and the cytokine cascade triggered by the disruption of the epithelial barrier prompt an inflammatory response in the intestinal mucosa. Among the different cells that participate in the pathology of IBDs, the most important are represented in this diagram. When bacterial organisms interact with dendritic cells (DC), or the intestinal epithelium is disrupted due to an inflammatory response, the production of proinflammatory cytokines and the release of DAMPs (Damaged- Associated Molecular Patterns) such as TNF-α and IL-33, respectively, is elicited. This leads to the infiltration of immune cells such as monocytes which differentiate into proinflammatory M1 macrophages (Mø) and lymphocytes T (T cells), along with mast cell activation. In the presence of glucocorticoids (GCs), the infiltrating monocytes differentiate into a regulatory M2 profile Mø and CD103^{+} DCs. These tolerogenic cells produce anti-inflammatory cytokines, skew the infiltrating naïve T cells toward a T regulatory phenotype, and control the inflammatory response. Also, the action of GCs blocks the production of proinflammatory cytokines by the M1 Mø, T cells and DCs, leading to the healing of the intestinal mucosa. Figure 1 was produced using Servier Medical Art from http://smart.servier.com. TGF-β: Transforming growth factor-β; IL: Interleukin.

Table 2 Molecular highlights on responses to steroidal treatment

Gene/protein	Function	Molecular alteration or genetic variant	Effect on GC response	Ref.
NR3C1	GC receptor	Increased expression of the isoform	Block signaling of the isoform/GC resistance	[58]
		rs6189 rs6190	Altered transactivation of GC receptor/GC resistance	[54,63]
		rs6195	Enhanced sensitivity to GC	[65]
		rs41423247	Alternative splicing of receptor/GC resistance	[54,63]
MDR1	Export of drugs from the cell	rs1045642	Decreased MDR1 levels/GC resistance in Brazilian CD patients	[68]
		rs1128503 rs1045642	Possible misfolding/GC dependence in Chinese CD patients	[75-77]
IL-10	Anti-inflammatory cytokine	rs1800896	Lowered production of IL-10/GC dependence in UC and CD patients	[45]
EBF3	Transcription factor	Decreased levels of EBF3	More severely inflamed phenotype/GC dependence	[72]
PAR2	G protein coupled receptor	Methylation of gene promoter	GC resistance/dependence in UC patients.	[82]

UC: Ulcerative colitis; CD: Crohn's disease; GCs: Glucocorticoids.

In 2009, a study was conducted to measure the expression of GRβ in intestinal mucosa of IBD patients that were refractory to treatment with GCs[55]. High levels of the GRα isoform were found in both GC-resistant and responsive patients; however, GC-resistant patients expressed higher levels of GRβ, in comparison with the GC-responder group. In the intestinal mucosa, the cells that primarily expressed the GRβ receptor were CD4^{+} and CD8^{+} T lymphocytes, macrophages and B cells. Fibroblasts and vascular endothelial cells expressed GRβ in a minor proportion. These results suggest that a higher ratio of GRβ/GRα in inflammatory mononuclear cells disturbs the effects of GCs resulting in refractory outcomes.

Single nucleotide polymorphisms (SNPs) in *NR3C1* gene, have been associated with GC resistance[54],

and these SNPs may result in different effects on the receptor structure and its signal transduction[54]. The most-studied SNPs that could induce a refractory phenotype in IBDs patients are: ER22/23EK (rs6189 and rs6190), N363S (rs6195), and BcII (rs41423247)[54]. The ER22/23EK is produced by a change in the amino acid residues 22 and 23 in the GR, where two glutamic acids (E) are replaced by an arginine (R) in position 22, and a lysine (K) in position 23[63]. These SNPs affect the transactivation process in *in vitro* assays, where the receptor is less likely to change its conformation and induce gene transcription in the presence of GCs[64]. The SNP N363S is in codon 363 of exon 2 and results in an amino acid change from asparagine (N) to serine (S). This SNP was reported to be associated with enhanced sensitivity to GCs, as was demonstrated by a Dex suppression test[65]. The BclI SNP was identified as a cytosine-guanine substitution 646 nucleotides downstream from exon 2, yielding 2.2 and 3.9 kb long fragments[63], which could suggest alternative splicing. A meta-analysis that included a mostly Caucasian population, analyzed the association of each of these 3 SNPs with GC resistance in IBDs patients[54]. Although no association was found, the analysis of the presence of all three SNPs, (or combinations of two SNPs) as a polygenic additive effect, could show risk associations of these variants with GC resistance.

Like the GR, the P-glycoprotein (P-gp) has also been associated with GC resistance in IBDs. P-gp is a trans-membrane glycoprotein with a molecular weight of 170 kDa encoded by the *MDR1* gene, which is located on the long arm of chromosome 7. P-gp is responsible for the absorption, distribution, metabolism and excretion of various drugs including GC[66]. A relationship between increased P-gp levels in circulating lymphocytes and intestinal epithelial cells with GC-resistant IBDs patients has been reported[67]. Polymorphisms in the *MDR1* gene have been shown to alter P-gp function, which in turn modifies the response to GCs[67]. A case-control study in a Brazilian population demonstrated that SNP C3435T (rs1045642), located in exon 26 of *MDR1* was associated with GC resistance in CD but not in UC patients[68].

Additionally, higher expression of proinflammatory cytokines has been associated with GC resistance. Overexpression of IL-6 and IL-8 has been associated with non-responsiveness to GC treatment[69]. In pediatric patients with UC, a similar correlation was found between the expression levels of IL-6 and unresponsiveness to GC[70]. In this study, the patients who did not respond to GC had higher levels of IL-6, compared to responders. Given the pleiotropic nature of IL-6, this correlation could reflect a cause-effect relationship between the signaling of the GC receptor and the expression of proinflammatory cytokines, but the real reason for the failure to respond remains unclear[70].

Similarly, in CD a correlation between cytokine production and resistance to GC was found. A clinical study showed that in intestinal mucosa from GC-resistant patients, the rate of apoptosis of T and B cells, presence of caspase-3 and IL-10 production were diminished compared to healthy and GC-responsive individuals[71]. These findings suggest a strong relationship between cytokine signaling and the inhibition of the GR effect in inflammatory diseases.

Analyses of gene expression using microarrays have been conducted to associate gene deregulation with GC resistance from colonoscopic biopsies of IBDs patients. *ATG16L1*, a gene related to the autophagy process was down-regulated in GC-resistant patients[72]. A low expression of the ATG16L1 protein has been associated with altered secretion of mucus by Paneth cells[73]. Furthermore, inhibition of autophagy led to Dex-resistance in a lymphoma cell line and in a mouse model[74]. This evidence indicates the importance of autophagy in the GC-resistant phenotype.

GC dependence: another genetic issue

Contrary to GC refractoriness, the molecular mechanisms behind GC dependence are poorly studied. There is evidence that associates *MDR1* SNPs (rs1128503 and rs1045642) with GC-dependent CD patients in a Chinese population[75]. Both SNPs correspond to a change from cytosine (C) to thymine (T) and are in coding regions of the gene (exon 12 and 26, respectively). Based on computational protein analysis, these SNPs, as haplotypes, seem to affect protein folding which could alter P-gp levels[76], but this mechanism has not been experimentally demonstrated. Nevertheless, the rs1045642 - CC was frequently found in individuals with significantly higher P-gp levels in plasma along with increased protein activity (measured by rhodamine efflux in $CD56^+$ natural killer cells), compared to carriers of the TT genotype[77]. This study suggests that increased GC transportation out of cells might drive a decreased GC response leading to the dependent phenotype[75].

SNPs in the *IL-10* gene have been associated with GC dependency as well. The SNP -1082 A/G (rs1800896) is located in the promoter sequence of the IL-10 gene, and a case control study of this variant showed an association with GC dependence in UC and CD patients[45]. This SNP was also related to a lower production of IL-10 in IBDs patients. Together, this evidence suggests that intrinsically inferior levels of IL-10 may generate GC dependency because exogenous GCs would be necessary to reach the anti-inflammatory IL-10 content necessary to achieve sustained remission. Other mechanisms, similar to those of refractoriness, such as altered expression of GR that impair intracellular signaling, have been proposed[78].

Downregulation of EBF3 was related to GC dependence in IBD patients[72]. The transcription factor EBF3 is a downstream target of SMAD2/3, which are components of the TGF-β signaling pathway[79]. This anti-inflammatory mechanism might be impaired in the context of low EBF3 expression[80]. Moreover,

EFB3 down-regulation has been associated with a low rate of apoptosis and increased cell proliferation[81]. This evidence suggests that decreased EBF3 levels might allow for a more severely inflamed phenotype making necessary a continual GC administration to maintain remission in IBD patients and driving their dependence.

Epigenetics knowledge in GCs dependence/resistance in IBDs

Epigenetic changes may also cause GC dependence/resistance. In the context of IBDs, higher methylation of the *Protease-Activated Receptor 2* (*PAR2*) gene, has been associated with steroid-dependent and resistance phenotypes in UC patients[82]. PAR2 is a G protein coupled receptor that is involved in pro-inflammatory responses and play a role in IBDs pathogenesis[82]. However, its specific role in GCs dependence/resistance has not been described.

Various studies relate the epigenetics changes such as hypermethylation of promoter genes with severe disease and risk of neoplasia[83-85]. There is evidence that epigenetic shifts can occur in the early stages of embryonic development in genes known as metastable epialleles, and these events can appear, as shown in rat and murine models, by environmental factors such as stress[86-88]. However, more analyses are needed to clarify the role of epigenetics in controlling the molecular mechanisms that underlie dependence or resistant to GCs in IBDs patients.

CONCLUSION

It has been over half a century since the first use of GC in IBD, yet amazingly, it currently remains a widely used therapeutic tool, specifically in disease exacerbations. The latest pharmacological applications, known as second-generation steroids, have contributed to the reduction of adverse reactions, however, increased effectiveness would need to be demonstrated in order for them to replace traditional GC therapy.

Despite findings showing positive GC responses, some patients remain resistant or become dependent to this drug. Deepening our understanding of the molecular basis of these undesired effects will help us generate novel, personalized GC therapies, depending on a patient's genetic background and cytokine profiles.

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ORIGINAL ARTICLE

Basic Study

Attenuation of MET-mediated migration and invasion in hepatocellular carcinoma cells by SOCS1

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Abstract

AIM

To investigate the role of suppressor of cytokine signaling 1 (SOCS1) in regulating MET-mediated invasive potential of hepatocellular carcinoma (HCC) cells.

METHODS

Stable derivatives of mouse (Hepa1-6) and human (hep3B, HepG2) HCC cell lines expressing SOCS1 or control vector were evaluated for their ability to migrate towards hepatocyte growth factor (HGF) in the transwell migration assay, invade extracellular matrix in response to HGF stimulation in a 3-D invasion assay by confocal microscopy, and to undergo anchorage-independent proliferation in semisolid agar. Following intravenous and intrasplenic inoculation into NOD.scid. gamma mice, the ability of Hepa cells to form othotopic tumors was evaluated. Following HGF stimulation of Hepa and Hep3B cells, expression of proteins implicated in epithelial-to-mesenchymal transition was evaluated by western blot and qRT-PCR.

RESULTS

SOCS1 expression in mouse and human HCC cells inhibited HGF-induced migration through matrigel. In the

3-D invasion assay, HGF stimulation induced invasion of HCC cells across type-Ⅰ collagen matrix, and SOCS1 expression significantly reduced the depth of invasion. SOCS1 expression also reduced the number and size of colonies formed by anchorage-independent growth in semisolid agar. Following intravenous inoculation, control Hepa cell formed large tumor nodules that obliterated the liver whereas the SOCS1-expressing Hepa cells formed significantly smaller nodules. Tumors formed by SOCS1-expressing cells showed reduced phosphorylation of STAT3 and ERK that was accompanied by reduced levels of MET protein expression. HGF stimulated Hepa cells expressing SOCS1 showed increased expression of E-cadherin and decreased expression of EGR1, SNAI1 and ZEB1. Comparable results were obtained with Hep3B cells. SOCS1 expressing HCC cells also showed reduced levels of EGR1 and SNAI1 transcripts.

CONCLUSION

Our findings indicate that loss of SOCS1-dependent control over epithelial-to-mesenchymal transition may contribute to MET-mediated migration, invasion and metastatic growth of HCC.

Key words: Migration; Invasion; MET; Hepatocellular carcinoma; Suppressor of cytokine signaling 1

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Core tip: The suppressor of cytokine signaling 1 (*SOCS1*) gene is frequently repressed in primary hepatocellular carcinoma (HCC) specimens, and mice lacking SOCS1 are highly susceptible to experimental HCC. The tumor suppressor functions of SOCS1 in HCC are not yet fully understood. We have shown that SOCS1 regulates hepatocyte growth factor signaling *via* the MET receptor in HCC cells and inhibits their growth. In this study, we characterize SOCS1 as a regulator of MET-mediated migration and invasion of HCC cells. We propose that *SOCS1* gene expression status could be exploited as a selection marker for precision therapies targeting MET in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers and a leading cause of worldwide cancer mortality[1]. HCC develops slowly over two to three decades and most cases present advanced disease at the time of diagnosis. Treatment options for HCC are constrained by the extent of disease, and are very limited and less effective in patients with advanced disease. Therefore, reducing HCC-associated mortality is critically dependent on the development of new treatment methods targeting molecular signaling pathways that promote HCC pathogenesis and diagnostic tools to facilitate targeted therapies[2-4].

Invasive intrahepatic dissemination is a key factor in malignant growth of HCC and its poor prognosis[5,6]. Up to 65% of HCC patients also present extrahepatic metastasis at autopsy[7-9]. HCC can also metastasize to stomach *via* direct invasion[10]. Detachment from the tumor mass and invasion of the extracellular matrix and the basement membrane are important steps in tumor cell invasion and metastasis. Cancer cells gain these abilities through epithelial-mesenchymal transition (EMT), a developmental genetic program that is crucial for embryogenesis and wound healing response[11,12]. A wide spectrum of paracrine (from the tumor stroma) and aurocrine cytokines and growth factors elicit and modulate the EMT program[12]. Even though TGFβ is the most important inducer of EMT, growth factor receptor tyrosine kinase (RTK) signaling induced by hepatocyte growth factor (HGF), epidermal growth factor and platelet-derived growth factor can also activate the EMT program in carcinomas[12,13].

The HGF receptor c-MET is overexpressed in many human cancers including HCC[14]. MET not only promotes neoplastic growth of HCC cells but also facilitates tumor metastasis by promoting EMT[14-16]. Recent studies have implicated the microRNAs miR-148a and miR-449a in regulating EMT in HCC cells by targeting the MET receptor[17,18]. Previously, we have shown that suppressor of cytokine signaling 1 (SOCS1) is an important regulator of HGF signaling hepatocytes. SOCS1 deficiency in primary mouse hepatocytes increases MET signaling and cell proliferation, whereas stable expression of SOCS1 in human HCC cells attenuates HGF-induced cell growth[19,20]. We have also shown that SOCS1 binds to the MET receptor and promotes its ubiquitination and proteasomal degradation[20]. The *SOCS1* gene is frequently repressed in HCC, and *Socs1*-deficient mice show high susceptibility to experimental HCC with larger and more numerous tumor nodules[21-23], suggesting a role for SOCS1 in controlling MET-mediated tumor cell invasion.

In this study, we examined the role of SOCS1 in MET-mediated migration and invasion of HCC cells and their growth following delivery to the liver. Our findings show that SOCS1 inhibits the invasive potential of HCC cells and reduces orthotopic tumor growth that could result at least partly from modulating the expression of proteins implicated in EMT.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

HCC cell lines of mouse (Hepa1-6; Hepa) and human origin (Hep3B, HepG2) were obtained from ATCC

and grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS, from Sigma). Cells stably expressing full-length HA-SOCS1 through lentiviral transduction have been previously described[20]. Recombinant mouse and human HGF were from Peprotech (Rocky Hill, NJ). Phospho-ERK (T202/Y204; #9106), phosphor-STAT3 (Y705; #9131), E-cadherin (24E10), ZO-1 (D7D12), N-cadherin (D4R1H), vimentin (D21H3), EGR1 (44D5), SNAI1 (C15D3) and ZEB1 (D80D3) antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies (Ab) against total ERK (sc-93, sc-153), STAT3 (sc-483) and MET (sc-161) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb against β-actin (A4700) was from Sigma Aldrich (Oakville, ON, Canada). Secondary antibodies were from Jackson Immuno-research Laboratories Inc. (Cedarlane, Burlington, ON, United States). Calcein was from Calbiochem (San Diego, CA, United States).

Orthotopic tumor growth

Male NOD.*scid.gamma* (NSG) mice (8-12 wk old) purchased from the Jackson Labs (Bar Harbor, ME, United States) were used to evaluate tumor growth *in vivo* under protocols approved by the Université de Sherbrooke ethical committee on animal care and use. To evaluate the growth of hepatoma cells in the liver, cells were injected *via* intravenous or intrasplenic/portal route. For intravenous inoculation, 10^6 Hepa-vector or Hepa-SOCS1 cells in 100 μL volume were injected *via* the caudal vein. For intrasplenic inoculation, mice were anesthetized with ketamine (10 mg/kg) and the spleen was exposed through a small abdominal incision[24]. Tumor cells (10^6 cells in 100 μL) were injected into the spleen and mice were splenectomized 2 min later. Tumor nodules in the liver were examined 20 d later when the animals began to show distress. The images of hematoxylin and eosin (H and E) -stained sections of the liver were acquired using Nanozoomer Slide Scanner and analyzed by the Nanozoomer Digital Pathology (NDP) software (Hamamatsu Photonics, Japan).

Soft agar colony formation

After adding the bottom layer of 0.6% agar in 2 × DMEM containing 20% FBS to 100 mm Petri dishes, 5000 HCC cells suspended in 0.3% agar in 2 × DMEM containing 10% were added FBS as the top layer. Cells were fed every 3-5 d by overlying fresh 500 μL of DMEM containing 10% FBS. After 3 wk culture, colonies were stained with 1 mg/mL methylthiazol tetrazolium for 3 h, photographed and counted using the NIH ImageJ software.

Transwell migration assay

Migration of cells was assessed using transwells (Corning) with 8 μmol/L pore polycarbonated inserts, coated with growth factor-reduced matrigel (BD Pharmingen Biosciences, Palo Alto, CA, United States). The upper chamber contained 2 × 10^4 cells and the lower chamber contained 0.7 mL of complete medium with or without HGF. Migration through the membrane was determined after 24 h of incubation at 37 ℃. Cells remaining on the top side of the transwell membrane were removed using a cotton swab, and cells migrated to bottom were stained with 0.5% crystal violet and examined by microscopy. Cells in five random fields of the lower side of the membrane were counted. Data represent means ± SEM of three independent experiments.

3-dimensional invasion assay

The wells of a 96-well microtiter plate was covered with 50 μL of 1% agarose containing 10% FBS as the bottom layer and overlaid with 50 μL of fibrillar type-Ⅰ collagen (3 mg/mL) (R and D Systems, Minneapolis, MN, United States). Control and SOCS1-expressing HCC cells were serum starved overnight and layered on the top (20000 cells/well in 100 μL volume) in the presence or absence of HGF. After 48 h incubation, the cells were labeled with CellTrace™ Calcein Green, AM (Invitrogen, CA, United States) for 1 h, washed with PBS, fixed with 3% glutaraldehyde and confocal images were acquired using the FV1000 Olympus confocal microscope. The collagen matrix was scanned along the Z-axis at incremental 5 μm depths in order to reconstruct 3-D images. The images were analyzed to assess the depth of cell migration, which is expressed as ratio of the fluorescence intensity at each 5 μm layer over the fluorescence intensity of the non-invaded cells at the top 5 μm layer.

Western blot

Lysates of liver tissues harboring the tumor nodules were prepared in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0 and protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN) for 2 min using the bead mill MM 400 (Retsch, Hann, Germany). After adding equal volume of the same buffer containing 0.2% SDS, 1% Triton X-100 and 1% sodium deoxycholate, the lysates were kept on a shaker at 4 ℃ for 30 min. Following centrifugation at 15000 *g* for 20 min, the supernatant was collected and protein concentration determined using the RC-DC Protein Assay Kit (Bio-Rad, Mississauga, ON, United States). Cultured cells were lysed directly in 1 × SDS-PAGE sample buffer. Aliquots of 30-50 μg proteins were analyzed by western blot. Secondary antibodies and enhanced chemiluminescence reagents were from GE Healthcare Life Sciences (Pittsburg, PA, United States). Western blot images were captured by the VersaDOC 5000 imaging system (Bio-Rad). Densitometric quantification of the western blot bands was carried out using the NIH ImageJ 1.62 software.

Real-time RT-PCR

For RT-PCR analysis, total RNA was isolated from 1 × 10^6 cells, either unstimulated or stimulated with HGF for the indicated periods of time, using RiboZol™ (AMRESCO,

Solon, OH, United States). After verifying the RNA quality by UV absorption, the first complementary strand was made from 1 μg total RNA using QuantiTect® reverse transcription kit (Qiagen). The primers for gene expression analysis are as follows: Mouse *Egr1* (NM_007913): AGCGCCTTCAATCCTCAAG and TTTGGC TGGGATAACTCGTC; *Snai1* (NM_011427): GTGAAGAGA TACCAGTGCCAG and AAGATGCCAGCGAGGATG; Human *EGR1* (NM_001964): TGTCACCAACTCCTTCAGC and TCCTGTCCTTTAAGTCTCTTGTG; SNAI1: TCTAGGCCCTG GCTGCTACAA and ACATCTGAGTGGGTCTGGAGGTG; housekeeping genes: mouse *Gapdh* (NM_008084.3): ATGACATCAAGAAGGTGGTGAA and GTCTTACTCCTTGG AGGCCATGT; Human *GAPDH* (NM_002046.5): GATGA CATCAAGAAGGTGGTGAA and GTCTTACTCCTTGGAGG CCATGT. All primers showed more than 90% efficiency with a single melting curve in the MyQi5® cycler (Bio-Rad, Mississauga, ON, United States). Expression levels of the housekeeping gene were used to calculate fold induction of the specific genes modulated by the presence or absence of SOCS1.

Statistical analysis

Statistical significance of the differences in cell proliferation, tumor growth and protein expression was evaluated by student's *t*-test.

RESULTS

Previously we have shown that SOCS1 inhibited HGF-induced MET signaling, cell proliferation and migration in mouse (Hepa) and human (Hep3B, HepG2, SNU-423) HCC cell lines[19,20]. Here, we investigated whether SOCS1 also inhibits HGF-induced invasion of the extracellular matrix in some of these cell lines. At the concentration previously found to be effective in inducing cell proliferation and migration[19], HGF induced cell scattering in murine Hepa cells and human Hep3B cells, which was attenuated by SOCS1 (Figure 1A). In a trans-well migration assay, Hepa cells expressing the control vector showed marked ability to invade the matrigel in response to HGF, whereas the SOCS1-expressing cells showed significantly reduced ability (Figure 1B). Similar reduction of HGF-induced matrix invasion by SOCS1 was also observed with the human HCC cell line Hep3B (Figure 1C).

Next, we used a 3-D matrix invasion assay using collagen as matrix, where the depth of invasion was evaluated by confocal microscopy and quantified (Figure 2A and B). HGF stimulation increased the ability of Hepa, Hep3B and HepG2 cells to invade the collagen matrix, and this invasive potential was markedly pronounced in Hep3B and HepG2 cells, as indicated by the maximum depth of invasion (Figure 2A and C). In all the three cell lines, SOCS1 reduced the HGF-induced invasive potential (Figure 2A and C). Even though the SOCS1-mediated inhibition of HGF-induced invasion was only marginal in Hepa cells, Hep3B-SOCS1 and HepG2-SOCS1 cells showed significant reduction in HGF-induced invasive potential (Figure 2D). These results indicated that SOCS1 is an important regulator of MET signaling that promotes cell migration and invasion.

Tumor dissemination requires not only the ability to invade but also the ability to survive and undergo anchorage-independent proliferation. Therefore, we compared the ability of control and SOCS1-expressing HCC cells to form colonies in semi-solid agar. As shown in Figure 3A, Hepa-SOCS1 cells form fewer and smaller colonies than Hepa-V cells. Similar reduction of colony forming ability was also observed in Hep3B-SOCS1 and HepG2-SOCS1 cells compared to controls (Figure 3B).

To evaluate whether SOCS1 attenuates invasive tumor growth within the liver, we delivered control and SOCS1-expressing HCC cells *via* parenteral inoculation to the liver. For this purpose, we used murine Hepa1-6 cells, which can utilize endogenous HGF and other mouse growth factors better than human hepatoma cells[25]. Following intravenous injection, the control cells formed numerous orthotopic tumor nodules that obliterated the liver, whereas Hepa-SOCS1 cells induced fewer nodules (Figure 4A). The lungs of these mice did not show macroscopically visible tumor, suggesting that Hepa cells preferentially colonized the liver. We also delivered the HCC cells to the liver *via* intrasplenic route through the portal circulation. In the latter setting, Hepa-vector cells obliterated the liver with numerous macroscopically visible nodules, whereas Hepa-SOCS1 cells formed markedly smaller nodules (Figure 4B and C). Orthotopic tumors formed by Hepa-SOCS1 showed markedly diminished phosphorylation of STAT3 and ERK (Figure 4D), presumably through SOCS1-mediated inhibition of HGF and other cytokine and growth factor signaling[19,26,27]. Moreover, the SOCS1 expressing tumors showed significantly reduced levels of MET expression (Figure 4E), in agreement with our previous report that SOCS1 targets the activated MET receptor to the proteasomal degradation machinery[20]. These results indicated that SOCS1 inhibits the invasive growth potential of HCC cells and that at least part of this invasive growth could result from attenuation of HGF-induced MET signaling.

To understand the mechanisms underlying SOCS1-mediated attenuation of the invasive growth of HCC cells, we evaluated the expression of proteins implicated in EMT that promote tumor cell spreading following HGF stimulation[28-30]. Even though HGF stimulation did not appreciably affect the expression of E-Cadherin in control or SOCS1-expressing Hepa and Hep3B cells, the SOCS1-expressing cells showed increased basal expression of E-Cadherin (Figure 5A). SOCS1-expresing Hepa cells but not Hep3B cells showed discernible increase in ZO-1 expression, whereas the expression of N-Cadherin was not affected by SOCS1 in both cell

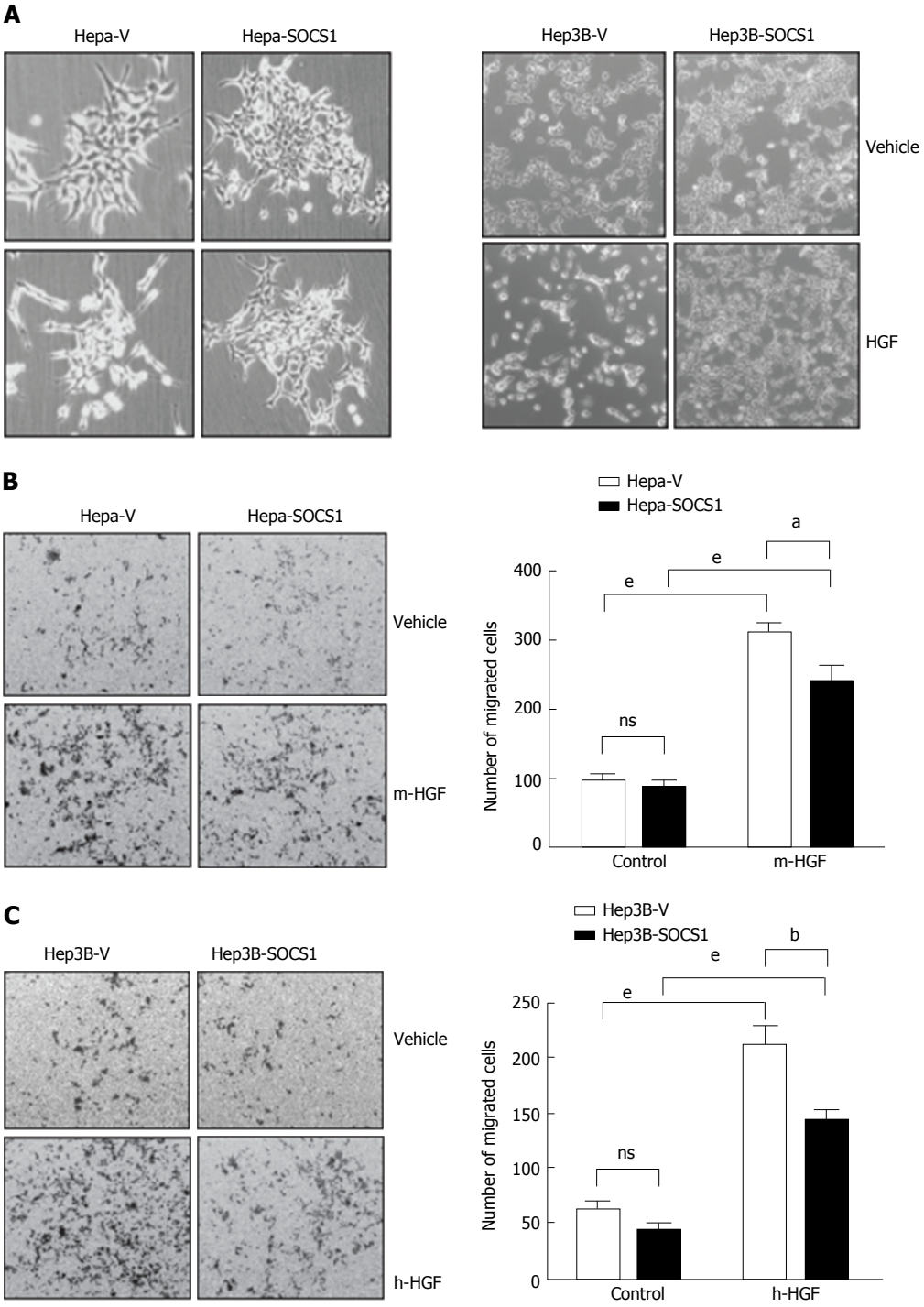


Figure 1 Suppressor of cytokine signaling 1 attenuates hepatocyte growth factor-induced scattering and migration of hepatocellular carcinoma cells. A: Hepa and Hep3B cells expressing SOCS1 (Hepa-SOCS1, Hep3B-SOCS1) or control vector (Hepa-V, Hep3B-V) were grown to less than 50% confluent state, starved in serum-free medium for 16 h and exposed to murine or human HGF (HGF, 25 ng/mL), respectively. After 48 h, the culture plates were examined by phase contrast microscopy to visualize HGF-induced cell scattering; B: Hepa-SOCS1 and Hepa-V cells were seeded onto Matrigel-coated trans-well migration chambers, and migration towards m-HGF was evaluated after 48 h. Representative images of cells that had migrated across the membrane and the number of migrated cells in five random fields from three separate experiments (mean ± SE) are shown; C: Migration of Hep3B cells expressing SOCS1 (Hep3B-SOCS1) or control vector (Hepa-V) towards human HGF (h-HGF, 10 ng/mL). $^{a}P < 0.05$, $^{b}P < 0.01$,$^{e}P < 0.001$. SOCS1: Suppressor of cytokine signaling 1.

lines. Analysis of gene expression showed marked reduction in the induction *Egr1* and *Snai1* genes in Hepa-SOCS1 cells, and *EGR1* and *SNAI2* genes in Hep3B-SOCS1 cells (Figure 5B). Western blot analysis showed reduced expression of EGR1, SNAI1 and ZEB1 in Hepa-SOCS1 cells compared to Hepa-V cells, whereas only SNAI1 showed appreciable reduction in Hep3B cells (Figure 5A). These findings suggest that SOCS1 may modulate diverse components of the EMT signaling machinery during MET-mediated invasion of

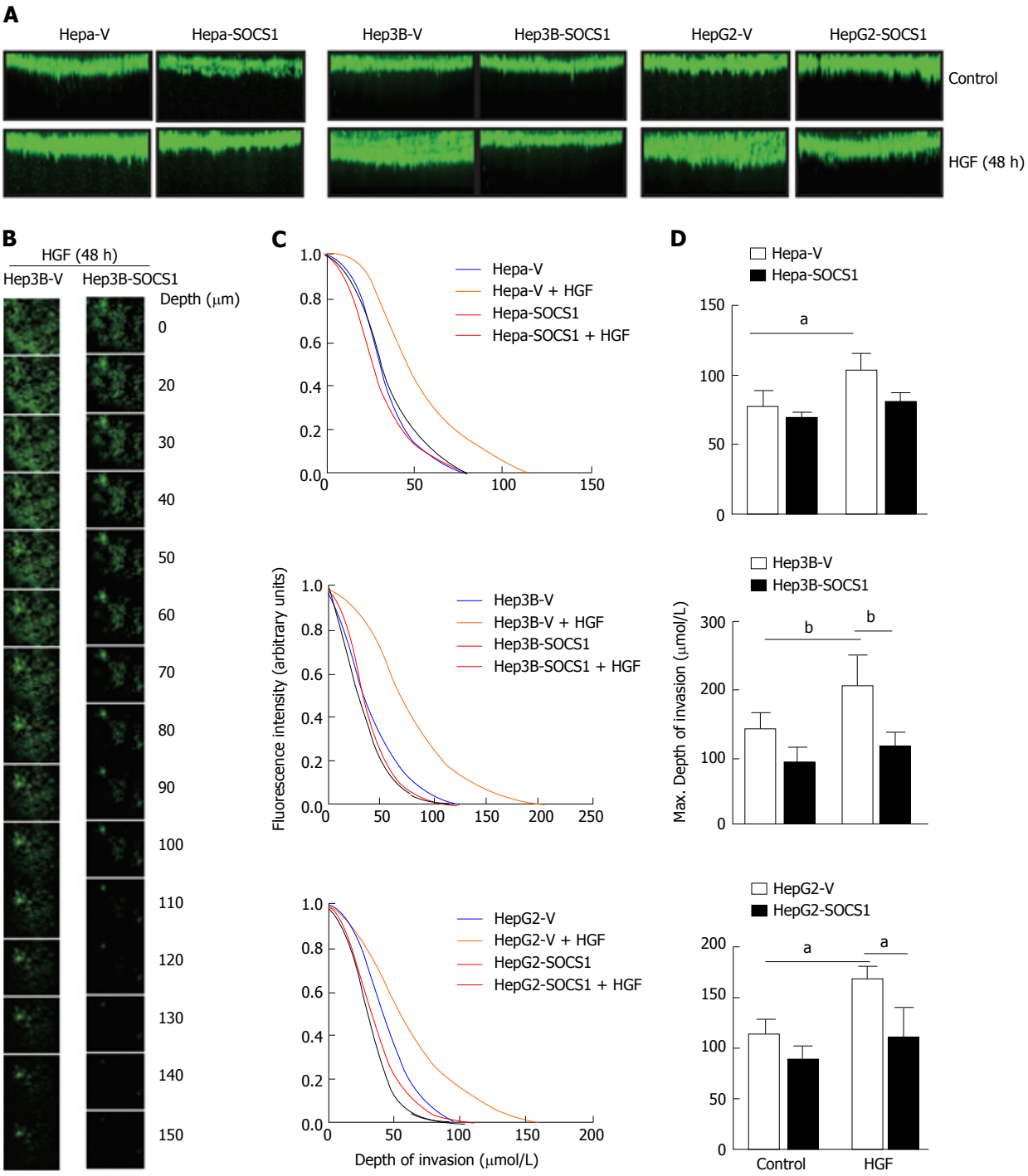


Figure 2 Inhibition of hepatocyte growth factor-induced invasion of collagen matrix by suppressor of cytokine signaling 1 in hepatocellular carcinoma cells. Hepa-V and Hepa-SOCS1, Hep3B-V and Hep3B-SOCS1, and HepG2-V and HepG2-SOCS1 cell lines were evaluated for their ability to invade collagen matrix in a 3-D invasion assay in the presence or absence of mouse HGF (25 ng/mL for Hepa cells) or human HGF (30 ng/mL for Hep3B, HepG2 cells). After 48 h, cells were fluorescently labeled with calcein Green, fixed and examined by confocal microscopy. A: The reconstructed cross-sectional images of cell migration, representative of two independent experiments, are shown; B: Representative images of Hep3B-V and Hep3B-SOCS1 cells migrated to different planes of the collagen-agarose matrix along the z-axis; C: Efficiency of migration (ratio of the fluorescence intensity at each 5 μm layer over the fluorescence intensity of the non-invaded cells at the top 5 μm); D: Maximum depth of invasion by control and SOCS1-expressing cells in the absence or presence of HGF. $^{a}P < 0.05$, $^{b}P < 0.01$. SOCS1: Suppressor of cytokine signaling 1.

HCC cells.

DISCUSSION

The *SOCS1* gene is frequently repressed in HCC by promoter CpG methylation[21,31,32]. Recent meta-analyses of published works on DNA methylation status of several HCC-associated genes have shown that *SOCS1* gene promoter hypermethylation is an important risk factor and predictive biomarker for HCC[33,34]. Consistent with the role of SOCS1 as a putative tumor suppressor in HCC, others and we

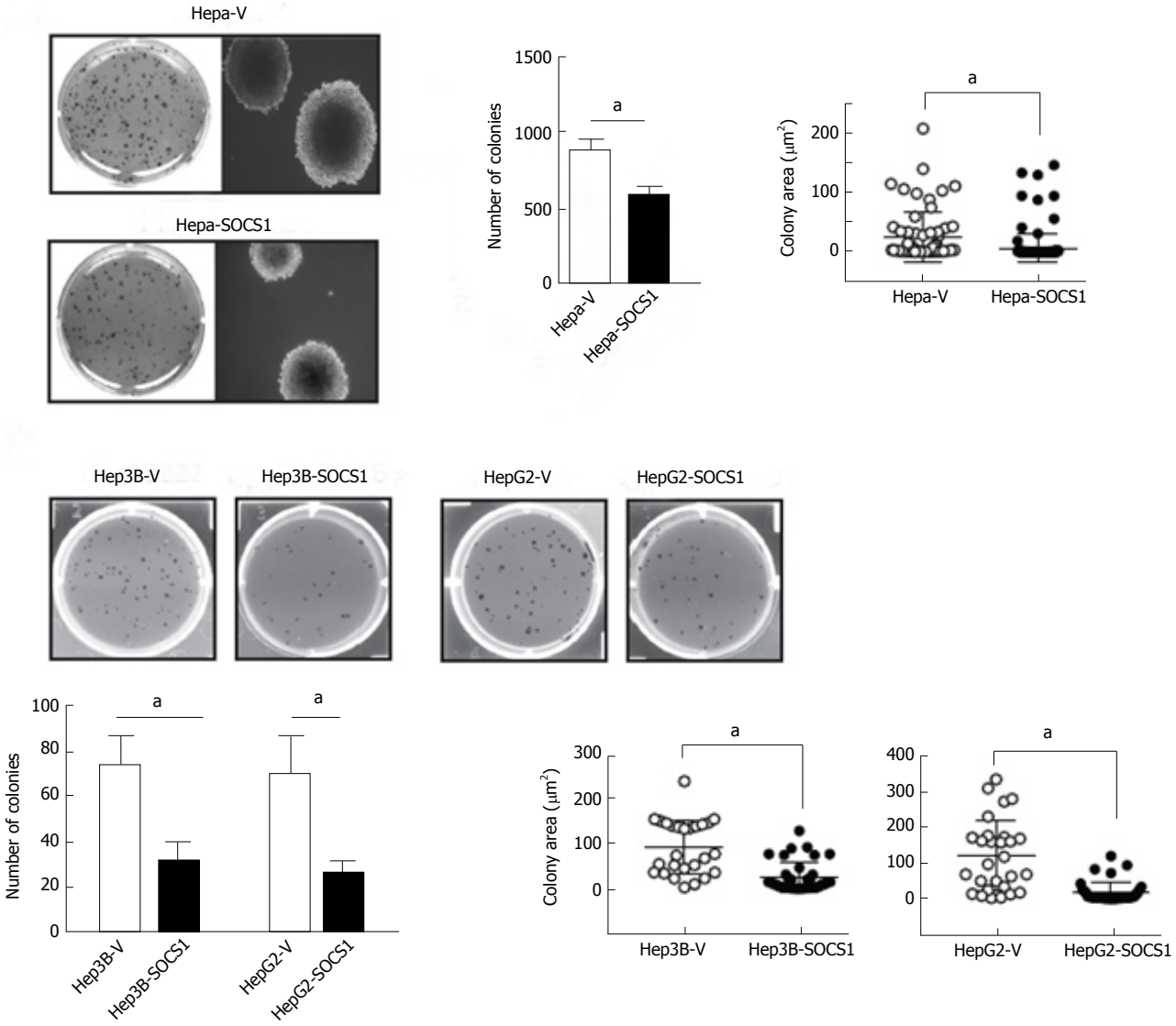


Figure 3 Reduced anchorage-independent proliferation of hepatocellular carcinoma cells expressing suppressor of cytokine signaling 1. SOCS1-expressing and control mouse Hepa (A) and human Hep3B and HepG2 (B) HCC cell lines were evaluated for their ability to form colonies in soft agar. After 3 wk culture, the colonies were photographed, and the number and size of colonies (area; mean ± SE) were evaluated from triplicates of two separate experiments. [a]$P < 0.05$. SOCS1: Suppressor of cytokine signaling 1.

have shown that loss of *Socs1* gene expression in mice increases susceptibility to HCC induction[22,23]. The loss of SOCS1 expression might contribute to HCC progression *via* multiple mechanisms. We have shown that SOCS1 is an important regulator of oncogenic MET RTK signaling and suppresses the oncogenic potential of P21CIP in hepatocytes[19,20,23]. These reports suggested that the loss of SOCS1 expression in HCC by epigenetic mechanisms could promote cancer cell survival, migration and proliferation. The findings of the present study indicate that the loss of SOCS1-dependent regulation of HGF/MET signaling may also contribute to invasive growth of HCC cells through, at least partly, *via* promoting EMT.

Aberrant MET signaling that facilitate tumor progression and metastasis occurs in many cancers mainly *via* receptor overexpression, gene amplification or through the loss of MET-targeting microRNAs, although activating mutations and increased autocrine HGF stimulation can also amplify MET signaling[14]. While gain-of-function mutations and gene amplification of MET are rare in HCC, MET overexpression has been frequently reported[14]. The elevated MET expression would not only lead to ligand-independent activation but also confer increased responsiveness to des-gamma-carboxy prothrombin, which is highly expressed in HCC[35]. The molecular basis of MET overexpression in HCC has not been elucidated yet[14]. The high frequency of SOCS1 promoter methylation in HCC[33,34] and the ability of SOCS1 to promote MET degradation by proteasomes[20] suggest that MET overexpression in HCC could result at least partly from the loss of SOCS1 mediated control of MET expression.

The MET signaling pathway is an important mediator of EMT in HCC progression *via* intra- and extra- hepatic metastases[36,37]. EMT is mediated *via* induction of transcription factors (*SNAI1, SNAI2, ZEB1, ZEB2, TWIST1*), which repress genes coding for proteins

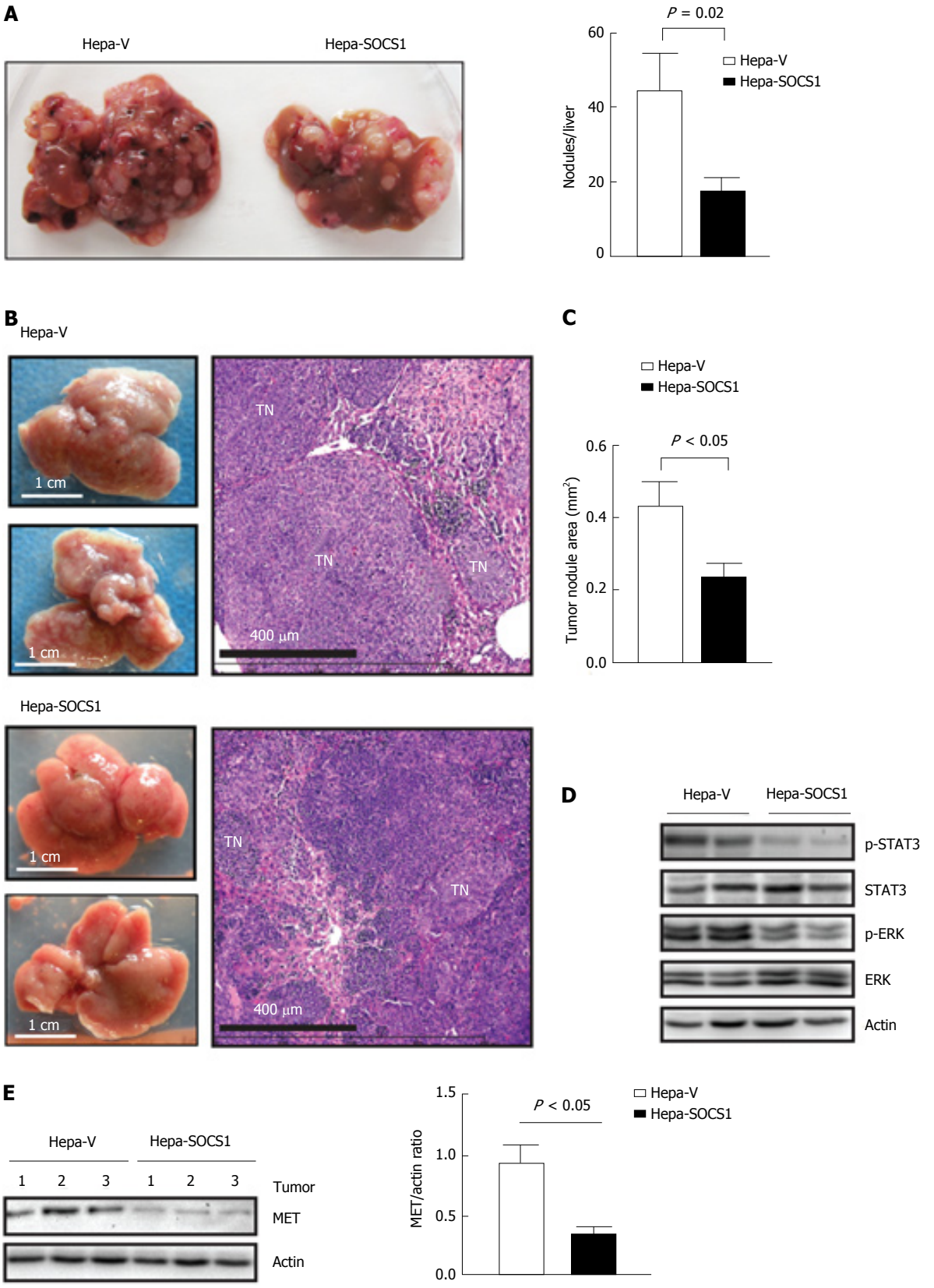


Figure 4 Suppressor of cytokine signaling 1 attenuates invasive growth of orthotopic Hepa cell tumors. A: Hepa-V and Hepa-SOCS1 cells were injected *via* intravenous route into NOD.*scid.gamma* (NSG) mice and the livers were examined macroscopically after 20 d. Representative images (left) and quantification of the liver tumor nodules (right) from four mice per group are shown; B-D: Hepa-vector and Hepa-SOCS1 were injected *via* intrasplenic route into NSG mice (1×10^6 cells per mouse; $n = 6$ per group from two separate experiments) and the mice were splenectomized. After 20 d, the liver tissues were examined macroscopically for tumor nodules. The dorsal and ventral views of representative livers (B, left) and H and E-stained sections of a representative liver for each group (B, right) are shown; C: For each group, the areas of thirty random tumor nodules from three different mice were measured digitally using the NDP software and compared by Student's paired *t* test; D: Western blot evaluation of STAT3 and ERK phosphorylation in the tumors from 2 mice per group; E: Western blot evaluation of MET expression and its quantification in the tumors from 3 mice per group. SOCS1: Suppressor of cytokine signaling 1.

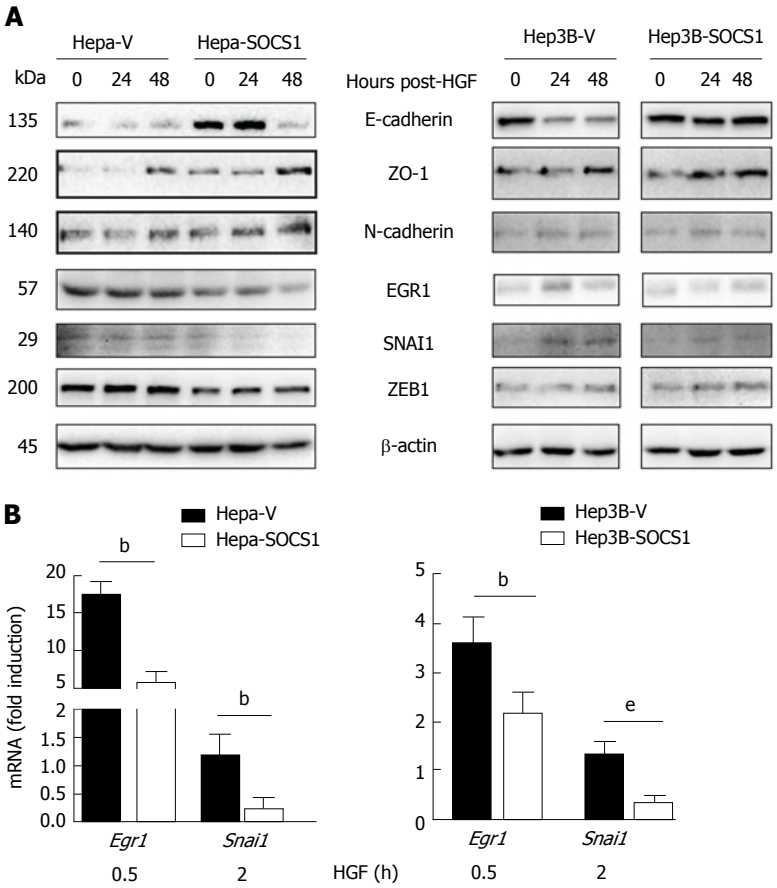


Figure 5 Effect of suppressor of cytokine signaling 1 on the expression of proteins modulated during epithelial-mesenchymal transition in hepatocyte growth factor-stimulated hepatocellular carcinoma cells. A: Hepa-V and Hepa-SOCS1, and Hep3B-V and Hep3B-SOCS1 were cultured in low-serum medium (0.25%) overnight and stimulated or not with m-HGF or h-HGF for 24 or 48 h. Unstimulated and HGF-stimulated cells were lysed in SDS sample buffer and analyzed by western blot for the indicated proteins. Representative data from two experiments are shown; B: Cells were stimulated with HGF for 30 min or 2 h and the expression of *Egr1* and *Snai1* genes was evaluated by qRT-PCR. Fold-induction relative to the housekeeping gene *Gapdh* was evaluated from triplicates of two separate experiments (mean ± SE). $^{b}P < 0.01$, $^{e}P < 0.001$. SOCS1: Suppressor of cytokine signaling 1.

that maintain epithelial cell integrity[12,13]. During EMT, progressive loss of epithelial markers (E-cadherin, occludin, ZO-1) and acquisition of mesenchymal markers (N-cadherin, vimentin) cause tumor cells to disrupt intercellular junctions, become motile, remodel cell-matrix interactions and become invasive. However, these changes may not be absolute and intermediate phenotypes may represent partial EMT[37]. Reduced expression of E-cadherin occurs in 50%-60% of primary HCC that correlates with increased *SNAI1* expression and poor prognosis[38]. Similar changes were reported in human and murine HCC cell lines with high invasive potential[36,38]. Indeed, elevated MET expression has been associated with mesenchymal phenotype characterized by reduced expression of E-Cadherin and increased expression of the *CDH1* gene repressor *ZEB2* in human HCC cell lines MHCC97-L and MHCC97-H that display increased metastatic potential[16]. Similar findings have been reported in murine HCC cell lines[15]. A murine HCC line-derived circulating tumor cells isolated following intrahepatic implantation displayed elevated MET expression with reduced levels of E-Cadherin, implicating the EMT process in HCC dissemination[39]. Our findings that SOCS1 expression reduces HGF-induced invasive growth in HCC cells that is accompanied by increased expression of E-cadherin and discernibly reduced expression of EGR1, SNAI1 and ZEB1, as well as the markedly diminished orthotopic growth of SOCS1-expressing HCC cells associated with reduced MET expression support the idea that SOCS1 is an important regulator of MET-mediated cancer progression *via* EMT. Thus, our findings add SOCS1 to the growing list of endogenous regulators of MET-mediated EMT, namely miR-148a, miR449a, SENP1[17,30,40].

Molecular mechanisms underlying MET-mediated EMT are not yet fully elucidated. In HepG2 human HCC cells, HGF-induced cell scattering requires ERK activation, leading to induction of *EGR1* (Early growth response factor), a transcriptional activator of SNAI1, which represses *CDH1* encoding E-cadherin[28]. The HGF-induced morphological and molecular changes of EMT in HCC cells could be reversed by blocking ERK activation using sorafenib that targets RTKs, or by U0126 that inhibits the ERK upstream kinase MEK1[29]. Given that the MET signaling pathways are implicated in multiple aspects of cancer progression, attenuating MET signaling will also disrupt MET-mediated EMT[37].

Several therapeutics targeting the MET signaling pathway including multikinase inhibitors, selective MET inhibitors, antibodies targeting HGF or MET, agents that neutralize HGF and molecules that disrupt MET signaling[14]. Given the possible emergence of drug resistance to agents that target signaling pathways, and that MET can be activated by alternate ligands such as des-gamma-carboxy prothrombin and heparin[35,41], alternate strategies are needed to effectively target MET-mediated HCC invasion and metastasis. In this context, anti-MET Ab such as DN-30 that promotes MET degradation[42] and strategies to disrupt MET receptor expression[14] could represent promising approaches. However, this would require identification of HCC patients who would benefit from aggressive MET-targeting therapy[14]. Although MET overexpression could be a direct marker, clinical grade MET Ab are still under development[43]. Given that SOCS1 is an important regulator of MET protein expression, and that loss of SOCS1 expression predominantly occur by promoter methylation in HCC, assessing SOCS1 methylation status could be a useful approach to identify HCC patients who would benefit from MET-targeting therapies.

In conclusion, our findings show that SOCS1 attenuates migration and invasion properties of HCC cells at least partly *via* modulation of MET-mediated EMT, and controls invasive tumor growth. Based on these findings, we propose that the *SOCS1* gene methylation and expression in primary HCC may serve as useful markers to identify patients for targeted MET therapy.

COMMENTS

Background

Invasive intrahepatic dissemination is a key factor in malignant growth of hepatocellular carcinoma (HCC) and its poor prognosis. HCC patients also present extrahepatic metastasis that may occur *via* direct invasion. Cancer cells gain invasive potential through epithelial-mesenchymal transition (EMT) induced by cytokines and growth factors that stimulate receptor tyrosine kinases (RTK). An important RTK implicated in EMT is the HGF receptor MET. Suppressor of cytokine signaling 1 (SOCS1) is an important regulator of MET signaling in hepatocytes.

Research frontiers

Emerging data implicate EMT in cancer growth and metastasis in HCC and many other cancers. Among the strategies to target EMT, the MET signaling pathway holds promise. The present study investigates the role of SOCS1 in regulating MET signaling involved in the invasive tumor growth of HCC.

Innovations and breakthroughs

This study shows that SOCS1 inhibits the invasive growth of HCC cells *in vitro* and *in vivo*, and adds SOCS1 to the growing list of endogenous regulators of MET-mediated EMT.

Applications

SOCS1 expression in HCC biopsies could be a useful biomarker of invasive and metastatic potential to identify patients suitable for treatment with MET-targeting therapies. Development of therapeutics to restore SOCS1 expression in cancer cells could be another approach to attenuate the invasive potential and metastatic growth of HCC.

Terminology

SOCS1 is a negative feedback regulator of cytokine and growth factor signaling. SOCS1 inhibits HGF-induced MET signaling pathways that promote invasive growth, which can be assessed *in vitro* using a 3-dimensional invasion assay and *in vivo* by orthotopic tumor growth.

Peer-review

In this study, the authors investigated the role of SOCS1 in the invasion ability of HCC. Extending the findings of their previous work, the authors show that SOCS1 inhibits the invasive growth of HCC cells by targeting MET signaling.

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ORIGINAL ARTICLE

Basic Study

Protective effects of oral glutathione on fasting-induced intestinal atrophy through oxidative stress

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Abstract

AIM

To determine whether oral glutathione (GSH) administration can alleviate the effects of fasting-induced intestinal atrophy in the small intestinal mucosa.

METHODS

Rats were divided into eight groups. One group was fed *ad libitum*, another was fed *ad libitum* and received oral GSH, and six groups were administrated saline (SA) or GSH orally during fasting. Mucosal height, apoptosis, and cell proliferation in the jejunum were histologically evaluated. iNOS protein expression (by immunohistochemistry), nitrite levels (by high performance liquid chromatography, as a measure of NO production), 8-hydroxydeoxyguanosine formation (by ELISA, indicating ROS levels), glutathione/oxidized glutathione (GSH/GSSG) ratio (by enzymatic colorimetric detection), and γ-glutamyl transpeptidase (*Ggt1*) mRNA levels in the jejunum (by semi-quantitative RT-PCR) were also estimated.

RESULTS

Oral GSH administration was demonstrated to drastically reduce fasting-induced intestinal atrophy in the jejunum. In particular, jejunal mucosal height was enhanced in GSH-treated animals compared to SA-treated animals [527.2 ± 6.9 for 50 mg/kg GSH, 567.6 ± 5.4 for 500 mg/kg GSH *vs* 483.1 ± 4.9 (μm), $P < 0.01$ at 72 h]. This effect was consistent with decreasing changes in GSH-treated animals compared to SA-treated animals for iNOS protein staining [0.337 ± 0.016 for 50 mg/kg GSH, 0.317 ± 0.017 for 500 mg/kg GSH *vs* 0.430 ± 0.023 (area of staining part/area of tissue), $P < 0.01$ at 72 h] and NO [2.99 ± 0.29 for 50 mg/kg GSH, 2.88 ± 0.19 for 500 mg/kg GSH *vs* 5.34 ± 0.35 (nmol/g tissue), $P < 0.01$ at 72 h] and ROS [3.92 ± 0.46 for 50 mg/kg GSH, 4.58 ± 0.29 for 500 mg/kg GSH *vs* 6.42 ± 0.52 (8-OHdG pg/μg DNA), $P < 0.01$, $P < 0.05$ at 72 h, respectively] levels as apoptosis mediators in the jejunum. Furthermore, oral GSH administration attenuated cell proliferation decreases in the fasting jejunum [182.5 ± 1.9 for 500 mg/kg GSH *vs* 155.8 ± 3.4 (5-BrdU positive cells/10 crypts), $P < 0.01$ at 72 h]. Notably, both GSH concentration and Ggt1 mRNA expression in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone [0.45 ± 0.12 *vs* 0.97 ± 0.06 (nmol/mg tissue), $P < 0.01$; 1.01 ± 0.11 *vs* 2.79 ± 0.39 (Ggt1 mRNA/Gapdh mRNA), $P < 0.01$ for 500 mg/kg GSH at 48 h, respectively].

CONCLUSION

Oral GSH administration during fasting enhances jejunal regenerative potential to minimize intestinal mucosal atrophy by diminishing fasting-mediated ROS generation and enterocyte apoptosis and enhancing cell proliferation.

Key words: Intestinal atrophy; Glutathione; Apoptosis; Cell proliferation; Inducible nitric oxide synthase

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Core tip: We have previously demonstrated that the intestinal mucosal atrophy consequent to fasting is due in large part to increased apoptosis in jejunal villi and decreased cell proliferation in jejunal crypts, with concomitant increased reactive oxygen species (ROS) and NO production and decreased glutathione (GSH). Here we demonstrate protection against fasting-induced intestinal mucosal atrophy by minimizing ROS induction in the intestinal mucosa through supplemental oral administration of an antioxidant such as GSH during fasting in rats. In particular, oral GSH administration during fasting enhances jejunal regenerative potential by diminishing enterocyte apoptosis and enhancing cell proliferation.

Uchida H, Nakajima Y, Ohtake K, Ito J, Morita M, Kamimura A, Kobayashi J. Protective effects of oral glutathione on fasting-induced intestinal atrophy through oxidative stress. *World J Gastroenterol* 2017; 23(36): 6650-6664 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6650.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6650

INTRODUCTION

Fasting induces small intestinal mucosal atrophy including increased epithelial permeability and compromised tight junctions, which can lead to bacterial translocation, particularly in patients receiving a prolonged course of total parenteral nutrition (TPN)[1,2]. Many surgeons involved in nutritional support therapy desire the identification of bioactive substances that may be efficacious in protecting against intestinal injury during long-term TPN administration, particularly with respect to intestinal barrier function and subsequent septic complications. Fasting and other states of malnutrition are associated with increased reactive oxygen species (ROS) formation, which has been implicated in the loss of intestinal mucosal structure and function under conditions of inflammation, injury, and shock[3,4]. In addition, these fasting states are also accompanied by depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa[5-7]. Therefore, GSH is the most prevalent and important low-molecular-weight thiol in mammalian tissues[8]. As GSH deficiency in tissues is associated with increased oxidative stress, aging, and increased risk of numerous chronic diseases as well as fasting[9], the maintenance of tissue levels of GSH is critical for maintaining health and preventing disease and age-related biological insults.

GSH sources in the intestinal mucosa include intracellular synthesis, biliary supply, and dietary intake. The intestinal lumen receives a large quantity of hepatic GSH from biliary secretion[10] and dietary GSH from fresh fruits, vegetables, and many types of meat[11]. As the only enzyme of the γ-glutamyl cycle located on the outer surface of the plasma membrane, γ-glutamyl transpeptidase (GGT) plays a key role in GSH homeostasis by breaking down extracellular GSH and providing cysteine, the rate-limiting substrate for intracellular *de novo* GSH synthesis[12]. The two intracellular enzymes, γ-glutamylcysteine synthetase and GSH synthetase, catalyze intracellular GSH synthesis from glutamate, cysteine, and glycine, which are translocated into cells by amino acid transporters on the surface of the plasma membrane following the resolution of extracellular GSH by GGT. Intracellular GSH plays an important role in antioxidant defense and the regulation of pathways essential for *in vivo* homeostasis through its catalysis by glutathione S-transferase (GST) and glutathione peroxidase[9]. Studies of oral GSH supplementation in humans and laboratory animals have shown that the enhancement of intestinal mucosal GSH levels by oral GSH

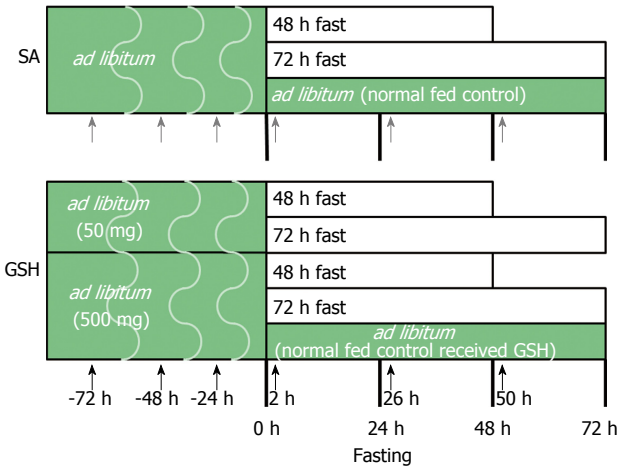


Figure 1 Experimental design. Rats were divided into eight groups, one of which was fed ad libitum, another of which was fed ad libitum and received oral glutathione (GSH), and six of which were administered saline (SA) or GSH orally prior to and after fasting. The large block arrows represent treatment with SA (closed gray arrow) and GSH (closed block arrow).

supplementation under conditions in which intracellular GSH status is compromised (*i.e.*, GSH deficiency-related biological insults) can restore tissue GSH and promote ROS metabolism[10,13,14]. Thus, it has been described that orally administered GSH acts as backup for GSH-deficient tissue.

As an alternative, the ability to control extracellular redox provides another possible role for intestinal luminal GSH. Recent studies have shown that the thiol/disulfide redox status in the extracellular compartment regulates the ROS that are generated primarily in the intracellular compartment and serve an important function in the activation of proteins and up-regulation of antioxidant and detoxification systems[15-17]. Furthermore, many proteins including transporters, receptors, and enzymes present on the surface of the plasma membrane and located in extracellular fluids contain cysteine and methionine residues that are subject to oxidation. Accordingly, these proteins respond to variations in the extracellular thiol/disulfide redox environment, which can alter their activity[18]. Notably, recent demonstrations that treatment with GSH leads to a significant increase in the number of free sulfhydryls on the cell surface have led to the conclusion that extracellular GSH modulates the interaction between bacteria and epithelial respiratory cells and inhibits bacterial invasion into these cells[19].

We previously suggested that fasting for 2-3 d causes intestinal mucosal atrophy resulting from increased apoptosis in jejunal villi, nitric oxide (NO) and ROS production following elevated inducible nitric oxide synthase (iNOS) expression, and decreased cell proliferation in jejunal crypts[20]. Conversely, these apoptosis mediators are all suppressed by treatment with aminoguanidine, a selective iNOS inhibitor, with consequent mucosal recovery, suggesting that iNOS, which induces the production of ROS, is likely to constitute a significant upstream factor promoting fasting-induced apoptosis in enterocytes. Furthermore, refeeding repaired fasting-induced jejunal atrophy by inhibiting these apoptosis mediators, including ROS, and also showed an association between ROS inhibition and increased cell proliferation for decreasing intestinal mucosal atrophy[21]. Therefore, we hypothesized that protection against intestinal mucosal atrophy might be provided through the elimination of induced ROS in the intestinal mucosa by introducing an antioxidant such as GSH during a fasting period in rats.

Therefore, the objective of the present study was to investigate the effects of oral GSH administration on the manifestation of fasting-induced intestinal atrophy through the reduction of oxidative stress, with a particular focus on the possible participation of intracellular GSH levels and GGT expression on the cell surface as parameters for *de novo* intracellular GSH synthesis.

MATERIALS AND METHODS

Animals and experimental design

The experimental protocol and design were approved by the Institutional Animal Care and Use Committee at the Life Science Center of Josai University. Male Wistar rats at 9 wk of age were purchased from SLC (Shizuoka, Japan) and housed in wire-bottomed cages. The rats were placed in a room illuminated from 7:00 am to 7:00 pm (12:12-h light:dark cycle). The animals were allowed free access to deionized water and standard rat chow (CE-2, CLEA Japan) until the study began. At 10 wk of age, 59 rats were randomly divided into eight groups (Figure 1). Each group received saline (SA) or GSH (50 or 500 mg/kg b.w./d) using oral gavage needles at 24, 48, and 72 h before fasting, including a normally fed control group and a normally fed control that received GSH, with the following fasting durations in each group: (1) SA + *ad libitum* (normal fed control); (2) SA + 48-h fast; (3) SA + 72-h fast; (4) 500 mg/kg GSH + *ad libitum* (normal fed control received GSH); (5) 50 mg/kg GSH + 48-h fast; (6) 50 mg/kg GSH + 72-h fast; (7) 500 mg/kg GSH + 48-h fast; and (8) 500 mg/kg GSH + 72-h fast. All rats received SA or GSH by oral gavage for 2, 26, and 50 h after fasting. The GSH (Setria® reduced glutathione) used in this work was provided by Kyowa Hakko Bio Co., Ltd. The rats were weighed daily.

Collection of intestinal mucosa

After fasting, the rats were anesthetized and then euthanized by exsanguination. The entire small intestine was carefully removed and placed on ice. Then, 10 cm of tissue from the oral (duodenum) side was removed and the remainder of the intestine was divided into two segments: proximal (jejunum) and distal (ileum). The segments used in analyses comprised the jejunum from 3 to 5 cm distal to the duodenum[22]. Samples with a

length of approximately 3 cm were fixed in 10% neutral buffered formalin for measurement of mucosal height and immunohistochemistry. The remaining segments were snap-frozen in liquid nitrogen and stored at -80 ℃.

Histopathological analysis of mucosal height and apoptotic index

Fixed tissue samples were embedded in paraffin and sectioned (2-3 μm thickness) prior to being stained with hematoxylin and eosin (H&E). Mucosal height (villous height plus crypt depth) was measured using a microscope (BX41; Olympus, Tokyo, Japan) and a digital camera system (Penguin 150CL; Pixera, San Jose, CA, United States). Mucosal height was measured for at least 30 villi per animal.

To detect enterocyte apoptosis in the jejunum, apoptotic index (AI) using conventional light microscopy of H&E-stained specimens was used. We followed previously published methods[20,23]. In brief, jejunal sections as used above for histopathological analysis were examined in a blinded manner for the typical attributes of apoptotic cells. We assessed 50 villus-crypt columns per rat. For each column, the number and position of apoptotic cells and the total number of cells were recorded. To account for the effects of fasting and GSH treatment on apoptosis, the average number of apoptotic cells in villi and crypts was determined along with AI. To identify the locations of apoptosis along the villi and crypts, AI distribution curves were constructed on the basis of group means, in which cell position *vs* AI was plotted at each position. Here, AI was defined as the total number of apoptotic cells at each cell position and is expressed as the percentage of the total number of cells counted at that cell position. Cell position 1 was set as the cell at the crypt-villus junction and the cell at the base of the crypt column for villus and crypt data, respectively.

Immunohistochemical assessment of iNOS expression and 5-bromo-2'-deoxyuridine (5-BrdU)-positive cells

Immunohistochemical staining was performed using a rabbit anti-iNOS polyclonal antibody[24]. The specimens were dewaxed and treated for antigen retrieval by boiling in 10 mmol/L citrate buffer (pH 6.0)[25]. After being washed with PBS, the specimens were incubated in 6% hydrogen peroxide and nonspecific binding was blocked with a 20% goat serum solution in PBS. Specimens were subsequently incubated with an anti-iNOS primary antibody (1:100; BD Transduction Laboratories, Lexington, KY, United States), except for the control sections, for which no primary antibody was used. A biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, United States) was used as a secondary antibody. The sections were then treated using the VECTASTAIN Elite ABC Kit (Vector Laboratories), and reaction products were detected using color development with diaminobenzidine. Finally, the sections were counterstained with hematoxylin and examined under a light microscope and a digital camera system. For each rat, 5 clearly dyed sections were chosen randomly and 5 random fields for each section were assessed (at 40 × magnification). The content of iNOS was quantitatively measured based on the average optical density using a digital camera and ImageJ software (National Institutes of Health, Bethesda, Maryland, United States)[26].

To assess cell proliferation in the crypt, the cell proliferation index was determined using conventional light microscopy of specimens immunohistochemically stained for 5-BrdU[27]. Rats were intraperitoneally injected with 100 mg/kg 5-BrdU prior to euthanasia. After paraffin embedding and sectioning, tissue sections were dewaxed and immersed in 3% hydrogen peroxide-methanol solution. The specimens were washed with PBS and denatured in 2 N hydrochloric acid. Following further PBS washing, the specimens were immersed in 0.1 mol/L boric acid buffer (pH 8.5), incubated with 20 μg/mL proteinase K at 37 ℃, and then the reaction was terminated with PBS containing bovine serum albumin. The sections were incubated with a mouse anti-5-BrdU monoclonal antibody (1:50; Chemicon International, Billerica, MA, United States), with the exception of control samples, for which the primary antibody was omitted. A biotinylated goat anti-mouse IgG (1:200; Vector Laboratories) was used as a secondary antibody. The sections were then treated using the VECTASTAIN Elite ABC Kit (Vector Laboratories) and antibody binding was detected by color development after addition of diaminobenzidine. Finally, the sections were counterstained with hematoxylin and examined under a light microscope with a digital camera system. The number of labeled cells in at least 10 well-oriented longitudinal crypts was determined for each rat. Results are expressed as the number of 5-BrdU-labeled cells per crypt.

Jejunal nitrite concentrations

Nitrite concentrations in the jejunum were measured using a dedicated high-performance liquid chromatography (HPLC) system (ENO-20; EiCom, Kyoto, Japan)[28]. Jejunal segments were homogenized with an equal volume of methanol and centrifuged for deproteinization at 12000 *g* at 4 ℃ for 5 min. The samples were then applied to the HPLC system. The nitrites and nitrates were separated using a reverse-phase column (NO-PAK; EiCom), after which nitrate was reduced to nitrite in a reduction column packed with copperized cadmium (NO-RED; EiCom). These nitrites were then mixed with the Griess reagent in a reaction coil and the change in absorbance was monitored at 540 nm.

DNA oxidation analysis

Oxidative stress in the jejunum was evaluated by quantifying 8-hydroxydeoxyguanosine (8-OHdG) present in DNA[29]. 8-OHdG is a product of oxidative

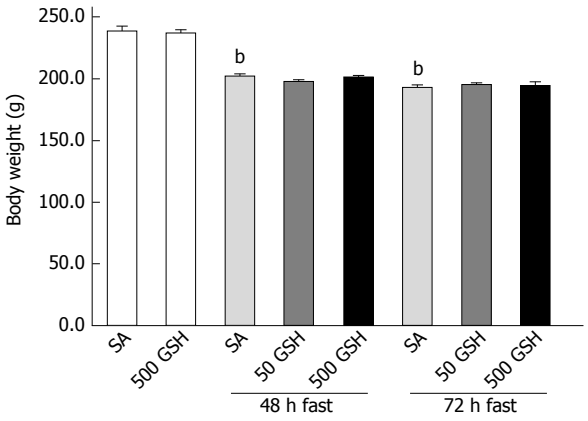


Figure 2 Effects of fasting and glutathione treatment on body weight. Fasting caused gradual decreases in body weight in both SA- and GSH-treated groups. There was no difference in weight loss between the GSH treatments and the respective SA-treated groups in each fasting period. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base and is thought to constitute a marker of oxidative DNA damage, reflecting the DNA repair rate[30]. Jejunal DNA was purified using the DNA Extractor TIS Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan)[31]. DNA samples were hydrolyzed to nucleosides by sequential incubation with 6 U of nuclease P1 (Wako) followed by 2 U of alkaline phosphatase (Wako). Hydrolysates were filtered through a VIVASPIN 500 MWCO 10000 filter (Sartorius Stedim Biotech, Gottingen, Germany). The levels of 8-OHdG in the filtered samples were determined using an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan).

Jejunal GSH and GSSG concentrations

GSH and GSSG concentrations were measured by using a GSSG/GSH Quantification Kit (Dojindo Molecular Technologies, Inc., Rockville, MD, United States). Jejunal segments were homogenized with 5% sodium sulfosalicylate (Wako) and centrifuged to remove proteins. For each sample, the supernatant was added to a well to which coenzyme working solution and enzyme working solution had been previously added, and incubated at room temperature. Then, the substrate working solution was added to the well and it was incubated at room temperature. The absorbance of samples and the GSH standard was measured at 405 nm using a microplate reader (TECAN GENios, Grodig, Austria). For measurement of GSSG, the supernatant was treated with masking solution and then added to a well as described above. The absorbance of samples and standard GSSG was measured at 405 nm.

Analysis of Ggt1 mRNA expression by semi-quantitative reverse transcription-PCR

Total RNA was purified using the TaKaRa RNAiso Reagent (TaKaRa Bio, Kusatsu, Japan). Reverse transcription (RT)-PCR was performed with total RNA using the RNA PCR kit (AMV) Version 3.0 (TaKaRa Bio): 1 cycle at 42 ℃ for 30 min, 99 ℃ for 5 min, and 5 ℃ for 5 min for reverse transcription, and 30 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min for PCR. The following primer pairs (synthesized by TaKaRa Bio) were used: *Ggt1* forward primer, 5'-ACCACTCACCCAACCGCCTAC-3'; *Ggt1* reverse, 5'-ATCCGAACCTTGCCGTCCTT-3' (product size: 317 bp)[12]. Expression of target mRNAs was measured relative to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), which was determined using the following primers: *Gapdh* forward, 5'-GGCACAGTCAAGGCTGAGAATG-3'; *Gapdh* reverse, 5'-ATGGTGGTGAAGACGCCAGTA-3' (143 bp, TaKaRa Bio ID: RA015380). A portion of each PCR mixture was electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer and DNA bands were visualized using ethidium bromide staining. PCR product intensity was measured using the Gene Genius Bioimaging System (Syngene, Cambridge, United Kingdom).

Statistical analysis

Statistical analyses were performed using SPSS ver. 22 for Windows. All values are expressed as the mean ± SE. One-way analysis of variance (ANOVA) followed by a Bonferroni multiple-comparison test was used for analyzing the statistical difference between the fasting periods in SA- or GSH-treated groups. Two-way ANOVA followed by a Bonferroni multiple-comparison test was used for analyzing the statistical difference between the SA- and GSH-treated groups for the fasting periods. Statistical significance was accepted at $P < 0.05$. The statistical analysis of this study was performed by Uchida H, who acquired biostatistics expertise during his training in public health.

RESULTS

Body weight changes

Figure 2 shows body weight changes in SA-treated and GSH-treated rats. Decreases in body weight in both groups were observed along with fasting. Rats fasted for 48 and 72 h with SA treatment showed approximately 15% ($P < 0.01$) and 19% ($P < 0.01$) body weight loss, respectively, compared with normally fed controls. There was no difference in weight loss between the GSH treatments and the respective SA-treated groups at each fasting period.

Histological characterization of jejunal mucosal atrophy

Jejunal mucosal atrophy was assessed by jejunal mucosal height. Decreased jejunal mucosal height in both SA- and GSH-treated rats was observed along with fasting (Figure 3). Rats fasted for 48 and 72 h with SA treatment showed a significant decrease ($P < 0.01$) in mucosal height compared with normally fed controls. However, significant differences ($P < 0.01$) in jejunal mucosal height were found between the SA-

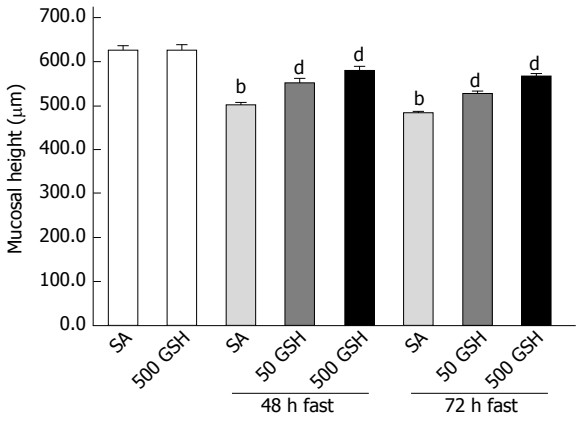


Figure 3 Effects of fasting and glutathione treatment on jejunal mucosal atrophy. Jejunal mucosal atrophy was assessed by jejunal mucosal height. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

and GSH-treated rats with 48 and 72 h of fasting.

iNOS protein expression in the jejunum

As shown in Figure 4A, iNOS protein staining was localized almost exclusively in the mucosal epithelial monolayer in the 48- and 72-h fasting periods compared with the normally fed controls, and a decreased level of staining occurred with GSH treatment. Quantitative measurement using the average optical density revealed that this expression significantly increased following fasting for 48 and 72 h with SA treatment ($P < 0.01$), whereas GSH treatments significantly decreased the fasting-induced enhancement of jejunal iNOS protein expression ($P < 0.05$ *vs* SA at 48 h fasting, $P < 0.01$ *vs* SA at 72 h fasting; Figure 4B).

Nitrite level in the jejunum

We next measured the levels of nitrite, which is a stable oxidation product of endogenous NO that is induced by iNOS. In SA-treated groups, fasting significantly increased the jejunal nitrite concentration with 48 and 72 h of fasting compared with the normally fed controls ($P < 0.01$; Figure 5). Conversely, GSH treatments significantly decreased the fasting-induced enhancement of the jejunal nitrite concentration compared with the respective SA-treated group for each fasting period ($P < 0.01$).

8-OHdG level in the jejunum

As we previously determined that fasting causes jejunal apoptosis *via* ROS production and induction of NO following increased iNOS expression, in the present study we measured levels of iNOS expression, nitrite (indicating NO production), and 8-OHdG (as a marker of ROS presence). Consistent with the abovementioned changes in iNOS expression, fasting increased intestinal nitrite levels, whereas the degree of increase was significantly reduced by GSH treatment. Furthermore, the elevated jejunal 8-OHdG levels observed after fasting were significantly diminished by both 48 h and 72 h of GSH treatment ($P < 0.01$ for 50 mg/kg GSH *vs* SA with 72 h of fasting, $P < 0.05$ for 500 mg/kg GSH *vs* SA with 72 h of fasting; Figure 6).

Evaluation of enterocyte apoptosis

Apoptosis was determined by histomorphometry, which is preferable to terminal deoxynucleotidyl transferase dUTP nick-end labeling for quantitative assessment. Using histomorphometric assessment of jejunal cells, we evaluated the contribution of reduced apoptosis to the recovery from fasting-induced mucosal atrophy mediated by oral GSH treatment. The representative apoptosis changes as visualized by conventional light microscopy of H&E-stained specimens are shown in Figure 7A. AI distribution profiles in the villi and the crypt are also shown in Figure 7B. From the AI distribution profiles, increased apoptosis in the lower half of the villus (cell positions 1 to 40) was evident with 48 and 72 h of fasting in the SA-treated groups, and this increase was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal villus AI with 48 and 72 h of fasting compared with that in the normally fed controls ($P < 0.01$; Table 1). GSH treatment significantly decreased the fasting-induced enhancement of villus AI compared with that in the respective SA-treated group for each fasting period ($P < 0.05$ for 50 mg/kg GSH *vs* SA with 48 or 72 h of fasting, $P < 0.01$ for 500 mg/kg GSH *vs* SA with 48 or 72 h of fasting). From the AI distribution profiles, increased apoptosis in the lower two-thirds of crypts (cell positions 1 to 20) was evident with 48 and 72 h of fasting in the SA-treated groups (Figure 7B), and this effect was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal crypt AIs with 48 and 72 h of fasting compared with the normally fed controls ($P < 0.01$; Table 1). GSH treatments significantly ameliorated the fasting-induced increase in crypt AI compared with the respective SA-treated group for each fasting period ($P < 0.01$).

Evaluation of cell proliferation

GSH is known to exhibit an important function related to the regulation of cell proliferation by protecting against the damaging effects of ROS[32]. To evaluate the effect of oral GSH administration on the reduced cell proliferation in fasting-induced jejunal mucosal atrophy, we assessed 5-BrdU incorporation, a proliferation indicator, in the jejunum. Although cell proliferation decreased with 48 and 72 h of fasting compared with that in the normally fed controls ($P < 0.01$; Figure 8), animals treated with GSH exhibited significantly higher levels of jejunal cell proliferation compared with those in the respective SA-treated group for each fasting period ($P < 0.01$ for 50 mg/kg GSH *vs* SA at 48 h fasting, and for 500 mg/kg GSH *vs*

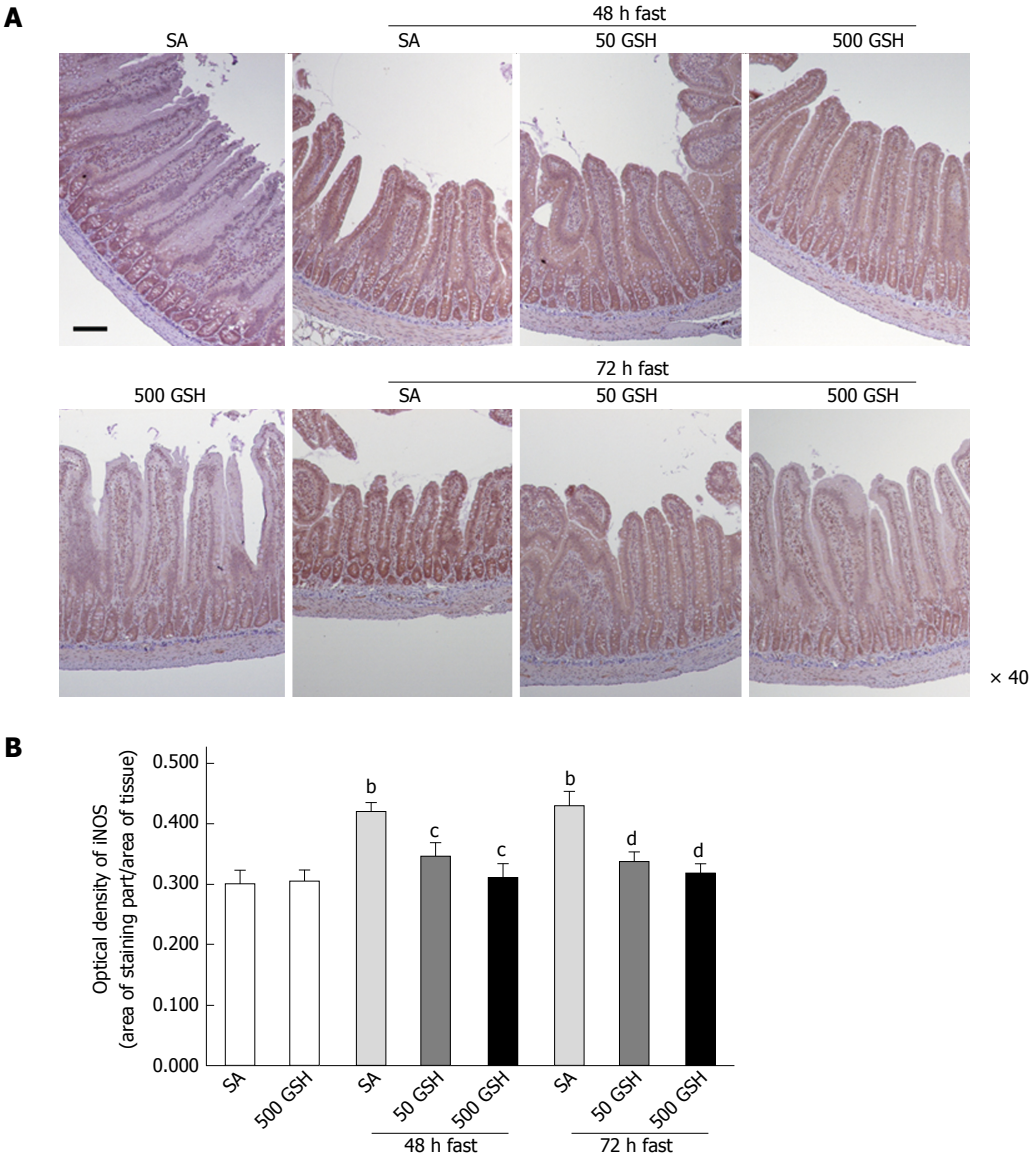


Figure 4 Effects of fasting and glutathione treatment on inducible nitric oxide synthase protein expression in the jejunum. A: Light micrographs of immunohistochemical staining for iNOS. In SA-treated groups, iNOS protein staining was localized almost exclusively in the mucosal epithelial monolayer with 48 and 72 h of fasting compared with the normally fed control. Decreased staining occurred with GSH treatment. Bar = 100 μm. B: Optical density of iNOS protein. The content of iNOS protein was quantitatively measured by averaging the optical density. Values represent the mean ± SE. $^{b}P < 0.01$ *vs* normally fed controls. $^{d}P < 0.01$, $^{c}P < 0.05$ *vs* the respective SA group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; iNOS: Inducible nitric oxide synthase.

SA at 48 and 72 h fasting).

GSH and GSSG levels in the jejunum

GSH is one of the most important scavengers of ROS; additionally, GSH and oxidized GSH (*i.e.*, GSSG) are used as markers of oxidative stress[33]. To evaluate GSH redox balance and GSH levels in the jejunum consequent to oral GSH administration during fasting, we assessed GSH and GSSG levels in the jejunum. Fasting significantly decreased the GSH concentration in the jejunum ($P < 0.01$; Figure 9A). GSH treatment significantly further decreased the fasting-induced decreases in the jejunal GSH concentration for each fasting period ($P < 0.01$). Conversely, GSSG concentration in the jejunum was similar to that of normally fed controls except for the 500 mg/kg GSH treatment group with *ad libitum* feeding (Figure 9B).

Ggt1 mRNA expression in the jejunum

Fasting significantly increased *Ggt1* expression in the jejunum at 48 h of fasting ($P < 0.05$) (Figure 10) whereas GSH treatment significantly reduced *Ggt1* elevation at 48 h of fasting ($P < 0.05$, $P < 0.01$ for 50 mg/kg GSH, 500 mg/kg GSH *vs* SA at 48 h of fasting, respectively). A similar tendency was obtained for the 72-h fasting group, although this difference did not reach statistical significance.

DISCUSSION

Previous reports have suggested the involvement of GSH in fasting-induced intestinal mucosal atrophy; in

Table 1 Enterocyte apoptosis of the jejunal villus and crypt evaluated by conventional light microscopy of hematoxylin and eosin-stained specimens

	SA	500 GSH	48 h fast			72 h fast		
			SA	50 GSH	500 GSH	SA	50 GSH	500 GSH
Villus								
Cells per villus column, *n*	70 ± 1	69 ± 1	56 ± 2[b]	64 ± 1[d]	67 ± 2[d]	56 ± 2[b]	63 ± 1[d]	65 ± 2[d]
Apoptotic cells per villus column, *n*	0.06 ± 0.01	0.09 ± 0.01	0.20 ± 0.03[b]	0.12 ± 0.01[d]	0.11 ± 0.01[d]	0.23 ± 0.03[b]	0.18 ± 0.01	0.14 ± 0.02[c]
Apoptotic index, %	0.09 ± 0.01	0.12 ± 0.01	0.38 ± 0.06[b]	0.19 ± 0.02[c]	0.18 ± 0.02[d]	0.42 ± 0.06[b]	0.29 ± 0.02[c]	0.21 ± 0.02[d]
Crypt								
Cells per villus column, *n*	28 ± 0.2	27 ± 0.4	22 ± 0.4[b]	25 ± 0.3[d]	27 ± 0.4[d]	22 ± 0.3[b]	23 ± 0.4[c]	26 ± 0.3[d]
Apoptotic cells per villus column, *n*	0.06 ± 0.01	0.06 ± 0.01	0.18 ± 0.01[b]	0.08 ± 0.01[d]	0.09 ± 0.01[d]	0.17 ± 0.02[b]	0.10 ± 0.01[d]	0.11 ± 0.01[d]
Apoptotic index, %	0.21 ± 0.04	0.22 ± 0.02	0.81 ± 0.05[b]	0.32 ± 0.03[d]	0.33 ± 0.03[d]	0.78 ± 0.10[b]	0.41 ± 0.06[d]	0.40 ± 0.05[d]

Apoptosis was measured as in Figure 7A. 7-8 rats were tested in each group. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the normally fed control; [d]$P < 0.01$ or [c]$P < 0.05$ *vs* the SA-treated group in each fasting period. GSH: Glutathione; SA: Saline.

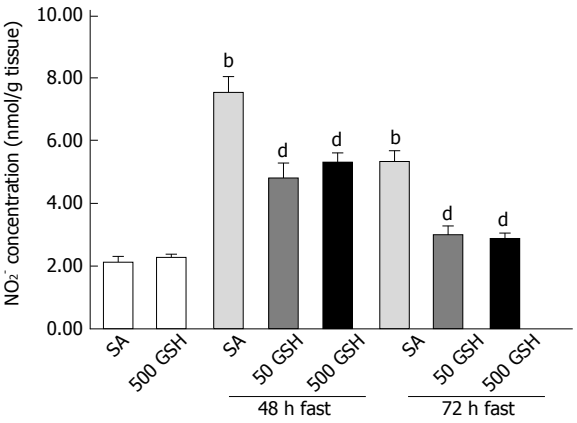


Figure 5 Effects of fasting and glutathione treatment on nitrite concentration in the jejunum. Nitrite concentrations were measured by HPLC using postcolumn derivatization with Griess reagent. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the SA-treated group. [d]$P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

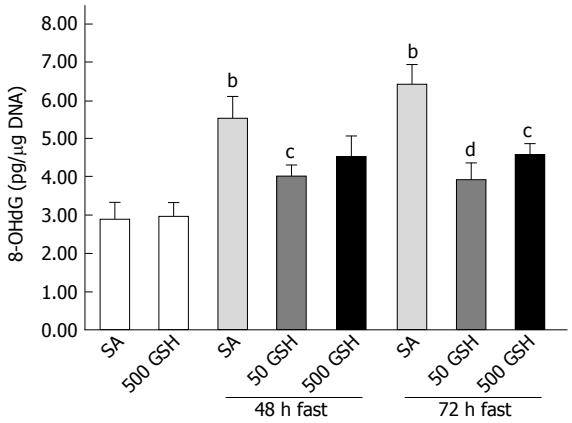


Figure 6 Effects of fasting and glutathione treatment on DNA oxidative damage in the jejunum. DNA oxidative damage was assessed by the level of 8-hydroxydeoxyguanosine (8-OHdG) in the jejunum. Values represent the mean ± SE. [b]$P < 0.01$ *vs* the normally fed control. [d]$P < 0.01$, [c]$P < 0.05$ *vs* the SA-treated group with 48 and 72 h of fasting. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

addition, fasting has been associated with increased ROS formation and depletion of the critical antioxidant GSH in the intestine[4,6,34-36]. However, the roles of GSH in intestinal mucosal recovery from this condition as mediated by oral GSH administration have not yet been described. The objective of the present study was to investigate the effects of oral GSH administration on the development of fasting-induced intestinal atrophy, with a particular focus on the possible participation of intracellular GSH level and GGT expression on the cell surface as potential parameters of intracellular *de novo* GSH synthesis.

During fasting, the intestinal lumen receives very little or no GSH from two of the three primary sources, *i.e.*, biliary secretion[10,37] and dietary intake[11]. Therefore, the overall GSH levels of the intestinal mucosa decrease during fasting because the remaining GSH source, intracellular GSH, is primarily synthesized from GSH in the intestinal lumen.

There is growing evidence that dysfunctional GSH homeostasis is involved in the etiology of several diseases. The previously reported conditions associated with GSH depletion include liver disease[38], immune disorders[39], neurodegenerative disease[40], cardiovascular disease[41], pulmonary disease[42], arthritis, diabetes[43], and the aging process itself[44]. Thus, oral supplementation with GSH to increase the depressed GSH level has been studied extensively as a potential means to prevent these diseases by countering the negative effects of oxidative stress, which is one of their underlying causes. Several studies have shown that GSH supplementation in laboratory animals is effective, with benefits including enhancement of immune function[45] and protection against carcinogenesis[46]. In addition, daily consumption of GSH supplements in humans was effective at increasing body compartment stores of GSH, which may be of importance as the maintenance of tissue levels of GSH might be critical for maintaining health and preventing disease as well as age-related biological insults[47]. Thus, it has been

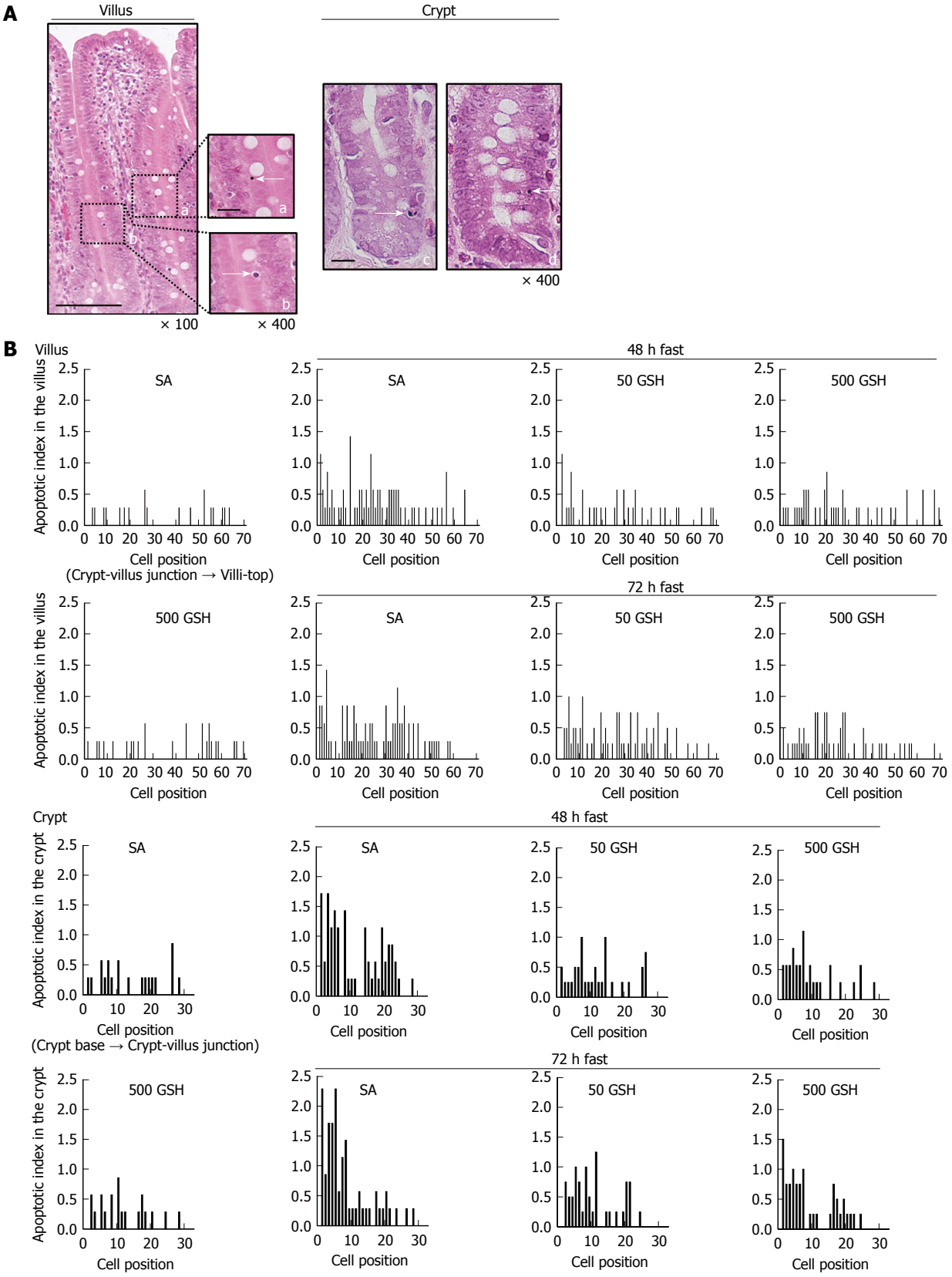


Figure 7 Effects of fasting and glutathione treatment on apoptotic index in the jejunal villus and crypt. Representative apoptotic changes determined by conventional light microscopy of hematoxylin and eosin (HE)-stained sections of the jejunal mucosa are shown in A. Left side: A jejunal villus from a 72-h fasted rat with 500 mg/kg GSH treatment. Apoptotic cells in the villus are indicated by an arrow showing an apoptotic corpuscle (a) and condensed chromatin (b). Bar = 100 μm (low magnification). Bar = 20 μm (high magnification). Right side: Jejunal crypts from 48- and 72-h fasted rats. Apoptotic cells in the crypt are indicated by an arrow showing an intensely eosinophilic cytoplasm and nuclear fragmentation (c) and condensed chromatin (d). Bar = 20 μm. AI distribution curves in the villus and the crypt are shown in B. AI is defined as the total number of apoptotic cells at each cell position and is expressed as a percentage of the total number of cells counted at that cell position. Cell position 1 is defined as the cell at the crypt-villus junction and the cell at the base of the crypt column for the villus and crypt data, respectively. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

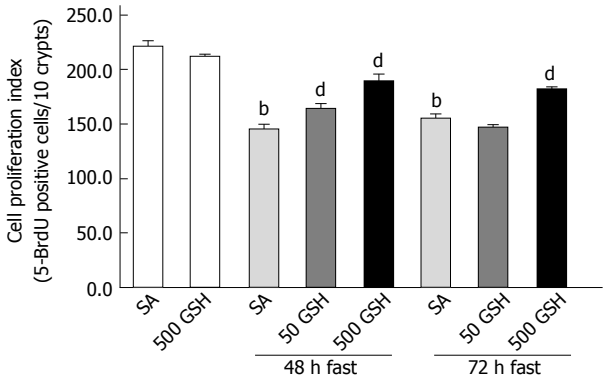


Figure 8 Effects of fasting and glutathione treatment on cell proliferation in the jejunum. Cell proliferation in the jejunal crypt was histologically assessed by 5-bromo-2'-deoxyuridine (5-BrdU) incorporation. The fraction of 5-BrdU-positive cells was expressed as the cell proliferation index (5-BrdU-positive cells/10 crypts). Values represent the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline.

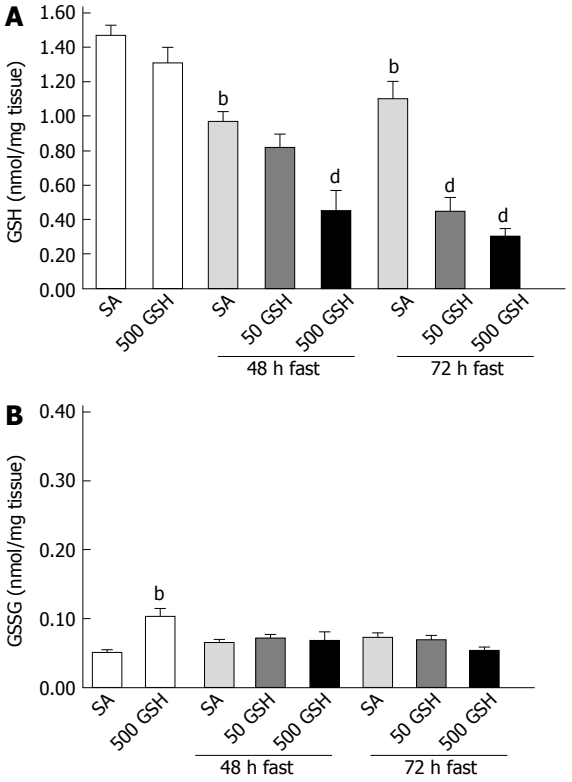


Figure 9 Effects of fasting and glutathione treatment on glutathione and oxidized glutathione concentration in the jejunum. GSH and GSSG concentration in the jejunum was determined by measuring the absorption derived from a colorimetric reaction with DTNB [5, 5'-dithiobis (2-nitrobenzoic acid)] coupled with the enzymatic recycling system. A: GSH concentration in the jejunum; B: GSSG concentration in the jejunum. Values are the mean ± SE. $^{b}P < 0.01$ *vs* the normally fed control. $^{d}P < 0.01$ *vs* the respective SA-treated group in each fasting period. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; GSSG: Oxidized glutathione.

suggested that orally administered GSH may act as a backup for intracellular GSH for GSH-deficient tissue. Therefore, the enhancement of intestinal mucosal GSH levels by oral GSH supplementation under conditions of intracellular GSH deficiency-related biological insults such as fasting may restore tissue GSH and promote ROS metabolism, thus facilitating the recovery from intestinal mucosal atrophy.

Our previous studies suggested that fasting-induced intestinal mucosal atrophy could be alleviated by directly inhibiting the fasting-mediated NO production by iNOS and subsequent ROS enhancement associated with increased jejunal apoptosis *via*, *e.g.*, iNOS inhibitor treatment[20] or refeeding[21]. Notably, an association was also observed between inhibition of ROS and increasing cell proliferation[21]. The intestinal epithelial layer is uniquely organized for rapid self-renewal, which is achieved by the well-regulated processes of crypt-to-villus apoptosis and crypt stem cell proliferation. The intestinal epithelium sits at the interface between the intestinal mucosa and the intestinal lumen, and as such is prone to oxidative damage induced by luminal and intracellular oxidants. A previous study[16] reviewed the GSH/GSSG redox mechanism, which modulates intestinal cell transition through cell proliferation, differentiation, or apoptosis and can govern the regenerative potential of the intestinal mucosa. Therefore, we hypothesized that protection against intestinal mucosal atrophy could be provided by controlling apoptosis and cell proliferation through the elimination of induced ROS in the intestinal mucosa by orally administering an antioxidant such as GSH during fasting in rats.

Here, we found that there was no difference in weight loss between fasting alone and oral GSH treatment during fasting (Figure 2) with the GSH levels used for this study. Conversely, jejunal mucosal atrophy was significantly increased by fasting but was remedied by oral GSH treatment during fasting (Figure 3), with significantly greater improvement resulting from the high *vs* low GSH dose. Furthermore, because the etiology of fasting-induced intestinal atrophy was shown to comprise ROS genesis from the NO produced by iNOS[20,21], we measured iNOS protein expression in the jejunum. We identified an inverse relationship between intestinal mucosal atrophy and iNOS protein expression (primarily in the mucosal epithelial monolayer) associated with oral GSH treatment during fasting, most particularly with high levels of GSH administration (Figure 4). This is supported by our previous observations that iNOS inhibitor treatment during fasting and refeeding after fasting decreases fasting-induced atrophy and suppresses iNOS expression in the jejunum.

Oral GSH treatment during fasting inhibited the fasting-mediated generation of the apoptosis mediators NO and ROS concomitant with decreased iNOS expression (Figures 5 and 6). To determine the importance of apoptotic changes in the jejunum for intestinal recovery by oral GSH treatment (Figure 7A), we performed a histomorphometric assessment including AI score (Table 1) and distribution profiles

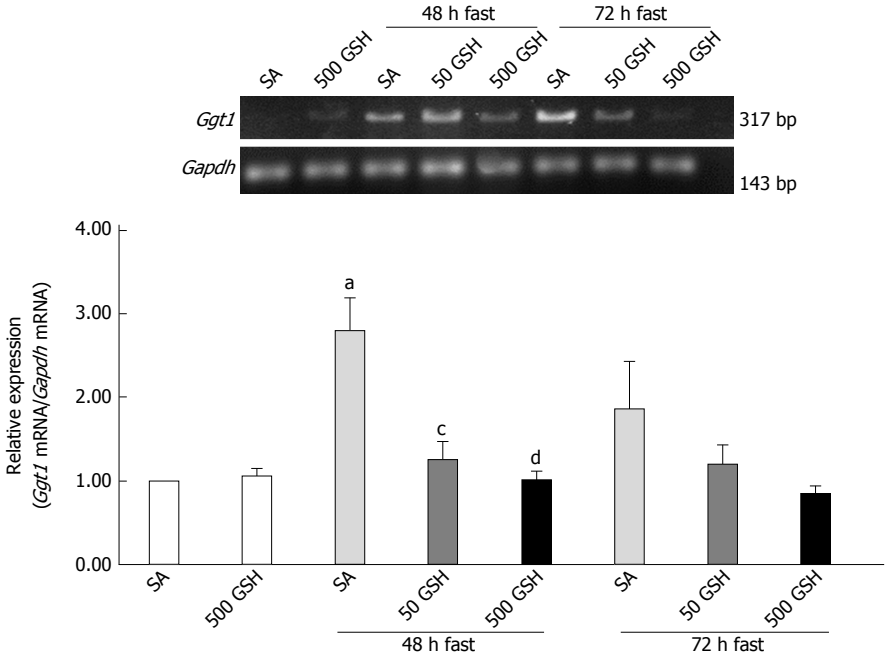


Figure 10 Effects of fasting and glutathione treatment on *Ggt1* mRNA expression in the jejunum. GGT is the only enzyme of the gamma-glutamyl cycle located on the outer surface of plasma membrane and plays key roles in GSH homeostasis by breaking down extracellular GSH. Values are the mean ± SE. $^{a}P < 0.05$ *vs* the normally fed control. $^{d}P < 0.01$, $^{c}P < 0.05$ *vs* the SA-treated group with 48 h of fasting. 7-8 rats were tested in each group. GSH: Glutathione; SA: Saline; GSSG: Oxidized glutathione.

(Figure 7B). The increased AI recorded in the villi and crypts after fasting decreased with oral GSH treatment (Table 1). Specifically, the lower half of the apoptotic enterocytes distributed across the entire jejunal villi and the apoptotic cells primarily located in the lower two-thirds of the crypt (Figure 7B) were particularly affected by oral GSH treatment during fasting. These results were broadly consistent with changes in iNOS, NO, and ROS as apoptosis mediators, suggesting that oral GSH administration during fasting may inhibit enterocyte apoptosis by diminishing the ROS in the jejunum generated by fasting.

GSH treatment also was found to alleviate the decreased jejunal cell proliferation mediated by fasting (Figure 8), with significantly greater improvement in fasting-induced jejunal mucosal atrophy resulting from high *vs* low dose GSH. These observations are consistent with the role of GSH in cell proliferation through protection against damage from ROS[16,32]. Together, these findings indicate that oral GSH administration leads to less fasting-mediated enhancement and therefore decreased overall ROS levels in the jejunal mucosa, thereby functioning to increase cell proliferation. This suggests that oral GSH administration may provide regenerative potential to the mucosa during fasting.

Notably, although our findings replicated prior results[6,34-36] that fasting significantly decreased jejunal GSH concentrations (Figure 9A), we found that the concentrations were further significantly decreased by oral GSH supplementation during fasting, with a greater effect from high *vs* low GSH dose. Conversely, the jejunal concentrations of the GSH oxidation product GSSG were not changed by any feeding regime (Figure 9B). Furthermore, oral GSH treatment during fasting significantly decreased the fasting-induced ROS levels in the jejunum (Figure 6), but we consider it unlikely that this effect depends on antioxidant activity given the depressed GSH concentration in the jejunum. These results demonstrate that oral GSH administration during fasting did not contribute to the supply of GSH in the jejunum, and an increase in GSH oxidation was not observed, suggesting that oral GSH administration during fasting does not provide a backup for the jejunum-depleted GSH.

The synthesis of intracellular *de novo* GSH, which is critical for ROS removal and maintenance of GSH homeostasis, relies upon the enzyme activity of the plasma membrane protein GGT to break down extracellular GSH[12]. In the present study, we demonstrated that oral GSH treatment significantly ameliorated the increased jejunal *Ggt1* mRNA expression observed following 48 h of fasting (Figure 10). In comparison, Jonas *et al*[4] indicated that fasting for 72 h did not change *Ggt1* mRNA levels in the ileum, although this was determined *via* ribonuclease protection assay rather than by semi-quantitative RT-PCR as used in the present study. The different findings may also be a consequence of the disparity in fasting duration. Notably, Ogasawara *et al*[34] showed that starvation causes a striking decrease of GGT activity in the jejunum, which is consistent with our finding of fasting-induced increases in *Ggt1* mRNA levels, as the mRNA expression of GGT might be upregulated by the

decline of GGT activity[12]. Furthermore, Zhang *et al*[12] described that *Ggt1* mRNA expression is increased during oxidative stress, which was considered to constitute an adaptation to such adverse conditions. Similarly, we found that fasting increased jejunal ROS levels and up-regulated *Ggt1* mRNA expression in the jejunum in this study. However, considering that oral GSH treatment during fasting significantly decreased both jejunal GSH concentration and *Ggt1* mRNA expression, the supplemented GSH in the intestinal lumen may not be involved in intracellular *de novo* GSH synthesis.

The ability of GSH to control extracellular redox provides another possible role for intestinal luminal GSH during fasting, as the present study demonstrated that intracellular GSH levels of the jejunum mucosa were not increased by intestinal luminal GSH administration during fasting, although the GSH administration attenuated the fasting-induced ROS in the intestinal mucosa. Reversible redox reactions of intracellular thiol/disulfide pairs such as GSH/GSSG regulate diverse biological processes *in vivo*, including signaling for apoptosis and cell proliferation, enzyme catalysis, gene expression, and molecular folding and trafficking[16-18,48,49]. Most studies have focused on the major intracellular thiols such as GSH[50,51]; however, relatively little is known regarding the effects of changes in extracellular thiol/disulfide pairs. Biliary GSH is an important intestinal luminal source of GSH and attenuates ROS of the intestinal mucosa by increasing the GSH levels of the intestinal mucosa. However, it has been suggested that biliary GSH controls the status of the intestinal lumen and contributes to protection of the intestinal mucosa[10]. The GSH of the intestinal lumen may act on the intestinal mucosa *via* both of these two pathways, and it is important to consider the control of the redox status of the intestinal lumen. In addition, the roles of extracellular (*i.e*., intestinal luminal) GSH in the recovery from fasting-induced intestinal mucosal atrophy enabled by oral GSH treatment have not yet been described.

A few studies have examined the ability of the extracellular thiol/disulfide redox state to regulate apoptosis and cell proliferation. Circu *et al*[16] demonstrated that the luminal/extracellular redox environment is determined by the cysteine/cystine (Cys/CySS) redox pair with contributions from the GSH system, with the majority of Cys in the intestinal lumen originating from GGT enzymatic hydrolysis of GSH obtained from dietary intake and biliary supply. Intestinal cell proliferation and apoptosis are associated with quantitative changes in the redox potential (Eh) of the extracellular GSH/GSSG or Cys/CySS redox pair. In the present study, the changes in apoptosis and cell proliferation in the intestinal mucosa resulting from oral GSH administration during fasting may derive from Eh changes mediated by GSH and Cys in the intestinal lumen, which in turn may ultimately control signaling proteins, enzyme catalysis, and gene expression. For example, Devadas *et al*[52] linked ROS formation with the progression of Fas-induced apoptosis *via* the protective effects of extracellular GSH, which are ascribed to its known capacity as an antioxidant. Specifically, extracellular GSH was deduced to inhibit Fas-mediated apoptosis following ROS reduction in cells because Fas crosslinking induced rapid generation of ROS well before the appearance of characteristic apoptotic changes. Furthermore, Fujise *et al*[22] suggested that fasting-induced apoptosis in the rat small intestine occurred *via* a receptor-mediated type I apoptotic pathway including induced expression of FasL, Fas, and TNFR1. Selleri *et al*[53] and Viard-Leveugle *et al*[54] provided evidence that iNOS expression and NO production are involved in the mechanism of FasL upregulation and Fas-mediated apoptosis. The iNOS-NO-ROS-FasL pathway represents a potential link between the apoptosis and intestinal atrophy observed in the fasting.

In the present study, fasting-induced intestinal mucosal atrophy resulting from increased apoptosis was caused by increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. Because the iNOS-NO-ROS-FasL pathway is part of the apoptotic mechanism in the intestinal mucosa atrophy occurring after fasting[22,52-54], oral GSH treatment during fasting might inhibit Fas-mediated apoptosis following reduction of ROS levels in the jejunal mucosa resulting from Eh changes mediated by GSH and Cys in the intestinal lumen. Additionally, because iNOS protein expression was also induced by ROS produced from NO in fasting-induced intestinal mucosal atrophy in a previous study[20], the decrease in ROS levels in the intestinal mucosa reduces iNOS protein expression and NO production. Moreover, Jonas *et al*[55] showed that the extracellular thiol/disulfide redox state modulates cell proliferation and that this system interacted with growth factor signaling in a human colon carcinoma cell line. Thus, the change in extracellular thiol/disulfide redox state in response to peptide growth factors indicated an interaction of growth factor-activated pathways and thiol/disulfide metabolism during intestinal cell proliferation.

Therefore, oral GSH administration during fasting may regulate the redox state of the intestinal lumen and consequently may both relieve Fas-mediated apoptosis and increase growth factor-mediated cell proliferation by ROS removal in jejunal epithelial cells (Figure 11). However, because our present study focused on intracellular GSH, further studies focusing on extracellular GSH are required to confirm this model. In particular, a study utilizing measurements of GSH/GSSG and Cys/CySS levels in the intestinal lumen along with Fas and growth factor signaling in the jejunum may be necessary to identify the mechanism underlying the recovery of the intestinal mucosal atrophy mediated by oral GSH administration during fasting.

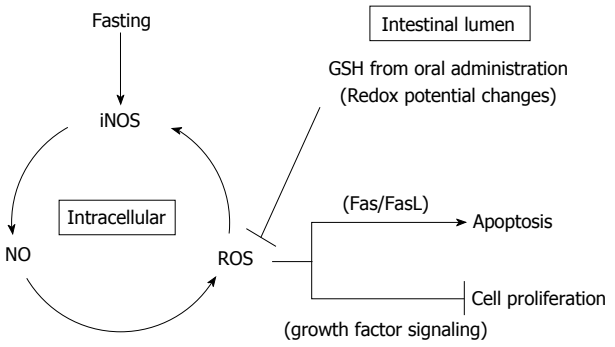


Figure 11 Schematic diagram of protective effects of glutathione in the intestinal lumen against fasting-induced intestinal atrophy, mediated through oxidative stress. The schematic diagram depicts a possible role of intestinal lumen redox status in the regulation of jejunal mucosa apoptosis and cell proliferation in fasting-induced intestinal atrophy, mediated through oxidative stress. Fasting causes increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. The changes in apoptosis and cell proliferation in the intestinal mucosa resulting from oral GSH administration during fasting may derive from intracellular ROS removal by redox potential changes mediated by GSH and Cys (originating from enzymatic hydrolysis of GSH) in the intestinal lumen. Intracellular ROS removal is considered to inhibit Fas-mediated apoptosis and increase growth factor-mediated cell proliferation. GSH: Glutathione; ROS: Reactive oxygen species.

In conclusion, oral GSH administration during fasting may provide regenerative potential to the jejunal mucosa through control of enterocyte apoptosis and cell proliferation. The GSH in the intestinal lumen provided through oral administration during fasting may not be involved in intracellular *de novo* GSH but may be useful to prevent intestinal mucosal atrophy by diminishing the levels of ROS in the jejunum generated by fasting.

COMMENTS

Background

Fasting induces small intestinal mucosal atrophy, which can lead to bacterial translocation, particularly in patients receiving a prolonged course of total parenteral nutrition (TPN), and is associated with increased reactive oxygen species (ROS) formation. This fasting state is also accompanied by depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa. As GSH deficiency in tissues is associated with increased oxidative stress and fasting, the maintenance of tissue levels of GSH is critical for protection of small intestinal mucosa.

Research frontiers

The roles of GSH in intestinal mucosal recovery from fasting as mediated by oral GSH administration have not been investigated.

Innovations and breakthroughs

GSH treatment in the intestinal lumen could be a promising strategy to improve clinical outcome in patients receiving a prolonged course of TPN.

Applications

Oral GSH administration during fasting enhances jejunal regenerative potential to minimize intestinal mucosal atrophy by diminishing fasting-mediated ROS generation and enterocyte apoptosis and enhancing cell proliferation.

Peer-review

The manuscript by Uchida *et al* describes effects of oral GSH administration on fasting-induced intestinal atrophy in the small intestinal mucosa. The authors found that oral GSH administration of rats during fasting significantly enhanced jejunal regenerative potential to minimize mucosal atrophy. This reduction of intestinal atrophy correlated with correction of histopathological outcomes and many biochemical parameters. The authors' conclusion that oral GSH improves intestinal atrophy is supported by convincing results.

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ORIGINAL ARTICLE

Basic Study

Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: Correlation with toll-like receptor 2/toll-like receptor 4 expression

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Abstract

AIM

To investigate the intestinal luminal microbiota (LM) and mucosa-associated microbiota (MAM) in Chinese patients with functional gastrointestinal disorders (FGIDs) and examine the association between these communities and the expression of toll-like receptor (TLR) 2 and TLR4.

METHODS

Thirty-two Chinese subjects who suffered from symptoms of FGIDs, as confirmed by gastroenterologists, were enrolled in this study. Fresh faecal samples and descending colonic mucosal biopsies were collected from the subjects before (faecal) and during (mucosal)

flexible colonoscopy. For analysis of the samples, we performed high-throughput sequencing of the V3-V4 region of the *16S rRNA* gene and reverse transcription (RT)-PCR to detect the expression of colonic TLR2 and TLR4. Differences in the stool and mucosal microbiota were examined and a correlation network analysis was performed.

RESULTS

The microbiota of faecal samples was significantly more diverse and richer than that of the mucosal samples, and the LM and MAM populations differed significantly. TLR2 expression showed a significant positive correlation with TLR4 expression. In the MAM samples, the genera *Faecalibacterium* and *Ruminococcus*, which belong to the family Ruminococcaceae, were inversely correlated with TLR4 expression (r = -0.45817, P = 0.0083 and r = -0.5306, P = 0.0018, respectively). *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression (r = -0.5573, P = 0.0010 and r = -0.5435, P = 0.0013, respectively). In the LM samples, examination at phylum, class, or order level revealed no correlation with TLR4 expression. *Faecalibacterium*, which belongs to Ruminococcaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression (r = -0.5743, P = 0.0058 and r = -0.3905, P = 0.0271, respectively).

CONCLUSION

Microbial compositions of LM and MAM in Chinese patients with FGIDs are different. Expression of TLRs may be affected by the type of bacteria that are present in the gut.

Key words: Gastrointestinal microbiota; *16S rRNA* gene; Toll-like receptors; High-throughput sequencing

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Core tip: To explore which bacteria regulate the expression of toll-like receptors (TLRs) and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA* gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders, and studied their association with the expression of colonic mucosal TLR2 and TLR4. Samples of luminal microbiota were different from those of mucosa-associated microbiota (MAM), and MAM samples were closely associated with TLR2/4 expression. The abundance of *Faecallibacterium* and *Ruminococcus* was lower in patients with gut disease, while the expression of TLRs is higher than in healthy controls. The presence of *Faecallibacterium* and *Ruminococcus* was significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria, which colonize on the colonic mucosa, play a key role in gut diseases by regulating mucosal TLR4 expression. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression. Because the two genera contained not only pathogenic species but also probiotic species, it will be important to establish a better understanding of the relationship between TLRs and bacterial strains in future studies.

Dong LN, Wang JP, Liu P, Yang YF, Feng J, Han Y. Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: Correlation with toll-like receptor 2/toll-like receptor 4 expression. *World J Gastroenterol* 2017; 23(36): 6665-6673 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6665.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6665

INTRODUCTION

The intestinal microbiota is a complex community of bacteria, archaea, viruses, and eukarya. A wide variety of bacterial species in the gastrointestinal tract exerts numerous effects on the host and influences a variety of gastrointestinal functions[1]. Faecal samples (representing the luminal niche) are examined in most studies of the intestinal microbiota because their collection is simple. However, recent studies have shown that the microbial compositions of the luminal microbiota (LM) and the mucosa-associated microbiota (MAM) differ, suggesting that these two distinct microbial populations play different roles within the intestinal microbiota ecosystem[2]. LM is present in the whole intestine, whereas MAM represents a special niche. Because MAM is in close contact with the host, it may play a more prominent role in the intestine, whereas LM may play a key role in metabolic activities and nutrient harvest[3]. Toll-like receptors (TLRs) are pattern recognition receptors expressed by various cells in the gastrointestinal tract. TLRs detect conserved microbial products and play a central role in the activation of innate and adaptive immune pathways. TLR2 and TLR4 are two of the best characterized TLRs that respond to microbial membrane components. A number of studies have examined the role of TLR signalling in microbiota-induced chronic inflammation and immunopathology[4]. The microbiota may directly interact with the TLRs and regulate gut immune responses[5].

In some intestinal diseases, such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS), the expression of TLR2 and TLR4 is increased and the dysbiosis is observed. However, limited data are available on the correlation between the intestinal microbiota and TLRs in humans. To explore which bacteria regulate the expression of TLRs and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA*

gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders (FGIDs), and studied their association with the expression of colonic mucosal TLR2 and TLR4.

MATERIALS AND METHODS

Study subjects

Thirty-two Chinese subjects aged between 18 and 65 years, who were diagnosed with FGIDs, were recruited at the Department of Gastroenterology, Shanxi Provincial People's Hospital from 2013 to 2014. None of the subjects enrolled in the study had taken corticosteroids, opioids, probiotic and prebiotic supplements or antibiotics in the 6 mo preceding the study, no one had systemic comorbidity, and no one had a history of excessive alcohol intake (> 20 alcoholic drinks per week). Patients with a prior history of gastrointestinal surgery or intestinal organic disease were excluded. All subjects provided signed informed consent before participation. The study was performed in accordance with the principles of the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Shanxi Provincial People's Hospital, China.

Stool sample processing and DNA extraction

Faecal samples were collected at home before bowel preparation, frozen immediately at -20 ℃, and transported within 6 h to the study centre, where they were stored at -80 ℃ until analysis. Bacterial DNA was extracted from the faecal samples with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA concentrations were quantified with an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany).

Mucosal sample collection and genomic DNA extraction

Colonic mucosal biopsies were collected from each subject during an unsedated flexible colonoscopy. Colonic mucosal biopsies were taken from the descending colon. After removal from the colon, the biopsies were immediately frozen at -20 ℃ and transported within 6 h to the study centre, where they were stored at -80 ℃ until analysis. Total genomic DNA was extracted from the colon samples with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The concentration and purity of the genomic DNA were measured with an Eppendorf BioSpectrometer.

PCR amplification of the V3-V4 region of bacterial 16S rRNA gene and Illumina sequencing

The bacterial genomic DNA was used as the template for amplification of the V3-V4 hypervariable region of the *16S rRNA* gene with the forward primer 5′-GACTA CHVGGGTATCTAATCC-3′ and the reverse primer 5′-CC TACGGGNGGCWGCAG-3′.

Bioinformatics analysis

Pairs of reads from the original DNA fragments were merged using FLASH, which was designed in case the original DNA fragments were shorter than twice the read length. The sequencing reads of each sample were given a unique barcode and analysed with the Quantitative Insights Into Microbial Ecology (QIIME) software and the UPARSE pipeline. In brief, the reads were filtered with the QIIME quality filters using the default settings for Illumina processing and the operational taxonomic units (OTUs) were selected using the UPARSE pipeline. The samples were sequenced on an Illumina MiSeq Benchtop Sequencer and the bioinformatic analysis was performed by Genesky Biotechnologies Inc. (Shanghai, China).

The size of the bacterial groups is expressed as percentage of the total bacteria.

Quantitative real-time polymerase chain reaction

Total mucosal RNA was extracted from the colonic biopsies using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan), according to the manufacturer's instructions. After reverse transcription with PrimeScript Reverse Transcriptase Mix (TaKaRa), the expression of *TLR2* and *TLR4* genes was determined by quantitative real-time polymerase chain reaction (qPCR) and SYBR Green technology on a Bio-Rad CFX96 Q-PCR instrument (Bio-Rad, United States) in duplicate. The specific primers for TLR2 were: 5′-TGATGCTGCCATTCTCATTC-3′ (forward) and 5′-CGC AGCTCTCAGATTTACCC-3′ (reverse); and for TLR4: 5′-CAGGGCTTTTCTGAGTCGTC-3′ (forward) and 5′-TG AGCAGTCGTGCTGGTATC-3′ (reverse). Each amplification reaction was run in duplicate in a final volume of 20 μL. All qPCR amplifications were optimized and performed in 0.2 mL 96-well plates with the following cycling program: initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s. Negative controls lacking the template DNA were included in triplicate. The relative amount of each mRNA was normalized to *GAPDH* (forward: 5′-CCATCAATGACCCCTTCATTG-3′, reverse: 5′-CTTGAC GGTGCCATGGAATT-3′) and data were analysed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analyses were performed with SPSS 22.0 for Windows (SPSS Inc., United States). To determine the statistical differences between the two groups, we used the independent *t* test and the Mann-Whitney test. Correlations were determined with Spearman's correlation test. Heat maps were generated by R-packages for TLR2 and TLR4 as environmental factors, to reveal the relationship between the diversity

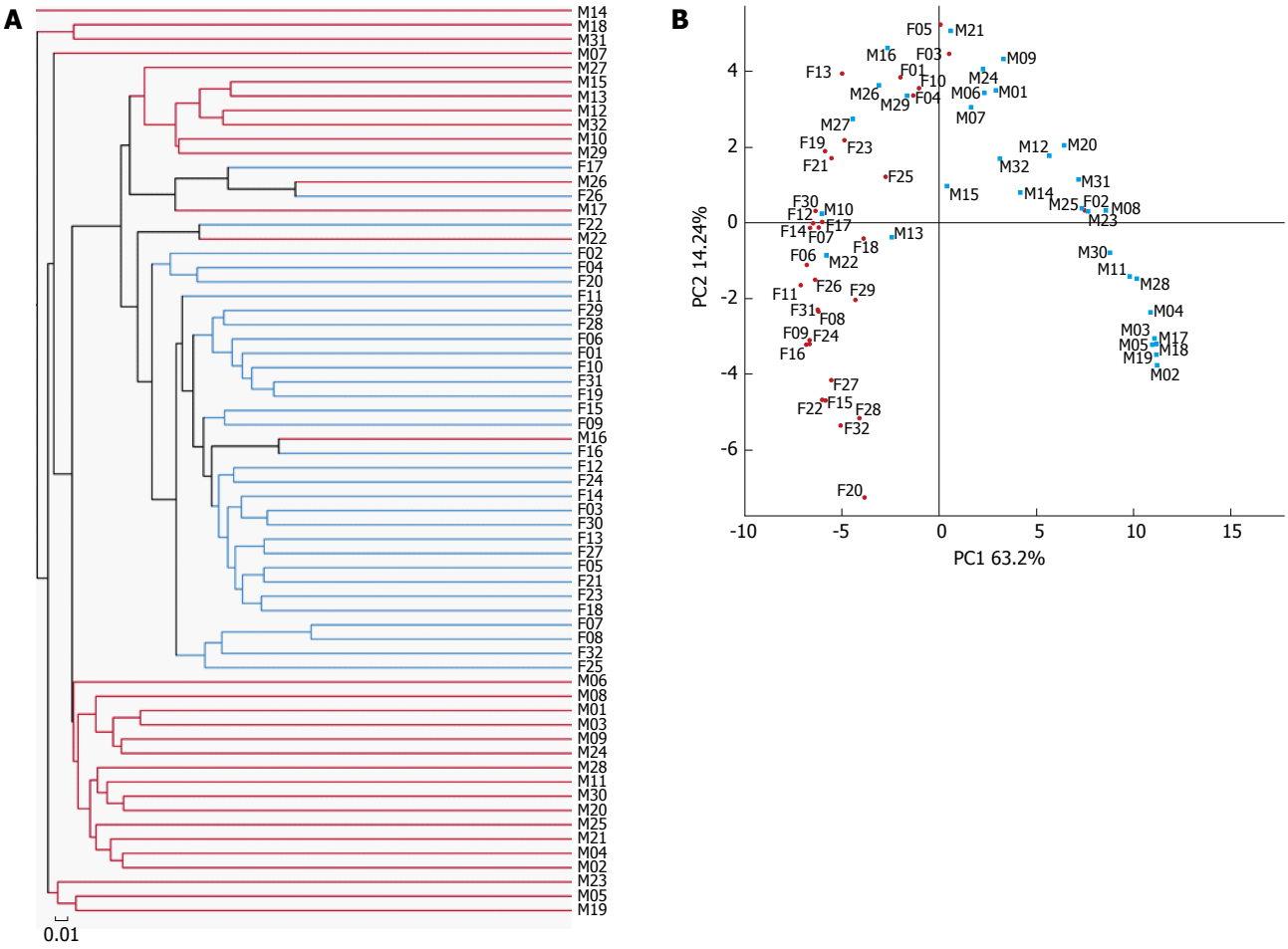


Figure 1 ***16S rRNA*** **gene surveys reveal a clear separation between luminal microbiota and mucosa-associated microbiota.** A: Dendrogram obtained from the complete linkage hierarchical clustering of the stool and mucosa samples based on the total operational taxonomic units; B: Principal coordinate analysis plot based on the weighted UniFrac metric.

Table 1 Comparison of the richness and diversity of mucosa-associated microbiota and luminal microbiota

	ACE	Chao	Shannon	Simpson
MAM	160.72 ± 51.62	151.56 ± 48.63	2.29 ± 1.24	0.33 ± 0.32
LM	195.50 ± 42.04	188.65 ± 42.70	2.901 ± 0.54	0.13 ± 0.07
P value	0.004[b]	0.002[b]	0.012[a]	0.001[b]

[a]$P < 0.05$, [b]$P < 0.01$, MAM *vs* LM. MAM: Mucosa-associated microbiota; LM: Luminal microbiota.

and environmental factors based on the Pearson's correlation analyses.

RESULTS

Study population

We investigated 64 samples from 32 subjects with FGIDs [14 with diarrhoea-predominant IBS, 14 with functional dyspepsia (FD), and 4 with other diseases]. All subjects provided both a faecal sample and a colonic mucosal sample. The study population consisted of 50% females, and had a mean age of 49 years (range, 20-65 years) and a mean body mass index of 23.24 kg/m^2 (range, 20-26 kg/m^2).

Characteristics of the pyrosequencing results

We obtained a total of 4351929 raw reads and 3545053 reads remained after filtering. The sequencing analysis of the 64 samples identified 1026 OTUs. The rarefaction curves had a tendency to approach a saturation plateau, indicating that the number of samples in this study was sufficient. The same tendency was found in the Shannon-Wiener curves, indicating that the number of *16S rRNA* gene sequences in the database was very abundant and reflected the vast majority of the microbiota. The gut faecal samples showed significantly more diversity and richness of the microbiota than the mucosal samples (Table 1) and samples of LM and MAM were significantly different (Figure 1A and B).

Significant differences between samples of LM and MAM were identified for almost all populated phyla. Bacteroidetes (44.7%) and Firmicutes (42.2%) were the most represented phyla in LM samples, followed by Proteobacteria (8.5%), whereas Proteobacteria (56.6%) was the most represented phylum in MAM samples, followed by Firmicutes (20.2%) and Bacteroidetes (12.7%) ($P < 0.05$; Figure 2).

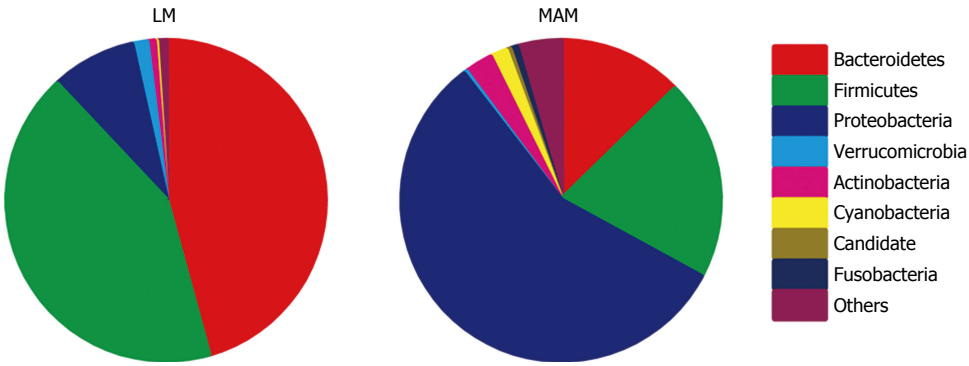


Figure 2 Relative abundance of bacterial genera in the stool and mucosa samples. Statistically significant differences were observed for all genera ($P < 0.05$). LM: Luminal microbiota; MAM: Mucosa-associated microbiota.

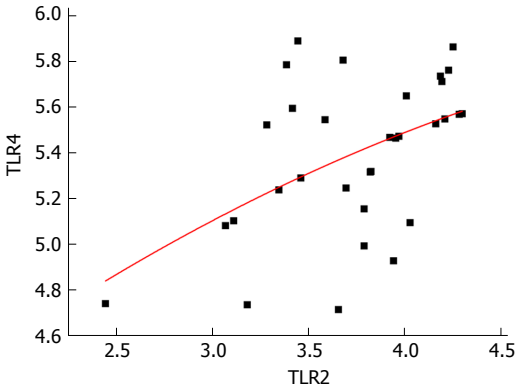


Figure 3 The expression of toll-like receptor 2 is significantly and positively correlated with toll-like receptor 4 expression ($r = 0.492$, $P = 0.004$). TLR: Toll-like receptor.

Examination at the genus level showed that the relative abundance of *Escherichia-Shigella*, *Streptococcus*, *Clostridium sensu stricto1*, *Sphingomonas*, *Acinetobacter*, *Brevundimonas*, and *Enhydrobacter* was significantly greater in samples of MAM than in LM samples, whereas abundance of *Bacteroides*, *Faecalibacterium*, *Incertae sedis*, *Subdoligranulum*, *Pseudobutyrivibrio*, *Megasphaera*, *Parasutterella*, *Akkermansia*, *Alistipes*, and *Lachnospira* was significantly lower in samples of MAM than in LM samples ($P < 0.05$).

Expression characteristics of mucosal TLR2 and TLR4

We measured the mRNA expression of TLR2 (3.7394 ± 0.43514) and TLR4 (5.3866 ± 0.33556) in all samples and expression of TLR2 was significantly and positively correlated with that of TLR4 ($r = 0.492$, $P = 0.004$) (Figure 3).

Correlation between gut microbiota and TLR4 expression

Regardless of phylum, class or order level, no bacterium was correlated with expression of TLR4 in LM samples.

At the phylum level, Firmicutes was inversely and significantly correlated with TLR4 expression in MAM samples ($r = -0.4676$, $P = 0.0070$). At the class level, Clostridia, which belongs to Firmicutes, was inversely and significantly correlated with TLR4 expression in MAM samples ($r = -0.3913$, $P = 0.0268$). At the order level among the class Clostridia, the order Clostridia was inversely and significantly correlated with TLR4 expression ($r = -0.3906$, $P = 0.0271$), as well as the families Defluviitaleaceae ($r = -0.4227$, $P = 0.0159$), Peptostreptococcaceae ($r = -0.3611$, $P = 0.0422$), and Ruminococcaceae ($r = -0.4740$, $P = 0.0061$), which belong to the order Clostridia. The genera *Faecalibacterium* and *Ruminococcus*, which belong to the family Ruminococcaceae, were also inversely and significantly correlated with TLR4 expression ($r = -0.45817$, $P = 0.0083$ and $r = -0.5306$, $P = 0.0018$, respectively) (Figure 4).

Correlation between gut microbiota and TLR2 expression

The presence of bacteria in both LM and MAM samples was correlated with TLR2 expression. However, the taxa of microbiota was very different in these two sample populations.

In LM samples, Clostridia and Bacilli at the class level were inversely and significantly correlated with TLR2 expression ($r = -0.4238$, $P = 0.0156$ and $r = -0.4482$, $P = 0.0101$, respectively), as well as Clostridiales at the order level, which belongs to Clostridia, and Lactobacillales, which belongs to Bacilli ($r = -0.4482$, $P = 0.0156$ and $r = -0.4207$, $P = 0.0165$, respectively). At the family level, Ruminococcaceae, which belongs to Clostridiales, and Streptococcaceae, which belongs to Lactobacillales, were inversely and significantly correlated with TLR2 expression ($r = -0.4437$, $P = 0.0110$ and $r = -0.3839$, $P = 0.0300$, respectively). And at the genus level, *Faecalibacterium*, which belongs to Ruminococcaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely and significantly correlated with TLR2 expression ($r = -0.5743$, $P = 0.0058$ and $r = -0.3905$, $P = 0.0271$, respectively) (Figure 4).

In MAM samples, Bacilli at the class level was inversely and significantly correlated with TLR2 expression ($r = -0.4676$, $P = 0.0070$), as well as Lactobacillales at the order level, which belongs to

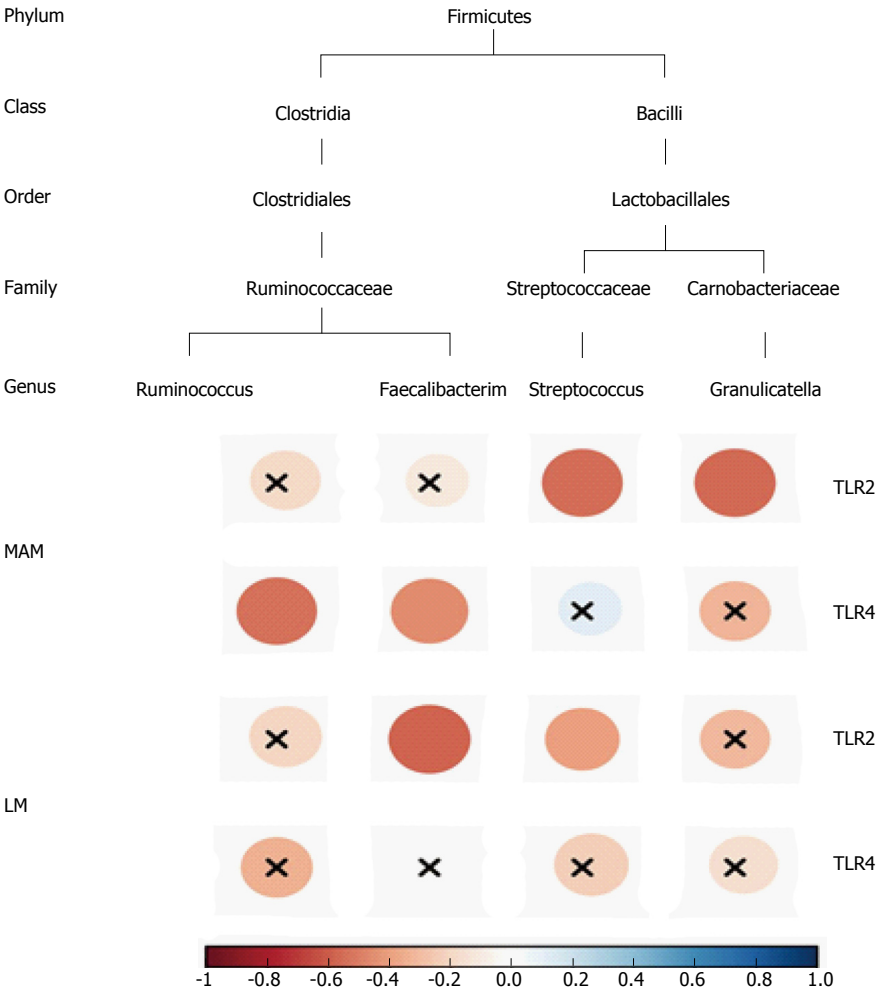


Figure 4 Correlogram showing the Pearson's correlation between key genera and toll-like receptors in both faecal and mucosa samples. Blue circles designate a positive correlation, while red ones designate a negative correlation. X means no significant result according to the significance level of 0.05. TLR: Toll-like receptor; MAM: Mucosa-associated microbiota; LM: Luminal microbiota.

Bacilli (r = -0.4574, P = 0.0085). At the family level, Carnobacteriaceae and Streptococcaceae, which belong to Lactobacillales, were inversely and significantly correlated with TLR2 expression (r = -0.5554, P = 0.0010 and r = -0.5445, P = 0.0013, respectively). And at the genus level, *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were correlated with TLR2 expression (r = -0.5573, P = 0.0010 and r = -0.5435, P = 0.0013, respectively) (Figure 4).

DISCUSSION

Deep sequencing showed that more than 95% of the sequences in all stool and mucosal samples belonged to the three most popular bacterial phyla, consisting of Firmicutes, Bacteroidetes, and Proteobacteria. This is consistent with the findings of previous studies[6], which showed that these phyla account for the majority of the gut microbiota in both stool and mucosal samples. The faecal samples displayed significantly greater bacterial diversity and richness than the mucosal samples, as in the study of Ringel *et al*[2]. Comparing the proportions of the dominant bacterial taxa in the faecal and mucosal samples revealed significant differences. In this study, Proteobacteria was the predominant phylum in MAM samples. This is different from other reports and might reflect geographical differences[7]. It is well known that the Chinese diet and genetics are very different from those in Western countries and these factors markedly influence the gut microbiota. Furthermore, MAM samples were taken from a distinct location in the colon, whereas LM samples displayed the whole intestinal microbiota. Therefore, MAM samples may reflect a stronger relationship with the host.

A correlation analysis was performed between the two bacterial populations at the phylum, class, order, family, and genus levels and the expression of TLR2 and TLR4. The results showed that although the Proteobacteria was the predominant phylum in MAM samples, the bacteria that correlated strongest with expression of TLRs were part of the phylum Firmicutes. The taxa that showed a significant correlation was selected and studied from the phylum to genus level. Finally, *Faecalibacterium, Ruminococccaceae*,

Streptococcus and *Granulicatella* genera were the bacteria that correlated strongest with TLRs expression. Although a positive correlation existed between the expression of TLR2 and TLR4, the types of bacteria associated with TLR4 expression were very different from those related to the expression of TLR2. However, this was in accordance with their distinct microbial compositions. The bacteria that correlated with TLR4 expression were only found in MAM samples. Bacteria that correlated with TLR2 expression originated from both LM and MAM. Both TLR4 and TLR2 expression were negatively correlated with MAM, however, TLR2 expression was mainly associated with bacteria belonging to the Bacilli class, whereas TLR4 expression was mainly associated with bacteria belonging to the Clostridia class.

The enteric commensal bacteria of the genus *Faecallibacterium*, which belongs to the group Clostridium, exert an anti-inflammatory effect[8]. In the present study, *Faecallibacterium* from LM samples was negatively correlated with the expression of TLR2 and *Faecallibacterium* from MAM samples was negatively correlated with the expression of TLR4. *Faecallibacterium* richness was reduced in patients with IBS and IBD[9,10]. TLR signalling, activated by pathogens, is involved in the pathogenesis of several infectious and inflammatory diseases. Imbalanced relationships among the environment, genetics, and host immunity may promote aberrant TLR signalling, thereby contributing on a large scale to acute and chronic intestinal inflammatory processes, such as IBD, colitis, and colorectal cancer[11]. In our previous study, the expression of TLR2 and TLR4 was increased in diarrhoea-predominant IBS patients and decrease of Clostridia was strongly associated with higher TLR2 and TLR4 expression[12]. In an animal study, *Faecallibacterium prausnitzii* and its supernatant (SN) had beneficial effects on intestinal epithelial barrier impairment in a chronic low-grade inflammation model[13]. Round *et al*[14] showed that unlike pathogens whose TLR ligands trigger inflammation, some commensal bacteria exploit the TLR pathway to actively suppress immune reactions. The anti-inflammatory effect of *Faecallibacterium* may be involved in the upregulation of the TLRs expression. *Ruminococcus* is another common bacterium that is decreased in patients with IBD or IBS. In addition, a reduction in the relative abundance of potentially immunomodulatory gut bacteria including *Ruminococcus* is associated with exaggerated inflammatory cytokine responses to TLR ligands and subsequent development of IgE-associated eczema[15]. In the present study, the decrease of *Faecallibacterium* and *Ruminococcus* in MAM samples was strongly associated with higher TLR4 expression, suggesting that the decrease of mucosal *Faecallibacterium* and *Ruminococcus* induced an increase in expression of TLR4 mRNA, which plays an important role in the causation of immune related gut diseases including IBD and IBS.

Streptococcus is a genus of (spherical) coccus Gram-positive bacteria belonging to the phylum Firmicutes phylum and the order Lactobacillales. Some streptococcal species, *e.g.*, *S. pneumoniae*, are pathogenic. Tomlinson *et al*[16] suggested that leukocyte responses to bacterial lipoproteins are required for TLR2- and IL-1R-associated kinase-4-mediated inflammatory responses to *S. pneumoniae*. However, many other streptococcal species, *e.g.*, *S. thermophilus*, are not pathogenic[17] and are part of the commensal human microbiota of the mouth, skin, intestine, and upper respiratory tract. S. *thermophilus* is considered a probiotic and is a key strain of VSL#3, which is considered a therapeutic immunomodulatory tool[18]. Streptococcaceae was negatively correlated with TLR2 expression in samples of both MAM and LM in our study. Although there is no direct evidence of inhibition of TLRs by Streptococcaceae, some beneficial streptococcal species may affect disease processes *via* the TLR2 pathway. Future research will explore which species belonging to Streptococcaceae can influence the expression of TLR2. Granulicatella is part of the normal body flora and is often difficult to culture in traditional media. A few reports mention that it can induce an infection similar to Streptococci[19].

The composition of LM samples was different than that of MAM; MAM samples showed a stronger correlation with the expression of TLR2 and TLR4. The abundance of *Faecallibacterium* and *Ruminococcus* was lower in IBD and IBS, while TLRs expression was higher in patients than in healthy controls. *Faecallibacterium* and *Ruminococcus* were significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria colonizing on the colonic mucosa play a key role in the pathogenesis of gut diseases by regulating the expression of mucosal TLR4. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2 expression.

One limitation of our study was the small biopsy from the colonic endoscopy and multiple sampling might cause bleeding, therefore we were not able to measure the expression of proteins. The other limitation of our study was the scattered distribution of the correlation between species levels, which might be explained by the restriction of pyrosequencing of species levels, therefore we did not report the results. It will be important to establish a better understanding of the relationship between TLRs and bacteria strains in future studies. Probiotics play an important role in the treatment and prevention of diseases. Further studies will have to verify the relationship between TLRs and bacteria[20,21].

COMMENTS

Background

Toll-like receptors (TLRs) detect conserved microbial products and play a

central role in the activation of innate and adaptive immune pathways. TLR2 and TLR4 are two of the best characterized TLRs that respond to microbial membrane components. Faecal samples (representing the luminal niche) were used in most studies on intestinal microbiota because their collection is easy. However, more recent studies revealed distinct microbial composition between luminal microbiota (LM) and mucosa-associated microbiota (MAM). To explore which bacteria may regulate the expression of TLRs and thereby affect intestinal functions, we performed high-throughput pyrosequencing of the bacterial *16S rRNA* gene, compared the microbial communities in the faeces and mucosa of Chinese patients with functional gastrointestinal disorders, and studied their association with the expression of colonic mucosal TLR2 and TLR4. LM differed from MAM; MAM showed a stronger association with the expression of TLR2 and TLR4.

Research frontiers

Recent advances in the understanding of intestinal immunology suggest that functional gastrointestinal disorders (FGIDs) may not all be 'functional', as considered for decades. This view was largely developed by the absence of active inflammation. Faecal microbiota and MAM play a vital role in gut immunity. It will be important to establish a better understanding of the relationship between TLRs and bacteria strains in FGIDs.

Innovations and breakthroughs

In some intestinal diseases, such as inflammatory bowel disease or irritable bowel syndrome, the expression of TLR2 and TLR4 is increased and dysbiosis is observed. However, limited data are available on the correlation between the intestinal microbiota and TLRs in humans. In this study, the abundance of *Faecallibacterium* and *Ruminococcus* was lower in many gut diseases, while the TLRs expression was higher than in healthy controls. *Faecallibacterium* and *Ruminococcus* were significantly and negatively correlated with TLR4 expression, suggesting that these two bacteria colonizing on the colonic mucosa play a key role in gut diseases by regulating the expression of mucosal TLR4. *Granulicatella*, which belongs to Carnobacteriaceae, and *Streptococcus*, which belongs to Streptococcaceae, were inversely correlated with TLR2.

Applications

Probiotics play an important role in the treatment and prevention of diseases. Further studies will have to verify the relationship between inflammatory proteins and bacteria. The bacteria may be used as probiotics in the treatment of diseases by regulating immunity.

Terminology

According to the literature, they described the microbiota from faecal samples as LM.

Peer-review

This is an interesting paper that investigated the intestinal LM and the MAM in Chinese patients with FGIDs and examined the association between these communities and the expression of TLR2 and TLR4. The manuscript is well written.

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ORIGINAL ARTICLE

Case Control Study

Genetic biomarkers for hepatocellular cancer risk in a caucasian population

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Author contributions: De Mattia E and Cecchin E designed the study and wrote the manuscript; Polesel J was involved in the statistical analysis and interpretation of data; Bignucolo A and Roncato R performed the majority of experiments; Lupo F, Crovatto M, Buonadonna A and Tiribelli C participated in the enrollment of cases and control groups; Toffoli G designed the study and edited the manuscript.

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Abstract

AIM

To uncover novel genetic markers that could contribute to predicting hepatocellular carcinoma (HCC) susceptibility in Caucasians.

METHODS

The present retrospective case-control study compared genotype frequencies between a cohort of HCC cases and two, independent, HCC-free, age/sex-matched control groups. The HCC cohort comprised 192 homogeneous patients that had undergone orthotopic liver transplantation. The first control group comprised 167 patients that were matched to the HCC cohort for the percentage of hepatitis B (HBV) and/or hepatitis C (HCV) infections. A second control group included 192 virus-free, healthy individuals that were used to evaluate the generalizability of the identified predictive markers. All cases and controls were Caucasian. The three study populations were characterized with a panel of 31 markers derived from 21 genes that encoded key proteins involved in hepatocarcinogenesis-related pathways. The study end-point was to assess the association between genetic variants and HCC onset.

RESULTS

Five genetic markers were identified as risk factors for HCC in high-risk patients infected with HBV/HCV. According to a dominant model, reduced HCC risk was associated with three polymorphisms: *ERCC1* rs3212986 (OR = 0.46, 95%CI: 0.30-0.71, P = 0.0005), *GST-P1* rs1138272 (OR = 0.41, 95%CI: 0.21-0.81, P = 0.0097), and *CYP17A1* rs743572 (OR = 0.50, 95%CI: 0.31-0.79, P = 0.0032). Conversely, according to a recessive model, increased HCC risk was associated with two polymorphisms: *XRCC3* rs1799794 (OR = 3.70, 95%CI: 1.02-13.39, P = 0.0461) and *ABCB1* rs1128503 (OR = 2.06, 95%CI: 1.18-3.61, P = 0.0111). These associations remained significant in a subgroup analysis, where patients were stratified according to viral status (HBV- or HCV-positive serology). Two variants exhibited a serology-specific effect: *ABCB1* rs1128503 (OR = 4.18, 95%CI: 1.55-11.29, P = 0.0048) showed an effect in the HBV-positive subgroup; and *ERCC1* rs3212986 (OR = 0.33, 95%CI: 0.18-0.60, P = 0.0003) showed an effect in the HCV-positive subgroup. Among the five markers identified, *ERCC1* rs3212986 (OR = 0.43, P < 0.0001) and *CYP17A1* rs743572 (OR = 0.73, P = 0.0310) had a different distribution in patients with HCC compared to healthy individuals. With a recursive partitioning approach, we also demonstrated that significant gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 predictive marker, played a role in the complex trait of HCC susceptibility.

CONCLUSION

We identified five polymorphisms and interactions that contributed crucially to predicting HCC risk. These findings represented an important step towards improving HCC diagnosis and management.

Key words: ERCC1; XRCC3; GST-P1; CYP17A1; MDR-1; Polymorphisms; Hepatocellular carcinoma risk; Early diagnosis; Antiviral therapy; Hepatitis B viral/hepatitis C viral

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Core tip: It is a great challenge to define new biomarkers of hepatocellular carcinoma (HCC) risk for HCC management. This work identified five genetic markers in key pathways linked to hepatocarcinogenesis (*ERCC1* rs3212986, *GSTP1* rs1138272, *CYP17A1* rs743572, *XRCC3* rs1799794, and *ABCB1* rs112850), which could predict individual HCC susceptibility, particularly in a high-risk [hepatitis B viral/hepatitis C viral (HCV)-infected] Caucasian population. We also identified potential gene-gene interactions that should be included in the definition of HCC risk. These findings could contribute to improved HCC surveillance, early cancer diagnoses, and potential curative therapies. For patients with HCV, these markers could be considered selection criteria for the personalized use of recently developed, highly expensive anti-viral therapies.

De Mattia E, Cecchin E, Polesel J, Bignucolo A, Roncato R, Lupo F, Crovatto M, Buonadonna A, Tiribelli C, Toffoli G. Genetic biomarkers for hepatocellular cancer risk in a caucasian population. *World J Gastroenterol* 2017; 23(36): 6674-6684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6674.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6674

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a particularly high prevalence in some areas of Asia and Africa. HCC represents the second leading cause of cancer-related deaths[1]. Although liver cancer is less frequent in Western developed countries, recent data indicate that, due to dissemination of hepatitis B (HBV) and C (HCV) viral infections, the HCC incidence is also dramatically rising, in both the United States and Europe[1,2].

Unlike most malignancies, the major risk factors for HCC development are well-defined. These risk factors are chronic HCV and HBV infections, liver cirrhosis, heavy alcohol intake, tobacco smoking, exposure to environmental and dietary carcinogens (*i.e.,* aflatoxin B1), genetic and metabolic liver disease (*i.e.,* hereditary hemochromatosis, non-alcoholic steatohepatitis), and other conditions capable of inducing liver damage[2]. Moreover, the prevalence of HCC increases with age and male sex[1-3].

In addition to environmental factors, recent insights into the biology of HCC have demonstrated that accumulations of genetic and epigenetic abnormalities can also play an essential role in liver carcinogenesis. In particular, data derived from candidate-gene investigations and, more recently, genome-wide associations studies have highlighted the possible role that genetic variants might play as significant determinants of HCC susceptibility[4-6]. Additionally, host genetic factors were shown to affect the clinical course of HBV or HCV infections, because they

influence the individual's predisposition to disease progression and hepatocarcinogenesis[7,8]. However, the currently available studies are highly heterogeneous in study design, sample size, ethnicity, and clinical-demographic features of the investigated population; these differences make it difficult to draw solid conclusions[4,6]. Moreover, the majority of case-control investigations were performed with Asian populations; thus, replication studies with different ethnicities are warranted[4-8].

It is of great clinical interest to identify genetic traits that can modify liver carcinogenesis, because they can potentially be used to improve preventive, diagnostic, and therapeutic strategies in HCC management[3,9,10]. To date, the majority of patients with HCC are diagnosed at an advanced tumor stage, which precludes potentially curative therapies, including orthotopic liver transplantation (OLT), surgical resection, and local ablation[3,9,10]. The HCC surveillance programs that could lead to early cancer detection and effective treatment are currently based on highly heterogeneous scoring systems that require costly measures[3,9,10]. The identification of additional molecular biomarkers predictive of individual HCC susceptibility, particularly in a high-risk population (*i.e.,* HBV/HCV-positive), could be important in improving current guidelines for genetics-based patient screening and preventive strategies. In some countries, including the United States and Europe, HCV-related HCC accounts for most liver cancer incidents[11]. Consequently, an increased understanding of the genetic contribution to the clinical course of viral infection could be essential in optimizing the use of newly developed, but highly expensive, direct acting antiviral (DAA) treatments[12,13].

We previously reported that the low-activity alleles, *UGT1A1**28, *UGT1A7**3, *UGT1A9**22, and related haplotypes may play a protective role against HCC development in Caucasian populations. These alleles positively modulate the serum levels of beneficial molecules, like bilirubin[14]. The present study aimed to extend genetic analyses by identifying additional molecular markers that could be integrated into the HCC risk stratification algorithms used in liver cancer prevention strategies. To that end, we analyzed a set of potential candidate HCC-risk markers with low penetrance. These candidate markers were based on functionally relevant polymorphic variants in genes that encoded proteins involved in key pathways underlying carcinogenesis[6,15-17]. The pathways we investigated were: (1) membrane transporters that play an essential role in xenobiotic defense and prevent the entry of toxic environmental compounds that might damage liver tissue; (2) DNA synthesis and repair mechanisms that modulate the cell's capacity to respond to DNA damage induced by toxic agents, and thus, these mechanisms affect the accumulation of mutations in DNA; (3) detoxification systems that facilitate the elimination of detrimental endo/exogenous compounds; and (4) systems that defend against oxidative stress and related cellular damage, which can play a crucial role in the development and progression of HCC. We designed a retrospective case-control study and enrolled a homogeneous group of patients with HCC that had received OLT and two age/sex-matched control groups (a group positive for HBV and/or HCV and a healthy population). We aimed to identify novel genetic markers that could contribute to the determination of individual HCC risk, particularly in Caucasian populations.

MATERIALS AND METHODS

Study population

We studied 192 patients with histologically confirmed HCC that had undergone OLTs between May 1991 and March 2006 at the Liver Transplantation Center of the S. Giovanni Battista Hospital (Turin, Italy). All patients were infected with HBV and/or HCV and had a cirrhotic liver. The first control group included 167 subjects with HBV and/or HCV infections, but no evidence of cancer (including HCC). These subjects were followed up for hepatitis infections between November 2009 and September 2010 at the Department of Laboratory Medicine, S. Maria degli Angeli Hospital (Pordenone, Italy). This control group was matched to the HCC group to ensure equivalent percentages of patients with HBV and/or HCV and similar distributions of gender and age (in quinquennia), when possible.

The second control group included 192 healthy subjects that were matched to patients with HCC for gender and age (in quinquennia). For this control group, individuals under 70 years of age were randomly selected from a pool of blood donors that visited the Centro di Riferimento Oncologico National Cancer Institute in Aviano, Italy. This control group also included individuals aged $\geqslant$ 70 years that were cancer-free without HBV/HCV infections. These individuals had been approached during occasional blood testing at the S. Maria degli Angeli Hospital, and they consented to provide a 5-mL blood sample for research purposes.

All cases and controls were Italian Caucasian individuals, and they all provided written informed consent for genetic analysis and for inclusion in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki, and it was approved by the Institutional Review Board of each participating institution (Ethics Committee of Azienda Ospedaliera Universitaria, S. Giovanni Battista, Torino).

Polymorphism selection and genetic analyses

Genomic DNA was extracted from peripheral blood with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The samples underwent standard proteinase K digestion and successive template purification. The genomic DNA solution was stored at 4 ℃. Positive and negative control samples were included in each analysis.

We considered a set of 31 molecular markers that

Table 1 Distribution of hepatocellular carcinoma patients, hepatitis B/hepatitis C -positive individuals and healthy subjects according to socio-demographic and clinical characteristics *n* (%)

Characteristics	HCC group	HBV/HCV group	Healthy group
Sex			
Men	166 (86.5)	141 (84.4)	166 (86.5)
Women	26 (13.5)	26 (15.6)	26 (13.5)
Age (yr)			
< 55	85 (44.3)	85 (50.9)	96 (50.0)
55-59	60 (31.3)	19 (11.4)	35 (18.2)
⩾ 60	47 (24.5)	63 (37.7)	61 (31.8)
Hepatitis infection			
None	0 (0.0)	0 (0.0)	192 (100.0)
HBV+ and HCV-	74 (38.5)	74 (44.3)	0 (0.0)
HBV- and HCV+	109 (56.8)	88 (52.7)	0 (0.0)
HBV+ and HCV+	9 (4.7)	5 (3.0)	0 (0.0)
Years of orthotopic liver transplantation or resection			
1991-1995	27 (14.1)		
1996-2000	60 (31.3)		
2001-2006	102 (54.7)		
Number of nodes			
1	110 (57.3)		
⩾ 2	72 (37.5)		
Unknown	10 (5.2)		
Maximal dimension of nodes (cm)			
⩽ 2	79 (41.2)		
2.1 to ⩽ 3	67 (34.9)		
> 3	37 (19.3)		
Unknown	9 (4.7)		

HCC: Hepatocellular carcinoma; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

represented functionally relevant polymorphisms (PubMed-MEDLINE search) in genes that encoded key proteins involved in hepatocarcinogenesis-related pathways. In particular, the biological processes evaluated (and related genes) were: membrane transport (*ABCB1, ABCC2*), DNA synthesis and repair mechanisms (*MTHFR, TYMS, ERCC1, ERCC2, XRCC1, XRCC3, APE1, hMLH1, hMSH2, hOOG1*), detoxifying systems (*CYP3A4, CYP3A5, CYP17A1, CYP2D6*), and oxidative stress response (*GST-M1, GST-T1, GST-P1; GSTA1, SOD2*).

All the genes, variants, and assays applied are listed in Supplementary Table 1. For pyrosequencing, we used PSQ96MA (Qiagen, Hilden, Germany). PCR amplifications were performed in an Eppendorf Mastercycler gradient, with TaqGold DNA Polymerase (AB Applied Biosystems, Foster City, CA, United States). The details of the pyrosequencing assays, primer sequences, and PCR conditions are available upon request. Pre-designed TaqMan single-nucleotide polymorphism genotyping assays were conducted with the Applera TaqMan Universal Master mix on an ABI 7900HT Real-time PCR system (AB Applied Biosystems, Foster City, CA, United States), according to the manufacturer's instructions for optimal allelic discrimination. All commercial TaqMan assays were purchased from the Applied Biosystems website (www.appliedbiosystems.com). Detailed protocols for genotyping methods, based on gel electrophoresis and enzymatic digestion, are available upon request.

Statistical analysis

In this study, we assessed associations between genetic markers and the individual risk of developing HCC. In healthy controls, deviation from Hardy-Weinberg equilibrium was tested for each polymorphism with the χ^2 test, and no deviation was found ($P < 0.05$).

The odds ratio (OR) and 95%CI were estimated with unconditional logistic regression, which was controlled for matching variables. We also investigated three genetic models (dominant, recessive, and additive) for associations, and we reported the most statistically significant one, based on the Wald χ^2-test. Statistical significance was set at $P < 0.05$ (two-sided). The analyses were performed with SAS 9.2, and a statistical review of the study was performed by a biomedical statistician.

Recursive partitioning was performed to elucidate high-order relationships among genetic factors, when stratifying patients between HCC cases and HBV/HCV group. The analysis was carried out with the five genetic variants that were identified in the present study as significant predictors of HCC risk and with the previously described *UGT1A* markers (*i.e., UGT1A1**28, *UGT1A9**22, *UGT1A7**3)[14].

RESULTS

Patient characteristics

The demographic, clinical, and serological characteristics of the 192 patients with HCC and the two control groups are reported in Table 1. The predominant gender was male (86.5% of patients with HCC, 84.4% of patients in the HBV/HCV group, and 86.5% of healthy subjects). The median ages at the time of enrolment were 56, 54 and 55 years for the HCC, HBV/HCV, and healthy groups, respectively. The HCC cohort was clinicopathologically homogeneous; all patients fulfilled the surgical intervention criteria (*i.e.,* T1/T2 primary tumor stage, evaluated with the TNM scale; well or moderately poorly differentiated grade at diagnosis; and no detectable vascular invasion).

Genetic polymorphisms and HCC risk

The average genotype call rates were 0.98 (range: 0.92-1.00), 1.00 (range: 0.99-1.00), and 0.99 (range: 0.95-1.00) for the HCC, HBV/HCV, and healthy groups, respectively. All case and control samples were included in the study, because they all reached the fixed call rate threshold of 90%.

HCC *vs* HBV/HCV group: Considering that chronic HBV and/or HCV infections represent a major risk factor for HCC development, a case/control analysis was initially performed to compare the genotype frequency distribution between patients with HCC and individuals

Table 2 Distribution of gene polymorphisms in patients with hepatocellular carcinoma, in hepatitis B/hepatitis C -positive patients, and in healthy controls *n* (%)

Gene	SNP	Genotype	HCC group	HBV/HCV group	Healthy group
ERCC1	rs3212986	GG	126 (67.7)	82 (49.1)	91 (47.4)
		GT	44 (23.7)	78 (46.7)	81 (42.2)
		TT	16 (8.6)	7 (4.2)	20 (10.4)
XRCC3	rs1799794	AA	128 (66.7)	108 (65.1)	137 (71.7)
		AG	52 (27.1)	55 (33.1)	49 (25.7)
		GG	12 (6.3)	3 (1.8)	5 (2.6)
GST-P1	rs1138272	CC	172 (92.5)	139 (83.2)	182 (94.8)
		CT	13 (7.0)	27 (16.2)	10 (5.2)
		TT	1 (0.5)	1 (0.6)	0 (0.0)
CYP17A1	rs743572	TT	72 (38.1)	39 (23.4)	54 (28.4)
		TC	84 (44.4)	88 (52.7)	91 (47.9)
		CC	33 (17.5)	40 (24.0)	45 (23.7)
ABCB1	rs1128503	CC	54 (28.1)	52 (31.1)	58 (30.2)
		CT	92 (47.9)	93 (55.7)	95 (49.5)
		TT	46 (24.0)	22 (13.2)	39 (20.3)

HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

without HCC, but with matched HBV/HCV infections. The frequency distributions of variant genotypes were significantly different between cases and controls (Tables 2 and 3) for five polymorphisms. No Hardy-Weinberg disequilibrium among healthy controls was detected ($P > 0.05$), which suggested that it was a representative sampling of the investigated population. The observed genotype frequency distribution in healthy controls was consistent with data published in the literature for a Caucasian population (www.ncbi.nlm.nih.gov/snp).

Specifically, *ERCC1* rs3212986 (OR = 0.46, $P = 0.0005$), *GST-P1* rs1138272 (OR = 0.41, $P = 0.0097$), and *CYP17A1* rs743572 (OR = 0.50, $P = 0.0032$) markers were observed more frequently in the HBV/HCV group than in the HCC group, and they were significantly associated with reduced liver cancer risk, according to the dominant model. In contrast, the frequency of *XRCC3* rs1799794 (OR = 3.70; $P = 0.0461$) and *ABCB1* rs1128503 (OR = 2.06; $P = 0.0111$) markers were higher in the HCC group than in the HBV/HCV group, and they were associated with increased HCC risk, according to the recessive model (Table 3).

Given the molecular and clinicopathological differences between HBV- and HCV- related hepatocarcinogenesis[18], a similar analysis was also carried out in subgroups, divided according to viral status (Table 4). In all cases, the ORs calculated for the HBV- and HCV-positive subgroups were concordant with those obtained for the entire combined group. The association was significant in both sub-groups for *CYP17A1* rs743572 (OR = 0.45, $P = 0.0315$ and OR = 0.51, $P = 0.0384$ in HBV-positive and HCV-positive subgroups, respectively). In contrast, *ABCB1* rs1128503 (OR = 4.18, $P = 0.0048$, HBV-positive subgroup) and *ERCC1* rs3212986 (OR = 0.33, $P = 0.0003$, HCV-positive subgroup) showed significant associations in only one sub-group.

HCC *vs* healthy group: We also conducted a case case/control analysis of the genotype frequency distribution in patients with HCC compared to individuals without HCC or virus infections to evaluate the extent of the predictive value of the selected five markers (Table 3). Two markers were significantly associated with HCC risk. Specifically, *ERCC1* rs3212986 (OR = 0.43, $P < 0.0001$) and *CYP17A1* rs743572 (OR = 0.73, $P = 0.0310$) were associated with reduced HCC risk, according to the dominant and additive models, respectively.

Classification and regression tree analysis: The Classification and Regression Tree (CART) method allowed us to identify 6 subgroups of subjects according to genotype features, each with a different probability of HCC occurrence. Three of the terminal nodes exhibited high-probability (59%-76%), two exhibited intermediate-probability (36% and 40%), and one exhibited low-probability (6%) of developing HCC (Figure 1). The ORs and 95%CIs were calculated for each high/intermediate-probability group, with respect to the reference low-probability group. The group with the highest percentage of HCC cases (group 5) was associated with an approximately 13-fold higher HCC risk compared to the reference low-probability group. The first node of the tree represented the *ERCC1* rs3212986 variant, which significantly interacted with *CYP17A1* rs743572, *GST-P1* rs1138272, and the *UGT1A7**3 marker in the generation of the tree (Figure 1).

DISCUSSION

HCC is a global healthcare problem. It is one of the most common malignancies worldwide, with an increasing incidence in Western developed countries. It represents the second cause of death attributable to cancer[1,2]. The genetic origin of HCC has been a focus of research in the past few years. Some studies found that genetic variants could be predictive of the clinical course of HBV or HCV and an individual's predisposition to developing liver cancer[4-8]. However, those studies were performed mainly in Asian populations; thus, few data are available on Caucasian populations. The ethnicity of the population being studied is a crucial factor in case-control studies, because polymorphism frequency varies greatly with geographical origin. This association could give rise to ethnicity-specific phenotypic effects[4,14]. The present study revealed some novel genetic markers that could be used as early diagnosis indicators for liver cancer susceptibility in Caucasians. The availability of these markers could improve HCC risk stratification algorithms and surveillance programs.

Table 3 Odds ratios and corresponding 95%CI for hepatocellular carcinoma compared to hepatitis B/hepatitis C infected patients and blood donors according to genetic polymorphisms

Gene	SNP	Allelic change	HCC *vs* HBV/HCV			HCC *vs* Healthy		
			Mod	OR (95%CI)[1]	*P* value	Mod	OR (95%CI)[2]	*P* value
ERCC1	rs3212986	G>T	Dom	0.46 (0.30-0.71)	0.0005[b]	Dom	0.43 (0.28-0.65)	< 0.0001[b]
XRCC3	rs1799794	A>G	Rec	3.70 (1.02-13.39)	0.0461[a]	Rec	2.44 (0.84-7.08)	0.1003
GST-P1	rs1138272	C>T	Dom	0.41 (0.21-0.81)	0.0097[b]	Add	1.58 (0.70-3.57)	0.2699
CYP17A1	rs743572	T>C	Dom	0.50 (0.31-0.79)	0.0032[b]	Add	0.73 (0.55-0.97)	0.0310[a]
ABCB1	rs1128503	C>T	Rec	2.06 (1.18-3.61)	0.0111[a]	Dom	1.22 (0.75-1.98)	0.4194

[1]Estimated through logistic regression model, adjusted for sex, age and HBV and/or HCV infection; [2]Estimated through logistic regression model, adjusted for sex and age. [a]*P* < 0.05, [b]*P*< 0.01. HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

Table 4 Odds ratios and corresponding 95%CI for hepatocellular carcinoma compared to hepatitis B/hepatitis C infected patients according to the viral status

Gene	SNP	Allelic change	Mod	HCC *vs* HBV/HCV			
				HBV-positive		HCV-positive	
				OR (95%CI)[1]	*P* value	OR (95%CI)[1]	*P* value
ERCC1	rs3212986	G>T	Dom	0.59 (0.30-1.15)	0.1231	0.33 (0.18-0.60)	0.0003[b]
XRCC3	rs1799794	A>G	Rec	2.23 (0.39-12.82)	0.3695	6.73 (0.82-55.03)	0.0753
GST-P1	rs1138272	C>T	Dom	0.40 (0.14-1.15)	0.0881	0.41 (0.16-1.02)	0.0558
CYP17A1	rs743572	T>C	Dom	0.45 (0.22-0.93)	0.0315[a]	0.51 (0.27-0.97)	0.0384[a]
ABCB1	rs1128503	C>T	Rec	4.18 (1.55-11.29)	0.0048[b]	1.49 (0.71-3.11)	0.2893

[1]Estimated through logistic regression model, adjusted for sex, age and HBV and/or HCV infection. [a]*P* < 0.05, [b]*P* < 0.01. HCC: Hepatocellular carcinoma; SNP: Single-nucleotide polymorphism; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

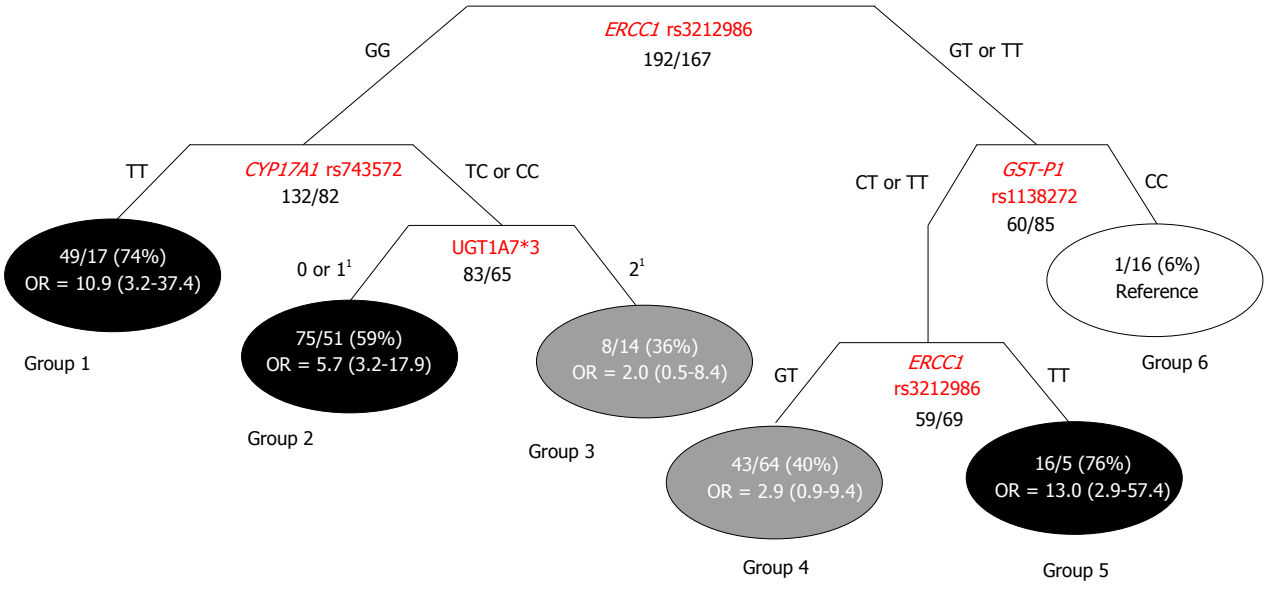


Figure 1 Classification and regression tree representation of the markers combination significantly predictive of hepatocellular carcinoma risk. Fractions indicate the number of HCC cases patients *vs* number of HBV/HCV infected patients (percentage of cases in parenthesis). Black circles represent terminal nodes with high probability to develop hepatocellular carcinoma (HCC) (ratio ⩾ 70%); gray circles represent terminal nodes with intermediate probability to develop HCC (30 ⩽ ratio < 70%); white circles represent terminal nodes with low probability to develop HCC (ratio < 30%). Odds ratios (OR) and 95%CI were calculated for each group in respect to the reference group (lower HCC risk) through logistic regression model, adjusted for sex, age and viral status. [1]Number of alleles carried by the patient. CART: Classification and regression tree; HCC: Hepatocellular carcinoma; HBV: Hepatitis B viral; HCV: Hepatitis C viral.

The main result of this work was the identification of five polymorphisms (*ERCC1* rs3212986, *GST-P1* rs1138272, *CYP17A1* rs743572, *XRCC3* rs1799794, and *ABCB1* rs1128503) that could identify sub-populations of patients among high-risk Caucasian individuals (HBV/HCV-positive), which had differential predispositions for HCC development. The *ABCB1* rs1128503 and *ERCC1* rs3212986 variants displayed different effects, based on the viral status of the patient (HBV- or HCV-positive serology). This result was consistent with the

molecular and clinical-pathological differences between HBV- and HCV-related hepatocarcinogenesis[18]. Two polymorphisms (*ERCC1* rs3212986, and *CYP17A1* rs743572) could also predict HCC risk among the healthy Caucasian population. A CART analysis provided evidence of gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 marker. These interactions defined subgroups of patients with genetic combinations that could further increase the risk of HCC.

The *ERCC1* rs3212986 (8092G>T) polymorphism was demonstrated to be a strong protective factor against liver cancer development in both high-risk (HCV/HBV-positive) and healthy individuals. Furthermore, in an analysis of subgroups with different viral statuses, a particularly significant effect was detected in the HCV-positive subgroup.

ERCC1 is one of several key rate-limiting enzymes in the nucleotide excision repair (NER) pathway. The NER mechanism of the DNA repair system maintains genomic integrity by removing inter-strand DNA crosslinks, and thus, it influences the cell's sensitivity to DNA-adducting carcinogens[19]. A NER-related reduction in DNA repair capacity has been associated with some types of tumors (*i.e.,* lung, head and neck, prostate, glioma, ovarian cancers). Accordingly, *ERCC1* gene variants were shown to influence susceptibility to cancer[20,21]. However, few studies have investigated the implication of the NER biological pathway and *ERCC1* polymorphisms, including rs3212986[20,21], in hepatocarcinogenesis, and no data are available in Caucasian populations. The *ERCC1* rs3212986 variant, located in the 3' untranslated region (3'UTR) of the gene, is thought to increase transcript stability, although further biochemical studies are required to clarify the effect of this polymorphism on gene transcription and protein translation[22,23]. Based on our data, we inferred that the rs3212986 variant allele was associated with elevated ERCC1 expression and/or activity, which could lead to enhanced protection against the accumulation of mutagenic DNA lesions, and consequently, against hepatocarcinogenesis. However, we could not exclude that this marker might also be closely linked to other functionally relevant genetic variants[21] that might alter ERCC activity and/or HCC onset.

The *XRCC3* rs1799794 (4541A>G) marker was associated with elevated HCC susceptibility in a high-risk (HCV/HBV-positive) population. XRCC3 is another DNA repair protein that participates in the DNA double-strand break/recombination repair machinery. XRCC3 was shown to be involved in the pathogenesis of many cancers, including HCC[24,25]. Reduced XRCC3 activity was associated with significantly elevated levels of bulky DNA adducts[26]. Furthermore, epidemiological evidence, derived mainly from studies in an Asian population, have shown that XRCC3 contributed to the repair of DNA damage induced by environmental toxins, such as aflatoxin B1, and that this effect depended on viral status and ethnicity[25,27]. The *XRCC3* rs1799794 variant, located in a regulatory region in the 5'UTR of the gene, was predicted to have a potential effect on protein expression levels[28]. The results of the present study suggested that this polymorphism could reduce the ability to repair DNA adducts, which would contribute to the accumulation of mutagenic lesions, and thus, increase the risk of HCC onset. However, the exact molecular mechanism of the rs1799794 variant is currently unknown, and we could not exclude a linkage with another functional marker[29].

The *GST-P1* rs1138272 (341C>T) polymorphism was found to have a protective effect on liver cancer development in a high-risk HCV/HBV-positive population. GST-P1 belongs to the GST multigene family. It is a phase Ⅱ enzyme involved in the inactivation of electrophilic xenobiotics by conjugation with glutathione[30], which facilitates the excretion of molecules that are potentially toxic to the liver[31]. This enzyme also contributes to protecting liver cells from damage related to reactive oxygen species generated, for instance, by chronic alcohol and tobacco abuse[32]. Several studies have clearly demonstrated that silencing GST-P1 expression by promoter methylation was positively correlated with the incidence of HCC[31] and with virus-related hepatocarcinogenesis[33]. In contrast, in meta-analyses, *GST-P1* genetic polymorphisms (*i.e.,* Ile105Val, rs1695) were not associated with HCC risk[34,35] although the few data available were insufficient for drawing any definite conclusions. The less investigated *GST-P1* rs1138272 polymorphism is a missense variant (Val114Ala) that lies in the electron binding, active domain of the protein. This variant was reported to affect substrate specificity by altering its ability to distinguish between planar and non-planar substrates[36,37]. Our results suggested that this functional change in GST-P1 activity may result in a lower risk of HCC development; further clinical experimental studies are required to clarify the role of the rs1138272 polymorphism in the response of GST-P1 to environmental xenobiotics and oxidative stress, particularly in a virus-infected liver.

The *CYP17A1* rs743572 (-34T>C) polymorphism was found to be a strong protective factor against HCC development in both the high-risk HCV/HBV-positive group and healthy individuals. CYP17A1 is an enzyme that plays a critical role in the biosynthesis of steroid hormones by catalyzing both 17a-hydroxylase and 17, 20-lyase activities[38]. Some data derived from animal models and human epidemiologic studies have demonstrated that estrogens play an essential role in hepatocarcinogenesis; however, the exact mechanism underlying the effect of estrogen levels on HCC development is currently controversial[39]. It was initially suggested that the *CYP17A1* rs743572 variant, located in the 5'UTR of the gene, served as a new Sp1-type (CCACC box) binding site, which provided an additional promoter, and thus, it could increase estrogen biosynthesis[40]. However, subsequent studies could not replicate the phenotypic impact of this

variant on CYP17A1 mRNA expression or estrogen levels[41]. Moreover, the involvement of the rs743572 marker on HCC development was not well defined[42,43]. From the present data, it could be speculated that the rs743572 variant might contribute to changing CYP17A1 activity, and its dependent steroid levels, to provide a protective effect against liver cancer development. Estrogen is known to participate in various biological functions. For example, an increase in sex hormone levels was suggested to suppress the inflammatory processes mediated by pro-inflammatory cytokines[44,45]. Additional work will be required to clarify the functional significance of the rs743572 marker and the molecular mechanism of CYP17A1 in hepatocellular carcinogenesis.

Finally, A*BCB1* rs1128503 (1236C>T) was associated with elevated HCC susceptibility in a high-risk (HCV/HBV-positive) population. Furthermore, an analysis of subgroups with different viral statuses showed that a particularly significant effect was detected in the HBV-positive subgroup. The *ABCB1* gene encodes MDR-1, also known as P-glycoprotein (P-gp). MDR-1 belongs to the ATP-binding cassette (ABC) transporter superfamily[46,47]. This membrane efflux pump is widely expressed in various organs, including the liver, and it is physiologically involved in the protection of normal tissue from environmental and endogenous toxins[46-48]. MDR1 is implicated in hepatic clearance of oxidative and other genotoxic products generated by chronic inflammation induced by HCC risk factors. Thus, it contributes to preventing the initiation of hepatocarcinogenesis[49,50]. Many studies have described MDR1 over-expression in various tumor tissues, including liver cancer[51]; thus, this membrane carrier and its functional genetic variants are good candidates for influencing liver susceptibility to HCC. *ABCB1* rs1128503 is a synonymous variant (Gly412Gly) of a residue located in the cytoplasmic domain of the transporter. It is reported to affect mRNA stability[52], but the exact phenotypic impact of this variant on protein expression/activity remains to be defined[53,54]. Several studies, performed in Asian populations, have investigated associations between *ABCB1* polymorphisms (including rs1128503) and the risk of developing HCC. A recent meta-analysis[55] showed that polymorphic alleles of the *ABCB1* gene significantly increased HCC risk. In analyses of subgroups with different types of variations, both synonymous and non-synonymous polymorphisms were associated with elevated liver cancer susceptibility. In particular, synonymous variants (*i.e.*, rs1128503) were suggested to be correlated with changes in transcriptional and translational processes. In addition, genetic markers located in the cytoplasmic domain of the transporter, like rs1128503, were shown to have the greatest predictive power for HCC risk. These data, although obtained in Asian cohorts, were consistent with the result of the present study, which provided the first evidence of a role of the *ABCB1* rs1128503 marker in HCC risk in a Caucasian population.

Due to the multifactorial nature of hepatocarcinogenesis, an exploratory CART analysis was applied to investigate interactions between the genetic factors identified as predictors of liver cancer susceptibility in the high-risk population (HBV/HCV-infected group). The polymorphism in the gene of the DNA repair system (*i.e., ERCC1* rs3212986) was demonstrated to significantly interact with variants of genes that encoded phase Ⅰ (*CYP17A1* rs74357) and Ⅱ (*GST-P1* rs1138272, *UGT1A7**3) metabolic enzymes; these interactions were important in defining individual HCC risk. The CART analysis generated a distinctive clustering of subjects, according to different probabilities of developing liver cancer. These data pointed out that previously acknowledged predictive markers, such as the *UGT1A*3* polymorphism[14], should be considered in association with novel identified genetic variants for properly defining the complex trait of HCC susceptibility. In particular, *ERCC1* rs3212986 was shown to be the most relevant polymorphism for HCC risk stratification, because it appeared twice in the generated tree. This marker significantly interacted with three additional genetic variants (*CYP17A1* rs74357, *GST-P1* rs1138272, and *UGT1A7**3) in defining individual susceptibility. The analysis of gene-gene interactions and the network shape uncovered specific genetic-context effects that could not be detected in evaluations of single markers. These findings highlighted the importance of combining genetic markers to obtain a better representation of the biological cooperation among multiple pathways in hepatocarcinogenesis.

The present study had some limitations. First, our case-cohort comprised a highly specific group of patients with HCC (patients that received OLT). These patients had definite clinical features that could limit the generalizability of the present findings. The validity of the present results was also limited to Caucasian individuals; the results remain to be verified in other populations, like Asians or Africans. Second, our work did not evaluate other confounding etiological factors, such as smoking and alcohol intake. However, the use of a control group matched to HCC cases for HBV/HCV viral status permitted us to control for this crucial confounding variable. Third, the exact functional and phenotypic properties of some of the genetic markers that we identified as predictors of HCC risk have not been well established. Moreover, we could not exclude any linkages with other functionally relevant genetic variants. Finally, considering the retrospective and exploratory nature of our case-control analysis, the results obtained should be interpreted with caution. Our findings will require independent validation in future prospective, large-scale epidemiological investigations that are performed in well-defined, homogeneous populations.

The validation of the present findings could have important clinical implications for improving HCC

management. The novel identified genetic markers and their gene-gene interactions, in combination with other well-known serum biomarkers (*e.g.*, alphafetoprotein) and diagnostic imaging (*e.g.*, ultrasonography)[9], could contribute to improving the HCC risk stratification algorithms and surveillance programs, particularly for patients at risk. Those improvements might facilitate early cancer detection and might lead to potentially curative therapies. Furthermore, an enhanced understanding of the genetic factors associated with the evolution of HCV infection could be essential for the optimized use of the recently developed, but highly expensive, anti-HCV DAAs[12,13]. Thus, improved screening could assist the health care system in containing costs and ensuring the most clinically appropriate treatments.

The present study identified five variants in genes that encode DNA repair system proteins (*i.e.*, ERCC1, XRCC3), phase Ⅰ (*i.e.*, CYP17A1) and Ⅱ (*i.e.*, GST-P1) enzymes, and a membrane transporter (*i.e.*, MDR-1). Our data suggested that these genes might play a role in predicting HCC susceptibility in a high-risk (HBV-HCV infected) population and in a healthy Caucasian population. The CART analysis pointed out the potential value of studying of gene-gene interactions for the stratification of individuals at different risks of developing HCC. Although validation is required, these preliminary results might have important clinical implications in the management of HCC prevention and patient care.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) represents a global health problem, due to its high incidence and mortality rates. Although the etiologies of this neoplasia are well-defined, the majority of patients are diagnosed at advanced-stage disease, which excludes them from receiving a potentially curative therapy. Hence, they urgently need the definition of new biomarkers of HCC susceptibility that can permit early cancer detection, particularly for individuals in a high-risk population [hepatitis B (HBV)/hepatitis C (HCV)-positive]. Moreover, in developed countries (*i.e.,* United States and Europe), HCV-related HCC can account for most liver cancers; therefore, increasing our knowledge of the genetic contribution to the clinical course of viral infections could be essential in optimizing the use of the newly developed, but highly expensive, direct acting antiviral treatments.

Research frontiers

Accumulating data derived from candidate-gene investigations and genome-wide association studies have highlighted the possible role of genetic variants as significant predictors of HCC susceptibility and as determinants of the clinical course of HBV or HCV infections. However, the high heterogeneity among published works has prevented drawing definite conclusions. Furthermore, the majority of case-control investigations were performed in Asian populations; thus, replication studies with different ethnicities are warranted. Consequently, there is a critical need to intensify research efforts in identifying effective molecular biomarkers predictive of individual HCC risk in a homogeneous, well-defined cohort study, with particular attention to the (currently) poorly investigated Caucasian population.

Innovations and breakthroughs

This work uncovered five markers in genes that encode key proteins involved in pathways underlying hepatocarcinogenesis (*ERCC1*-rs3212986, *GSTP1*-rs1138272, *CYP17A1*-rs743572, *XRCC3*- rs1799794, and *ABCB1*-rs112850). These markers were predictive of individual HCC susceptibility in a high-risk Caucasian population (HBV/HCV-positive). Two variants also exhibited serology-specific effects when a subgroup analysis was performed in patients stratified according to HBV or HCV positive serology. That analysis showed that *ABCB1* rs1128503 specifically affected the HBV-positive subgroup and *ERCC1* rs3212986 specifically affected the HCV-positive subgroup. Among the five markers identified, *ERCC1* rs3212986 and *CYP17A1* rs743572 retained predictive value for HCC risk among healthy individuals. In addition, they found gene-gene interactions between *ERCC1* rs3212986, *CYP17A1* rs743572, *GST-P1* rs1138272, and the previously described *UGT1A7**3 marker, which were included in the definition of the complex trait of HCC susceptibility.

Applications

The definition of genetic traits that confer susceptibility to liver carcinogenesis is of potential clinical interest. This information can facilitate improvements in preventive, diagnostic, and therapeutic strategies for HCC management. If validated, these markers and their interactions should be considered for improving HCC risk stratification algorithms and surveillance programs. Their inclusion in screening could facilitate early cancer detection and might lead to potentially curative therapies. For patients with HCV infections, the biomarkers we discovered could also contribute to optimizing the use of recently developed, but highly expensive, anti-viral therapies. These findings address a great challenge in HCC management and might help enhance care for patients with HCC.

Terminology

Polymorphism: a variation in the DNA sequence that occurs in a population at a frequency of 1% or higher; CART: classification and regression tree analysis; this is a statistical method, based on recursive partitioning, that is applied to elucidate potential high-order gene-gene and gene-environmental relationships among discriminating factors that contribute to a specific end-point; ERCC1: a key rate-limiting enzyme that acts in the nucleotide excision repair pathway. This pathway represents a DNA repair mechanism designed to maintain genomic integrity by removing inter-strand DNA crosslinks; XRCC3: a DNA repair protein that participates in DNA double-strand break/recombination repair machinery; GST-P1: a phase Ⅱ enzyme involved in the inactivation of electrophilic xenobiotics; it acts by conjugating with glutathione, which facilitates excretion of xenobiotics from the body; CYP17A1: an enzyme that plays a critical role in the in the biosynthesis of steroid hormones; MDR-1 (encoded by *ABCB1*): A widely expressed membrane efflux pump, physiologically involved in the protection of normal tissue from environmental and endogenous toxins.

Peer-review

The aim of this study was to find novel genetic markers that could contribute in predicting HCC susceptibility in Caucasians. The authors identified five genetic markers in key pathways linked to hepatocarcinogenesis (ERCC1-rs3212986, GSTP1-rs1138272, CYP17A1-rs743572, XRCC3- rs1799794, ABCB1-rs112850) that could predict the individual HCC susceptibility, especially in high-risk (HBV/HCV-infected) Caucasians population. The article provides the novel informations that could contribute to improving HCC surveillance, allowing early cancer diagnosis and possible curative therapy.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic value of lymphovascular invasion in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma

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Abstract

AIM

To assess the prognostic value of lymphovascular invasion (LVI) in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma (HC) patients.

METHODS

A retrospective analysis was performed on 142 consecutively recruited type Ⅳ HC patients undergoing radical resection with at least 5 years of follow-up. Survival analysis was performed by the Kaplan-Meier method, and the association between the clinicopathologic variables and survival was evaluated by log-rank test. Multivariate analysis was adopted to identify the independent prognostic factors for overall survival (OS) and disease-free survival (DFS). Multiple logistic regression analysis was performed to determine the association between LVI and potential variables.

RESULTS

LVI was confirmed histopathologically in 29 (20.4%) patients. Multivariate analysis showed that positive resection margin (HR = 6.255, 95%CI: 3.485-11.229, P < 0.001), N1 stage (HR = 2.902, 95%CI: 1.132-7.439, P = 0.027), tumor size > 30 mm (HR = 1.942, 95%CI: 1.176-3.209, P = 0.010) and LVI positivity (HR = 2.799, 95%CI: 1.588-4.935, P < 0.001) were adverse

prognostic factors for DFS. The independent risk factors for OS were positive resection margin (HR = 6.776, 95%CI: 3.988-11.479, $P < 0.001$), N1 stage (HR = 2.827, 95%CI: 1.243-6.429, $P = 0.013$), tumor size > 30 mm (HR = 1.739, 95%CI: 1.101-2.745, $P = 0.018$) and LVI positivity (HR = 2.908, 95%CI: 1.712-4.938, $P < 0.001$). LVI was associated with N1 stage and tumor size > 30 mm. Multiple logistic regression analysis indicated that N1 stage (HR = 3.312, 95%CI: 1.338-8.198, $P = 0.026$) and tumor size > 30 mm (HR = 3.258, 95%CI: 1.288-8.236, $P = 0.013$) were associated with LVI.

CONCLUSION

LVI is associated with N1 stage and tumor size > 30 mm and adversely influences DFS and OS in type Ⅳ HC patients.

Key words: Bismuth-Corlette classification; Disease-free survival; Lymphovascular invasion; Overall survival; Hilar cholangiocarcinoma

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Core tip: Previous studies have reported that lymphovascular invasion (LVI) provokes an adverse impact on the long-term survival of several malignances, including breast, gastric, and esophageal carcinoma, among many others. However, the correlation between LVI and hilar cholangiocarcinoma remains unclear. In our study, LVI was found to be an independent risk factor for overall survival and disease-free survival in Bismuth-Corlette type Ⅳ hilar cholangiocarcinoma patients. To our knowledge, this report indicates for the first time that LVI is an adverse predictor of long-term survival in the setting of type Ⅳ hilar cholangiocarcinoma.

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INTRODUCTION

Hilar cholangiocarcinoma (HC), also known as Klatskin tumor, is a neoplasia arising from the biliary epithelium at the common hepatic duct bifurcation and may extend to the intrahepatic biliary tree and liver[1,2]. The only clinical approach that is considered to provide patients with an opportunity for a curative outcome and importantly long-term survival is radical surgical resection[2-6]. The Bismuth-Corlette classification is the most widely used preoperative system of evaluation that can predict resectability of the lesion and assist in the design of an appropriate surgical approach. A Bismuth type Ⅳ lesion is defined as a tumor that can invade the secondary biliary radicals of both hepatic ducts. During the past decades, accompanied liver resection has been increasingly but gradually recognized as the mainstay of surgical approaches for targeting a Bismuth type Ⅳ tumor[7-9], however, there are relatively few studies that have reported in any detail the factors that might affect long-term survival of type Ⅳ HC patients. The over-arching aim of the current study was to identify prognostic factors for long-term survival of patients following radical surgery for type Ⅳ HC patients, especially in the setting of lymphovascular invasion (LVI).

MATERIALS AND METHODS

Patient selection

One hundred and forty-two consecutive patients who underwent radical resection for a pathological diagnosis of type Ⅳ HC at the West China Hospital between January 2000 and February 2012 were enrolled in this study and then reviewed retrospectively. The inclusion criteria included the following: (1) patients who were confirmed with Bismuth type Ⅳ HC by pathological examination; and (2) patients who had undergone radical resection (R0 and R1 resection). The exclusion criteria included the following: (1) patients with gallbladder or intrahepatic cholangiocarcinoma extending to the hilum; (2) presence of a recurrent or metastatic tumor; and (3) R2 resection.

Preoperative workup

Preoperative assessment consisted of acquiring a medical history, physical examination, laboratory tests and radiographic analyses. All patients were evaluated by contrast-enhanced ultrasound, contrast-enhanced computed tomography or magnetic resonance cholangiography with magnetic resonance cholangiopancreatography with the intention of determining the Bismuth type, the location and extent of the tumor. Biliary drainage, including endoscopic retrograde cholangiopancreatography (ENBD) and percutaneous transhepatic cholangiodrainage (PTCD), was applied in the setting of patients presenting with obstructive jaundice that exceeded 85 μmol/L total bilirubin.

Surgical characteristics of the patients

Based on the relative location and extent of the tumor, different types of resection were performed, which included extrahepatic bile duct resection and *en bloc* resection of the caudate lobe combined with left hemihepatectomy, right hemihepatectomy and trisectionectomy. In addition, standard regional lymph node dissection should be performed. However, under conditions where tumor metastases to the distant lymph nodes was confirmed during surgery, the surgical intervention was abandoned. According to

the American Joint Committee on Cancer (AJCC, 7th edition), the location of regional lymph nodes was defined as follows: along the common bile duct, cystic duct, portal vein and proper hepatic artery[10]. Vascular resection and reconstruction was only performed when vessels could not be detached from the tumor.

Pathological examination

The pathological evidence of cancer was determined by examination of paraffin sections. All included Bismuth type Ⅳ HC cases were histopathologically confirmed by an experienced pathologist. The presence of tumor emboli within peritumoural endothelial lined spaces was defined as LVI. Resection margin was defined as ductal (*i.e*., proximal and distal ducts) and with evidence of radial margins. The radial margin was defined as the vertical margin between the tumor edge and dissected periductal structures (*e.g*., liver parenchyma, blood vessels and adjacent fat tissues). An R0 resection was defined as the presence of a microscopically tumor-free resection margin. An R1 resection was defined as microscopic evidence of tumor tissue at the resection margin, and an R2 resection was defined as macroscopic evidence of tumor tissue at the resection margin. In this study, radical resection was defined as an R0 and R1 resection, a negative resection margin indicated R0 resection, and a positive resection margin indicated an R1 resection.

Follow-up

Whether or not chemotherapy and radiotherapy can benefit HC patients was controversial. None of the patients received postoperative routine chemotherapy or radiotherapy. All enrolled patients had routine follow-ups every 3 mo in the first year and every 6 mo subsequently until at least 5 years after the surgery. The tumor markers [serum levels of carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen], liver functions and ultrasonography were conducted. If there was a suspicion of recurrence, contrast-enhanced computed tomography or magnetic resonance imaging was further performed. Tumor recurrence was diagnosed on the basis of the combined findings of typical radiological appearance, quantification of CA19-9 levels, and clinical presentation. The date of the first suspicious radiological finding was recorded as the date of initial disease recurrence.

Statistical analysis

Patient data were retrospectively collected and statistical analyses were performed with SPSS version 19.0 (SPSS Inc. Chicago, IL, United States). Survival was described using the Kaplan-Meier method and differences between subgroups were reviewed by the log-rank test. Multivariate analysis for prognostic factors was performed using a Cox proportional hazards model to analyze variables whose *P*-value was less than 0.1 in the univariate analysis. Multiple logistical regression analysis was performed to determine the association between LVI and potential variables. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Patients' characteristics and operative outcomes

Patients' characteristics and operative outcomes are shown in Table 1. Altogether 142 patients had a radical resection for type Ⅳ HC, including 75 men and 67 women with a median age of 59 years (range: 23-78). Pre-operative biliary drainage was carried out in 105 of the 123 obstructive jaundice (total bilirubin > 85 μmol/L) patients, wherein 71 patients underwent PCTD and 34 patients underwent ENBD. Preoperative portal vein embolization was performed in 6 patients.

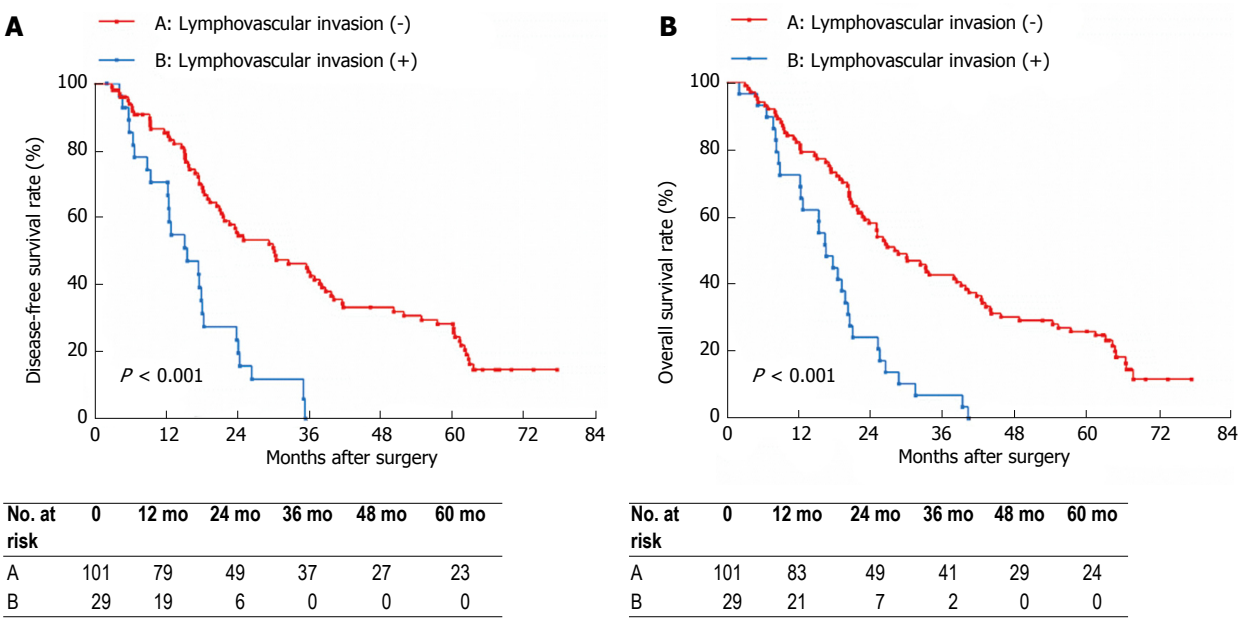
Radical resection included extrahepatic bile duct resection and *en bloc* resection of the caudate lobe combined with left hemihepatectomy (*n* = 73, 51.4%), extended left hemihepatectomy (*n* = 5, 3.5%), left trisectionectomy (*n* = 6, 4.2%), right hemihepatectomy (*n* = 51, 35.9%), extended right hemihepatectomy (*n* = 5, 3.5%), and right trisectionectomy (*n* = 2, 1.4%). Regional lymph node dissection was conventionally performed. The R0 resection rate was 75.6%.

Clinicopathological variables influencing DFS and OS

As shown in Table 2, potential factors that might influence the DFS and OS were analyzed. Univariate analysis demonstrated that age (*P* = 0.039), preoperative ALB (*P* = 0.005), resection margin (*P* < 0.001), histologic grade (*P* = 0.023), T stage (*P* = 0.004), N stage (*P* < 0.001), AJCC stage (*P* < 0.001), LVI (*P* < 0.001), tumor size (*P* < 0.001), portal vein invasion (*P* = 0.003) and hepatic artery invasion (*P* = 0.008) significantly influenced DFS. By contrast, patient gender, preoperative CA19-9, surgical method, perineural invasion and transfusion did not significantly influence DFS. Preoperative ALB (*P* = 0.009), resection margin (*P* < 0.001), histologic grade (*P* = 0.026), T stage (*P* = 0.001), N stage (*P* < 0.001), AJCC stage (*P* < 0.001), LVI (*P* < 0.001), tumor size (*P* < 0.001), portal vein invasion (*P* = 0.002) and hepatic artery invasion (*P* = 0.013), but not patient age, gender, preoperative CA19-9, surgical methods, perineural invasion or transfusion, significantly influenced the OS. Multivariate analysis indicated that positive resection margin, higher N stage, tumor size > 30 mm and LVI were adverse factors that influenced DFS and OS.

Association between LVI and N stage and tumor size

LVI was confirmed histologically in 29 (20.4%) of the 142 patients. Table 3 shows the association of LVI with tumor size, N stage and AJCC stage. On univariate analysis, N stage (*P* < 0.001) and tumor size (*P* = 0.001), but not AJCC stage, were significantly associated with LVI. Multivariate analysis using a



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	101	79	49	37	27	23
B	29	19	6	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	101	83	49	41	29	24
B	29	21	7	2	0	0

Figure 1 Disease-free survival and overall survival based on lymphovascular invasion in type Ⅳ hilar cholangiocarcinoma patients with radical resection. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

Table 1 Demographics and operative outcomes

Characteristic	*n* (%)
Age (yr)[1]	59 (23-78)
Gender	
Male	75 (52.8)
Female	67 (47.2)
Albumin level (g/L)[2]	36.74 ± 4.93
CA19-9	
> 200	82 (57.7)
< 200	60 (42.3)
Radiological examination	
Contrast-enhanced US	8 (5.6)
Contrast-enhanced CT	43 (30.3)
MRI + MRCP	91 (64.1)
Operative time (min)[2]	429.47 ± 134.19
Blood loss (ml)[1]	600 (180-4000)
Transfusion	76 (53.5)
R0 resection	107 (75.4)
Differentiation degree	
Well differentiated	14 (9.9)
Moderately differentiated	90 (63.4)
Poorly differentiated	38 (26.8)
Perineutral invasion positive	69 (48.6)
N stage	
N0	89 (62.7)
N1	53 (37.3)
Tumor diameter (mm)[1]	30 (12-55
Hospital stay[1]	19 (5-115)

[1]Parameters are presented as median and range; [2]Parameters are presented as mean ± SD.

logistic regression model indicated that N1 stage (P = 0.026) and tumor size > 30 mm were significant factors that were associated with LVI in Bismuth type Ⅳ HC. DFS and OS based on LVI status are shown in Figure 1. The 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (22.8% *vs* 0%, P < 0.001) as was the OS (23.8% *vs* 0%, P < 0.001). Based on the LVI status, N0 stage patients and patients with tumor size ⩽ 30 mm were divided into two subgroups, respectively. In the N0 stage subgroups, the 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (31.0% *vs* 0%, P = 0.002), and the 5-year OS rate in the LVI negative group was also significantly higher than that in the LVI positive group (32.4% *vs* 0%, P = 0.001). In the tumor size ⩽ 30 mm subgroups, the 5-year DFS rate in the LVI negative group was significantly higher than that in the LVI positive group (31.8% *vs* 0%, P = 0.003), and the 5-year OS rate in the LVI negative group was significantly higher than that in the LVI positive group (34.3% *vs* 0%, P = 0.006).

DISCUSSION

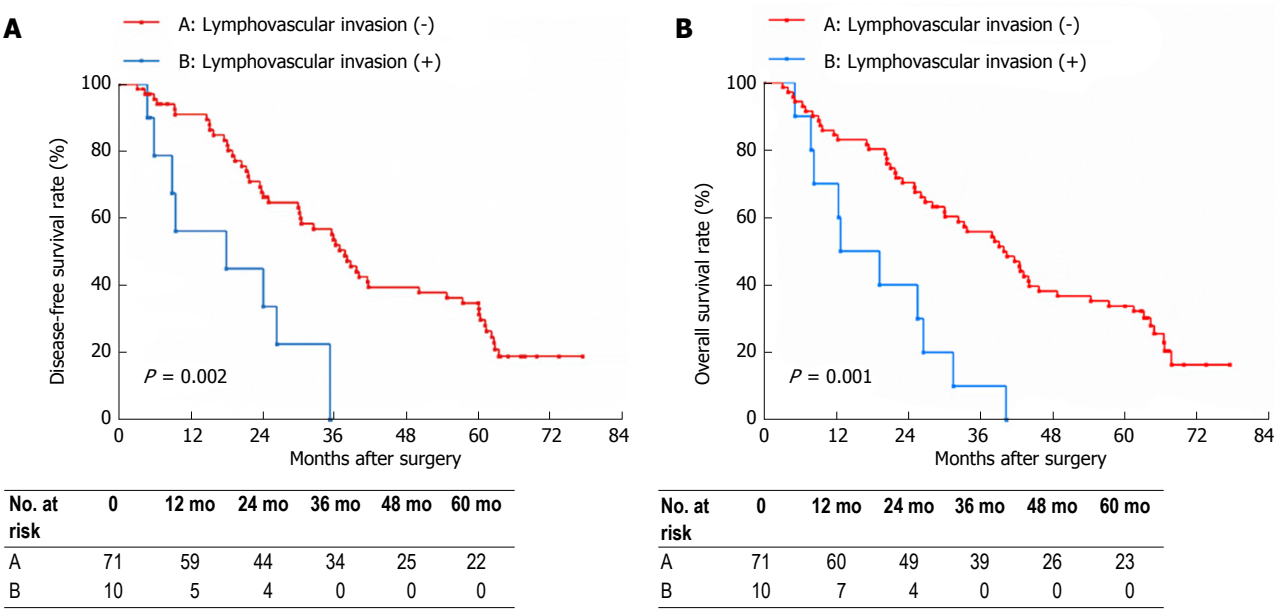
LVI is recognized as a dismal prognostic factor for OS in patients with breast cancer, colorectal cancer, and esophageal cancer[11-14]. However, no studies have yet been published on whether LVI affects the prognosis of Bismuth type Ⅳ HC. Thus, the current study was undertaken to clarify the significance of LVI in type Ⅳ HC patients who had a radical resection. LVI is defined as the involvement of arterial vessels, venules and lymphatic channels[11], but it is histologically difficult to distinguish, and the American Joint Committee on Cancer/Union Internationale Contre Cancer staging guidelines use the term lymphovascular to refer to those structures[11]. Furthermore, LVI can be confirmed specifically on HE-stained specimens[13]. In our series, LVI was confirmed histopathologically in 29 patients.

It is well recognized that resection margin status is

Table 2 Potential prognostic factors for disease-free survival and overall survival after radical resection for type Ⅳ hilar cholangiocarcinoma patients excluding operative mortality *n* (%)

Variable	Disease-free survival				Overall survival			
	5-yr survival	Univariate analysis	Multivariate analysis		5-yr survival	Univariate analysis	Multivariate analysis	
		P value	HR (95%CI)	*P* value		*P* value	HR (95%CI)	*P* value
Gender		0.682				0.965		
Male	13 (18.8)				14 (20.3)			
Female	10 (16.4)				10 (16.4)			
Age (yr)		0.039	1.291 (0.680-2.453)	0.435		0.230		
< 65	19 (19.2)				20 (20.2)			
> 65	4 (12.9)				4 (12.9)			
CA19-9		0.287				0.301		
< 200	13 (23.2)				14 (25)			
> 200	10 (13.5)				10 (13.5)			
ALB (g/L)		0.005	0.669 (0.413-1.085)	0.103		0.009	0.760 (0.492-1.173)	0.215
< 35	3 (7.1)				3 (7.1)			
> 35	21 (23.9)				20 (22.7)			
Surgical method		0.847				0.684		
Left-sided hepatectomy	13 (15.1)				13 (15.1)			
Right-sided hepatectomy	10 (17.2)				11 (19.0)			
Resection margin		< 0.001	6.255 (3.485-11.229)	< 0.001		< 0.001	6.776 (3.988-11.479)	< 0.001
Positive	0				0			
Negative	23 (23.7)				24 (24.7)			
Histologic grade		0.023	1.594 (0.994-2.554)	0.053		0.026	1.294 (0.830-2.017)	0.256
Well/moderate	20 (21.5)				21 (22.6)			
poor					3 (8.1)			
Perineural invasion		0.211				0.417		
Present	14 (22.6)				15 (24.2)			
Absent	9 (13.2)				9 (13.2)			
T stage		0.004	1.582 (0.390-6.415)	0.521		0.001	2.399 (0.734-7.836)	0.147
T1/2	22 (22)				21 (21)			
T3/4	2 (6.7)				2 (6.7)			
N stage		< 0.001	2.902 (1.132-7.439)	0.027		< 0.001	2.827 (1.243-6.429)	0.013
N0	22 (27.2)				23 (28.4)			
N1	1 (2.0)				1 (2.0)			
AJCC stage		< 0.001	0.673 (0.289-1.567)	0.358		< 0.001	0.844 (0.351-2.028)	0.704
Stage Ⅰ / Ⅱ	20 (32.8)				21 (34.4)			
Stage Ⅲ/Ⅳ	3 (4.9)				3 (4.9)			
Lymphovascular invasion		< 0.001	2.799 (1.588-4.935)	< 0.001		< 0.001	2.908 (1.712-4.938)	< 0.001
Present	0					0.000		
Absent	23 (22.8)				24 (24.8)			
Tumor size (mm)		< 0.001	1.942 (1.176-3.209)	0.010		< 0.001	1.739 (1.101-2.745)	0.018
⩽ 30	15 (20)				16 (21.3)			
> 30	8 (14.5)				8 (14.5)			
Portal vein invasion		0.003	1.759 (0.534-5.800)	0.353		0.002	1.130 (0.408-3.127)	0.815
Present	1 (4.2)				1 (4.2)			
Absent	22 (20.8)				23 (21.7)			
Hepatic invasion		0.008	1.499 (0.612-3.668)	0.376		0.013	1.196 (0.526-2.719)	0.669
Present	1 (9.1)	1 (9.1)			1 (9.1)			
Absent	22 (18.5)	22 (18.5)			23 (19.3)			
Transfusion		0.445				0.199		
Yes	11 (16.4)				11 (16.4)			
No	12 (19.0)				13 (20.6)			

the most important factor affecting long-term survival outcomes of HC patients[14,15], and positive resection margin remains a major dismal prognostic factor for type Ⅳ HC patients. Furthermore, since type Ⅳ HC had been considered unresectable[16], radical resection performed in such patients is technically challenging. Bracingly, in our center the R0 resection rate is 75.4%. Regional lymph node involvement represents another important dismal prognostic factor in HC patients who had undergone radical resection[17,18]. Concordantly, the multivariate analysis showed that lymph node metastasis was an adverse factor affecting DFS and OS in type Ⅳ HC. Furthermore, DeOliveira *et al*[19] proposed a new staging system, in which tumor size > 30 mm was defined as the T3 stage. The choice of 30 mm as a cutoff value for T3 is based on increasing evidence that the smaller the tumor, the better the prognosis[20,21]. Our results indicated that tumor size > 30 mm had an unfavorable impact on both DFS and OS. More importantly, we found that LVI is a significant adverse prognostic factor influencing DFS and OS in multivariate analysis. To our knowledge, no other



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	71	59	44	34	25	22
B	10	5	4	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	71	60	49	39	26	23
B	10	7	4	0	0	0

Figure 2 Disease-free survival and overall survival based on lymphovascular invasion in type Ⅳ hilar cholangiocarcinoma patients without lymph node metastasis. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

reports have shown the correlation between LVI and the prognosis of type Ⅳ HC.

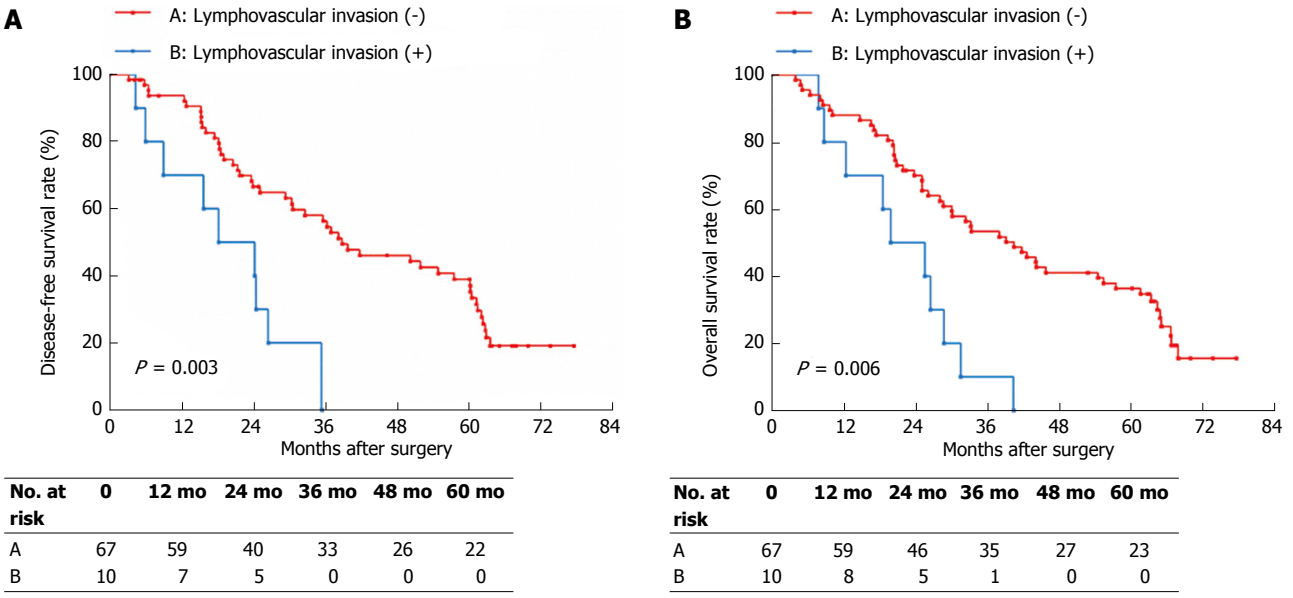
Previous studies suggested that LVI may interact with other adverse risk factors, which then have a dismal impact on the OS of esophageal cancer patients[22,23]. Lee *et al*[24] found that the presence of LVI correlated with the presence of lymph node metastasis in patients with gastric carcinoma. For different types of cholangiocarcinoma, the effect of LVI on prognosis was also different. Kim *et al*[25] reported that LVI did not influence the survival of patients with distal cholangiocarcinoma. Fisher *et al*[26] reported that LVI had an adverse influence on survival of patients with intrahepatic cholangiocarcinoma. Both of studies found that LVI was associated with lymph node metastasis[25,26]. In our analysis, N1 stage was a significant factor that was associated with LVI in type Ⅳ HC patients. It is generally known that tumor cells and tumor stromal cells (such as macrophages and thrombocytes) can produce pro-lymphangiogenic factors, which increase lymphovascular density in and around the tumors[27,28], mainly peritumoral regions. Lymph node metastases often occurred in tumors lacking intratumoral functional lymphatics, suggesting that functional lymphatics at the peritumoral regions are the route of lymphatic dissemination. Increased peritumoral lymphovascular density is considered to increase the flow of lymphatic fluid and provide an opportunity for invasive tumor cells to access the lymphatic vessels[29]. This may elucidate our results that LVI is closely related to lymph node metastasis. Moreover, we speculated that LVI may be the precursor of lymph node metastasis. Thus, we divided patients without lymph nodes metastases into LVI positive and LVI negative subgroups, and found that the 5-year DFS and OS rates in the LVI negative group were significantly higher than those in the LVI positive group (Figure 2A and B). This result indicates that LVI is an admirable prognostic predictor for patients with type Ⅳ HC when lymph node metastasis is absent.

Additionally, tumor size > 30 mm was another significant factor correlated with LVI in our series. Tumor size is recognized as a staging basis for many malignant tumors, including thyroid carcinoma, breast carcinoma, and liver carcinoma among others. Gurleyik *et al*[30] reported that LVI correlated with tumor size, and the rate of LVI positive increased with tumor size in patients with breast carcinoma. The significant correlation between LVI and tumor size could be explained through two potential aspects: (1) as the tumor size increases, the peritumoral areas increase. Thus, the tumor is endowed with the potential to make contact with an increasing lymphovasculature, and thus the possibility of LVI increases; and (2) tumor size is proportional to the time of growth: the larger the tumor, the greater the duration of time that the tumor can continue developing and growing in size. During a relatively longer growth time, a tumor has increasing opportunities to develop LVI. In our study, patients with tumor size ⩽ 30 mm were also divided into LVI positive and LVI negative subgroups. Here, the 5-year DFS and OS rates in the LVI negative group were significantly higher than those found in the LVI positive group (Figure 3A and B). This result indicates that LVI is also an excellent prognostic predictor in type Ⅳ HC patients with smaller tumor size.

Some limitations of the study should also be taken into account when interpreting the results. First, our study was retrospective with inherent limitations in its design. Thus, some clinical bias was inevitable. Next, LVI

Table 3 Logistic regression analysis for factors associated with lymphovascular invasion

Variable	Lymphovascualr invasion (+), *n* = 29	Lymphovascualr invasion (-), *n* = 113	Univariate analysis (*P* value)	OR (95%CI)	Multivariate analysis (*P* value)
AJCC stage			0.063	0.223 (0.026-1.927)	0.173
Ⅰ/Ⅱ	8	27			
Ⅲ/Ⅳ	21	86			
Tumor diameter (mm)			0.001	3.258 (1.288-8.236)	0.013
≤ 30	10	77			
> 30	19	36			
N stage			< 0.001	3.312 (1.338-8.198)	0.026
N0	10	79			
N1	19	34			



No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	67	59	40	33	26	22
B	10	7	5	0	0	0

No. at risk	0	12 mo	24 mo	36 mo	48 mo	60 mo
A	67	59	46	35	27	23
B	10	8	5	1	0	0

Figure 3 Disease-free survival and overall survival based on lymphovascular invasion in type Ⅳ hilar cholangiocarcinoma patients with tumor size ≤ 30 mm. A: The 5-yr DFS rate in the LVI negative group was significantly higher than that in the LVI positive group; B: The 5-yr OS rate in the negative group was significantly higher than that in the LVI positive group. LVI: Lymphovascular invasion; DFS: Disease-free survival; OS: Overall survival.

was confirmed by hematoxylin and eosin (HE) staining alone without application of an immunohistochemical staining with D2-40 antibody, which may improve the detection rate of LVI[31]. Third, N1 stage and tumor size > 30 mm might serve as potential confounding factors that could affect the association between LVI and the eventual prognosis. Finally, we did not carry out preclinical medical experiments to elaborate the specific molecular mechanism that could play a key role in the capacity of LVI to affect the prognosis of type Ⅳ HC patients. Future research should take into account this topic with a greater sample size. Prospective studies, even randomized controlled trials, are also urgently needed. Of course, the specific molecular mechanism responsible for LVI affecting the prognosis of type Ⅳ HC patients will be determined by empirical research.

In conclusion, the presence of LVI may be regarded as an indicator of biologically aggressive behavior, metastatic ability, and regional and systemic risk of metastasizing the primary malignancy. LVI is associated with N1 stage and tumor size > 30 mm and imparts an adverse influence on OS and DFS in type Ⅳ HC patients who received radical resection.

COMMENTS

Background

Despite advances in surgical techniques and that resection rates for Bismuth type Ⅳ hilar cholangiocarcinoma (HC) continue to increase, the prognosis of patients with type Ⅳ HC remains unsatisfactory. The reasons for this remain unclear and seem to be complex and multifactorial. Lymphovascular invasion (LVI) is associated with a poorer prognosis in patients with various malignances. This study sought to investigate whether LVI could predict type Ⅳ HC prognosis.

Research frontiers

This study estimates prognostic factors that might be associated with overall survival (OS) and disease-free survival (DFS) after radical resection in type Ⅳ HC patients. To our knowledge, this study represents the first clinical insight indicating that LVI is associated with the prognosis of type Ⅳ HC.

Innovations and breakthroughs

This findings confirmed that R1 resection, N1 stage, presence of LVI and tumor size > 30 mm were adverse prognostic predictors for type Ⅳ HC patients after radical resection. Further, LVI was found to be associated with N1 stage and tumor size > 30 mm and adversely influence OS and DFS.

Applications

Observations from the present study might formally indicate novel factors for predicting post-surgical survival in HC. Moreover, LVI might present a potentially novel target for developing anti-cancer strategies.

Terminology

HC is a neoplasia arising from the biliary epithelium at the common hepatic duct bifurcation that might extend to the intrahepatic biliary tree and liver. Bismuth-Corlette classification is the most commonly used HC typing system, which is often used by surgeons to develop preliminary surgical protocols.

Peer-review

This is a retrospective study evaluating the effect of LVI on the prognosis of Bismuth-Corlette type Ⅳ HC. The authors concluded that LVI had an adverse influence on the prognosis of patients with Bismuth-Corlette type Ⅳ HC. This manuscript is very interesting and well written.

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ORIGINAL ARTICLE

Retrospective Study

Gastrointestinal symptom prevalence depends on disease duration and gastrointestinal region in type 2 diabetes mellitus

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Abstract

AIM

To unravel relationships between gastrointestinal (GI) symptoms impairing quality of life (QOL) and clinical profiles of diabetes mellitus (DM) patients.

METHODS

We enrolled 134 outpatients with type 2 DM. Mean

age was 64.7 years, mean body mass index was 24.7 kg/m^2, mean glycated hemoglobin was 7.1%, and mean DM duration was 13.7 years. GI symptom-related QOL was determined using the Izumo scale, based on five factors, *i.e.*, heartburn, gastralgia, postprandial fullness, constipation and diarrhea. The sum of scores obtained for the three questions in each domain was calculated, and subjects with a score of 5 or higher were considered to be symptomatic with impaired QOL. JMP Clinical version 5.0 was used for all statistical analyses.

RESULTS

Lower abdominal symptoms were found to be more frequent than those affecting the upper abdomen. Diabetic duration and medications showed associations with GI symptoms. We identified differences in peak prevalences of the five symptoms. Gastralgia (P = 0.02 *vs* 10-14 years) and total GI symptoms (P = 0.01 and P = 0.02 *vs* 5-9 years and 10-14 years, respectively) peaked at a diabetes duration of 15-19 years. Heartburn (P = 0.004) and postprandial fullness (P = 0.03) tended to increase with disease duration. Constipation and diarrhea showed bimodal peaks, with the first early and the second late (*e.g.*, P = 0.03 at 15-19 years *vs* 10-14 years for diarrhea) in the disease course. Finally, GI symptoms showed clustering that reflected the region of the GI tract affected, *i.e.*, constipation and diarrhea had similar frequencies (P < 0.0001).

CONCLUSION

Our study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially in the early and the late periods of diabetes.

Key words: Gastrointestinal symptoms; Questionnaire survey; Disease duration; Type 2 diabetes; Quality of life; Gastrointestinal tract regions

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Core tip: We describe the results of a questionnaire survey of 134 type 2 diabetes mellitus outpatients experiencing gastrointestinal symptoms. The novel finding is that symptom frequencies differed among disease durations and according to affected gastrointestinal regions. Lower abdominal symptoms not only manifested during the late but also in the early stage of diabetes when there were no organ complications related to this disease. Our study highlights the importance of not underestimating gastrointestinal symptoms and of questioning patients about quality of life impairment due to abdominal symptoms, especially in both the early period and after a diabetes duration of 10 or even 15 years.

Fujishiro M, Kushiyama A, Yamazaki H, Kaneko S, Koketsu Y, Yamamotoya T, Kikuchi T, Sakoda H, Suzuki R, Kadowaki T. Gastrointestinal symptom prevalence depends on disease duration and gastrointestinal region in type 2 diabetes mellitus. *World J Gastroenterol* 2017; 23(36): 6694-6704 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6694.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6694

INTRODUCTION

The dramatic increase in the incidence of type (T)2 diabetes mellitus (DM) over the last century has become a major public health concern worldwide[1]. Both micro- and macrovascular complications of diabetes damage a variety of organs, leading to various symptoms, and impair quality of life (QOL) in patients with T2DM.

Among these symptoms, those affecting the gastrointestinal (GI) tract occur frequently in patients with diabetes[2]. Various GI symptoms are associated with this disease[3-5], especially diabetic neuropathy which develops with longstanding diabetes[6,7]. GI hyper- and hypofunction lead to delayed gastric or esophageal emptying, diabetic gastroparesis, constipation and diarrhea[6,8,9]. Other GI-related disorders and symptoms are also known to accompany diabetes, such as obesity with gastroesophageal reflux disease[10,11], and aging with gastric mucosal injury[12].

GI disorders in diabetes have thus far been poorly characterized, despite their high frequencies in both T1DM and T2DM patients[13]. Hyperglycemia-associated neuropathy was long considered to be a major mechanism underlying the pathogenesis of GI symptoms, probably *via* oxidative stress and inflammation, as with other microangiopathies. Levels of enteral hormones, such as incretin-related peptides [*e.g.*, glucagon-like peptide-1 (GLP-1), GLP-2, pancreatic polypeptide (PPY)][14] and serotonin[15], and enteric neurotransmitters, such as vasoactive intestinal peptide (VIP)[16], are altered in patients with DM, and affect GI motility and neural fiber growth. Furthermore, smooth muscle cells, interstitial cells of Cajal[17], gut microbiota[18] and intestinal stem cells[19], may be altered in DM and these changes are potentially related to GI symptoms. The relevance of these mechanisms in the development of human GI symptoms in T2DM remains uncertain.

As for the pathophysiological mechanisms underlying the development of complications, hyperglycemia and the resultant glycation products[20], oxidative stress[21] and inflammation[22], are the main candidates. GI symptoms in DM are associated with both poor glycemic control and diabetic complications, as shown in a large number of subjects[23], although we found no relationships between GI symptoms and glycemic control, probably due to the long and varied disease durations of our subjects.

There has been little systematic and comprehensive research focusing on GI conditions in patients with

T2DM. The Izumo Scale, a validated and useful tool for QOL assessment, is a scale for assessing GI symptoms[13,14]. Applying this scale, we investigated actual GI symptoms employing a questionnaire and determined whether the symptoms identified are related to the clinical profiles of patients with T2DM.

MATERIALS AND METHODS

Patients

Patients who visited the department of diabetes and metabolic diseases in our hospital, including those with T1DM and T2DM, from December 2011 to March 2014, were enrolled in this study. We asked 200 consecutive patients to answer the questionnaire. Of the 170 patients who gave informed consent and responded to the questionnaire, data from 134 were analyzed after exclusion of 31 non-T2DM patients and 5 with a past history of GI tract surgery. This protocol was approved by our institutional ethics review committees (approval number 3643). Patient QOL was assessed by asking the subjects if they suffered from GI symptoms and then scoring the symptoms according to the Izumo scale, as described below.

Patient profiles, including diabetic microangiopathy, were collected from medical records. We considered subjects to have "diabetic autonomic neuropathy (DAN)" if they had any autonomic signs or symptoms. Distal symmetric polyneuropathy (DSP) includes symmetric neurosensory signs and symptoms, such as tingling, numbness or cramps in the legs, based on the attending physician's assessment. Diabetic retinopathy (DR) includes non-proliferative DR diagnosed by the presence of microaneurysms and retinal hemorrhages[24], and all DR cases were confirmed by an ophthalmologist. Diabetic nephropathy was clinically diagnosed by attending physicians based on microalbuminuria or overt proteinuria with no evidence of other kidney or urological disease[25].

Izumo Scale

The Izumo Scale was developed and validated by Furuta *et al*[26]. The Izumo Scale is a self-administered questionnaire designed to assess the effects of abdominal symptoms on QOL and includes 15 items in five domains with three items in each domain: heartburn (questions 1-3), gastralgia (questions 4-6), postprandial fullness (questions 7-9), constipation (questions 10-12), and diarrhea (questions 13-15)[27]. Each question is rated on a 6-point Likert scale from 0 to 5, with higher values indicating more severe symptoms. This scale has been shown to have good internal consistency, reproducibility, as well as good correlations with the visual analogue scale of abdominal symptoms and the Gastrointestinal Symptoms Rating Scale (GSRS), and is thus widely utilized in Japan[28-31]. The sum of scores obtained for the three questions in each domain was calculated, and subjects with a score of 5 or higher were considered to be symptomatic with impaired QOL[30]. When the five symptoms were classified by GI region, heartburn was considered to involve the esophagus, gastralgia and postprandial fullness the upper GI tract, and constipation and diarrhea the lower GI tract.

Statistical analysis

JMP Clinical version 5.0 (SAS Institute, Cary, NC, United States) was used for all statistical analyses. ANOVA was used to compare scores among different groups. Frequencies were compared using Fisher's exact test. Trend comparisons for frequencies among disease duration, age, body mass index (BMI), and glycated hemoglobin (HbA1c) were performed using a Cochran-Armitage test. Hierarchical cluster analysis was performed to assess the relationships among GI symptoms. All P values are two-sided, and $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Characteristics of the enrolled patients

The characteristics of enrolled patients are listed in Table 1. We enrolled 134 patients with T2DM (87 males and 47 females, mean age: 64.7 years, range: 29-88 years). Mean diabetes duration was 13.7 (0.3-33.0) years, BMI was 24.7 (16.5-42.5) kg/m^2 and HbA1c was 7.1 (5.2-11.6)%. As to incidences of diabetic microangiopathy, 32 patients (24%) had DAN, 20 (15%) had DSP, 31 (23%) had DR and 64 (48%) had nephropathy. As to antidiabetic drugs, 8 patients (6%) received no medications, 34 (25%) used various forms of insulin including 22 (16%) also taking oral antidiabetic drugs (OADs), the details of which are presented in Table 1.

Eighty-six (64%) patients received OAD only, including 63 (47%) with one OAD, 30 (22%) with 2 OADs, 22 (16%) with three OADs, and 11 (8%) with four or more OADs. In detail, 38 patients (28%) used α-glucosidase inhibitors, 67 (50%) used biguanides, 26 (19%) used thiazolidinediones, 54 (40%) used sulfonylureas, 3 (2%) used glinides and 53 (40%) used dipeptidyl peptidase-4 (DPP4) inhibitors, at various daily doses, as indicated in Table 1. Six patients (4%) used GLP-1 agonists, 3 of whom were also taking an OAD. Sixty-two patients (46%) were receiving diet therapy with daily intakes ranging from 1200-1800 kcal (21-29 kcal/IBW) and, in 51 patients (38%), salt was restricted to less than 6 g/d.

As to antithrombotic or anti-inflammatory agents, 29 patients (22%) used antiplatelet agents, 7 (5%) used anticoagulants, 8 (6%) used steroids or non-steroidal anti-inflammatory drugs and 7 (5%) used other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid. As to GI agents, 29 patients (22%) used antacids, 13 (10%) used mucosal protectives, 11 (8%) used antimicrobials, and 18

Table 1 Characteristics of the study population

Variable	*n* (%)	mean (95%CI)
Sex, male/female	87/47 (65/35)	
Age of 65 yr or older	72 (54)	64.7 (62.8-66.6)
BMI of 25 kg/m^2 or more	56 (42)	24.7 (24.0-25.4)
HbA1c of 7% or more	69 (51)	7.1 (6.9-7.3)
Duration of diabetes, yr		13.7 (12.4-14.9)
⩽ 4	12 (9)	
5-9	27 (20)	
10-14	35 (26)	
15-19	31 (23)	
⩾ 20	29 (22)	
Incidence of diabetic microangiopathy		
Autonomic neuropathy	32 (24)	
Distal symmetric polyneuropathy	20 (15)	
Retinopathy	31 (23)	
Nephropathy	64 (48)	
Use of antidiabetic drugs		
None	8 (6)	
Insulins	34 (25)	
Insulins with OAD	22 (16)	
OAD only	86 (64)	
1 OAD	63 (47)	
2 OAD	30 (22)	
3 OAD	22 (16)	
⩾ 4 OAD	11 (8)	
GLP-1	6 (4)	
GLP-1 with OAD	3 (2)	
Insulins	34 (25)	
Range of injections per day	1-5	
Range of total daily insulin doses, IU	2-114	
Insulin glargine	12	
Insulin detemir	7	
Human NPH insulin	14	
Insulin aspart	2	
Insulin glulisine	4	
Insulin lispro	2	
Premixed human insulin 30/70	9	
Biphasic insulin aspart 30/70	1	
α-Glucosidase inhibitors	38 (28)	
Acarbose (150/200/300 mg)	3 (1/1/1)	
Miglitol (50/75/100/150 mg)	22 (1/12/18)	
Voglibose (0.6/0.9 mg)	13 (5/8)	
Biguanides	67 (50)	
Buformin 150 mg	1	
Metformin (500/750/1000/1500/2250 mg)	66 (12/20/14/19/1)	
Thiazolidinediones	26 (19)	
Pioglitazone (15/30 mg)	26 (18/8)	
Sulfonylureas	54 (40)	
Glimepiride (0.5/1/2/3/4 mg)	26 (10/10/2/1/3/)	
Gliclazide (20/40/120 mg)	21 (15/5/1)	
Glibenclamide (1.25/2.5/5 mg)	7 (1/4/2)	
Glinides	3 (2)	
Repaglinide 0.75 mg	1	
Mitiglinide (10/30 mg)	2 (1/1)	
Dipeptidyl peptidase-4 inhibitors	53 (40)	
Alogliptin 25 mg	12	
Sitagliptin (50/100 mg)	17 (15/2)	
Vildagliptin (50/100 mg)	24 (5/19)	
Glucagon-like peptide-1 agonists	6 (4)	
Liraglutide (0.6/0.9 mg)	4 (1/3)	
Exenatide 20 mg	2	
Dietary information		
Receiving dietary therapy	62 (46)	
With salt restriction to < 6 g per day	51 (38)	
Range of total energy intake, kcal	1200-1800	
Range of total energy intake per IBW, kcal/IBW	21-29	
Use of antithrombotic or anti-inflammatory agents		
Antiplatelet agents	29 (22)	
Anticoagulants	7 (5)	

Steroids/NSAIDs	8 (6)
Others[1]	7 (5)
Use of GI agents	
Antacids	29 (22)
Mucosal protectives	13 (10)
Antimicrobials	11 (8)
GI stimulants	18 (13)
Anti-diarrheals	0 (0)
Baseline blood parameters, mean ± SD	
WBC, × 1000/μL	6.1 ± 1.9
RBC, × 10000/μL	439 ± 78.1
Hb, g/dL	13.4 ± 2.4
Hct, %	40.8 ± 6.9
Plt, × 10000/μL	21.9 ± 6.6
TP, g/dL	7.1 ± 0.5
Alb, g/dL	3.8 ± 0.3
AST(GOT), U/L	24.4 ± 13.4
ALT(GPT), U/L	24.5 ± 17.7
γ-GTP, U/L	34.1 ± 24.3
CK, U/L	113 ± 63.6
T-Cho, mg/dL	180 ± 39.2
HDL-C, mg/dL	60.3 ± 18.3
TG, mg/dL	128 ± 67.2
cLDL, mg/dL	95.4 ± 26.2
BUN, mg/dL	17.6 ± 11.9
Cre, mg/dL	0.9 ± 0.8
eGFR, mL/min per 1.73 m^2	66.6 ± 22.8
UA, mg/dL	5.5 ± 1.4

[1]Others' includes other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid. Data are expressed as means with the 95%CI in parentheses, with numbers for each sex or numbers with the percentage of the total in parentheses. BMI: Body mass index; GI: Gastrointestinal; HbA1c: Hemoglobin A1c; IBW: Ideal body weight; NPH: Neutral protamine Hagedorn; NSAIDs: Nonsteroidal anti-inflammatory drugs; OAD: Oral antidiabetic drug.

(13%) used GI stimulants, but none were taking anti-diarrheal agents. Baseline blood parameter findings were unremarkable.

GI symptoms and diabetes duration

In the QOL assessment employing the Izumo Scale, the scores of total GI symptoms peaked during the 15- to 19-year diabetes duration period (Figure 1). When GI symptoms were analyzed separately, lower GI symptoms were found to be most frequent. Prevalence patterns differed between the esophagus/upper GI tract and lower GI tract regions. Scores for heartburn (P = 0.004) and postprandial fullness (P = 0.03) consistently and significantly rose as disease duration increased (Figure 2A).

Scores for gastralgia, like total GI symptoms, peaked during the 15- to 19-year diabetes duration period. Interestingly, only the scores for constipation and diarrhea showed bimodal peaks, the first in the early period of diabetes and the second late in the disease course. Age showed a similar but weaker relationship to diabetes duration. There were no apparent relationships of GI symptoms with age or BMI, but postprandial fullness, alone among the five symptoms, was slightly associated with high HbA1c levels (Figure 2B-D).

Relationships of GI symptoms with diabetic complications and medications

Next, we examined whether there were any relationships between GI symptoms and patient characteristics such as diabetic microangiopathy and the drugs administered (Figure 3A-D). None of the GI symptoms showed significant relationships with any of the forms of diabetic microangiopathy (Figure 3A), though symptoms tended to be more frequent in patients with than in those without DAN and/or DSP. As shown in Figure 3B, the rate of DPP4 inhibitor use was lower in patients suffering from constipation (P = 0.04). Insulin therapy and oral hypoglycemic agents other than DPP4 inhibitors were not related to GI symptoms. The numbers of patients using glinides or GLP-1 agonists were too small to allow statistically meaningful evaluation. As shown in Figure 3C, of the 29 patients taking antiplatelet agents, 38% (n = 11) had constipation (P = 0.046) and 62% (n = 18) had diarrhea (P = 0.03). Of the 18 patients using GI stimulants, 44% (n = 8) had constipation (P = 0.03) (Figure 3D).

We next investigated the effect of diabetic duration on constipation, excluding users of DPP4 inhibitors, antiplatelet agents or GI stimulants. We focused on the association of diabetes duration with constipation (Figure 4A), and on that with diarrhea, except in antiplatelet agent users (Figure 4B). When users of these medications were excluded, the early and late peaks in constipation and diarrhea remained.

Clustering and overlap of GI symptoms

A cluster analysis tree is presented in Figure 5. Gastralgia and postprandial fullness are more similar to

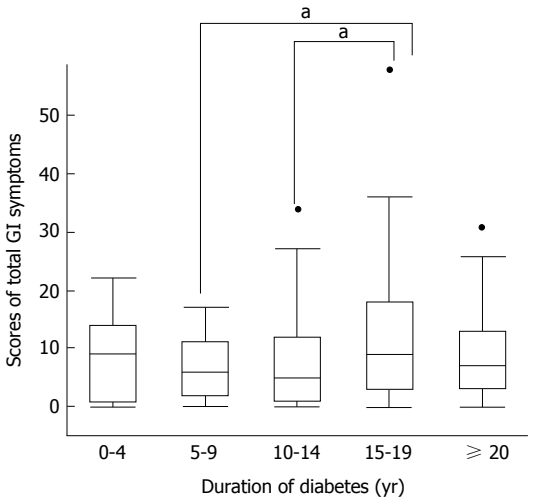


Figure 1 Distribution of the sum of scores of symptoms related to all GI areas by duration of diabetes. [a]*P* < 0.05, significantly different. Sums of symptom scores peaked during the 15- to 19-yr diabetes duration period, compared by Student's *t*-tests for paired periods (*P* = 0.01 and *P* = 0.02 when compared with 5-9 yr and 10-14 yr disease durations, respectively).

each other than to constipation and diarrhea. Heartburn is closer to upper than to lower GI symptoms. The relationship between constipation and diarrhea was found to be closer than the other associations identified in this study. The overlaps between two different symptoms on the Izumo Scale are shown in Table 2. Significant differences, determined by Fisher's exact test, between the presence and absence of two distinct symptoms, were demonstrated.

As in the cluster analysis, heartburn was associated with gastralgia (*P* = 0.03) or postprandial fullness (*P* = 0.002). Gastralgia and postprandial fullness (*P* < 0.0001) were strongly related to each other, as were constipation and diarrhea (*P* < 0.0001). Postprandial fullness was found to be related to all GI tract regions, showing modest interactions with both constipation (*P* = 0.004) and diarrhea (*P* = 0.001). Furthermore, all 31 patients with constipation also had episodes of diarrhea, while only 52.5% of those complaining mainly of diarrhea also had constipation (Table 2).

DISCUSSION

We investigated whether outpatients with T2DM actually have GI symptoms and, if present, the clinical severity of such symptoms. However, patients do not usually complain of GI symptoms. Overall, 10%-20% of adults have functional GI disorders[32-35]. In patients with diabetes, symptoms involving all portions of the GI tract are reportedly more common than in the general population[36]. However, we found that total symptom scores did not increase linearly with disease duration. In fact, rates peaked during different periods, according to GI regions. Lower abdominal symptoms were prominent early in the disease course, while diabetic complications were still mild, as well as in the late stage.

Significant confounding factors include aging, glycemic control and BMI reduction[5,8-10,37]. Japanese patients have become increasingly obese over the past 20 years and those in the younger generation have poor glycemic control at the diagnosis of T2DM, independent of hyperglycemic symptoms[38]. Therefore, symptom prevalence may change in the future.

We found no significant relationships between GI symptoms and either DAN or DSP. There might, however, be a detection bias or low sensitivity for DAN and DSP, since a previous report noted that symptoms tend to be related to lifestyle factors, rather than to either glycemic control or peripheral neuropathy[36]. Another study found no association of GI symptoms with either diabetic neuropathy or psychiatric illnesses[39]. Early symptoms might well be related to psychiatric illnesses and/or lifestyle factors but we did not obtain data pertaining to such factors in the present study.

Among anti-hyperglycemic medications, only the relationship between DPP4 inhibitors and constipation was significant. Constipation is a well-known side effect of these drugs[40], and physicians often discontinue or do not start DPP4 inhibitors due to this adverse effect[41]. Oral medications are generally prescribed to patients with poor glycemic control[42] and are frequently used to manage longstanding diabetes[43]. Administration of anti-platelet agents was associated with a variety of symptoms, as expected. These agents were still preferentially used despite apparently being harmful to the upper, middle and lower GI tracts[44,45], since the treatment of cardiovascular diseases has the highest priority. However, the bimodal peaks for constipation and diarrhea late and early in the course of diabetes were maintained and were independent of medication use.

The mechanisms underlying rapid development of lower GI complications, such as diarrhea and constipation, are unknown. In our cluster analysis, the affected GI tract region was found to be an important factor. Furthermore, the mechanisms underlying the bimodal early and late disturbances are suggested to not be the same. Early diabetic neuropathy is reportedly multifactorial, being triggered by impairment of insulin signaling, abnormal blood flow, and oxidative stress including N-acetylglucosamine, activation of protein kinase C, activation of the polyol sugar pathway and glucose autoxidation, as well as non-enzymatic protein glycation[46]. Insulin growth factors (IGFs) possess multiple neurotrophic functions, including neuronal survival, neurite outgrowth and regeneration. C-peptide also exerts IGF-like activity, such that endogenous proinsulin production might be related to the pathogenesis of GI symptoms[47]. One possible explanation of mechanistic differences between early lower GI symptoms and upper GI symptoms might involve cellular composition. The lower GI tract contains colonic stem cells and their functions are disrupted in states of diabetic enteropathy[19].

Table 2 Overlapping of gastrointestinal symptoms

Overlapping symptom	Heartburn, *n* = 9	Gastralgia, *n* = 11	Postprandial fullness, *n* = 17	Constipation, *n* = 31	Diarrhea, *n* = 59
Heartburn	-	3/11[a]	5/17[b]	5/31	6/59
Gastralgia	3/9[a]	-	8/17[b]	4/31	7/59
Postprandial fullness	5/9[b]	8/11[a]	-	9/31[b]	14/59[b]
Constipation	5/9	4/11	9/17[b]	-	31/59[b]
Diarrhea	6/9	7/11	13/17[b]	31/31[b]	-

[a]$P < 0.05$ or [b]$P < 0.01$, significantly different.

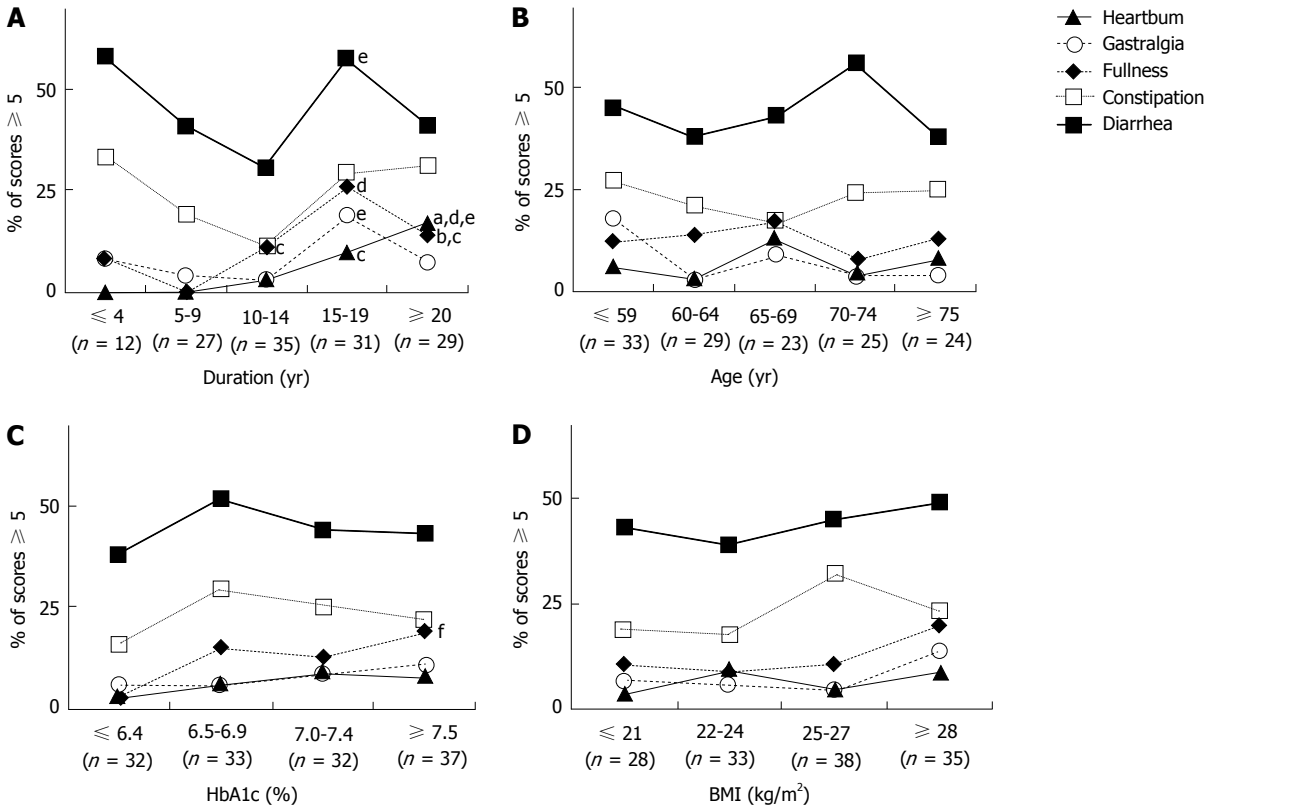


Figure 2 Relationships of abdominal symptoms with duration of diabetes, age, body mass index and hemoglobin A1c. The lines show the percentages of subjects with a score of 5 or higher, for each GI region, among those considered to be sufficiently symptomatic to impair quality of life. Comparisons among groups with five disease duration periods (A), five age ranges (B), four HbA1c levels (C) and four BMI levels (D) were performed using a Cochran-Armitage-trend-test. [a]$P < 0.05$ or [b]$P < 0.01$, significantly different. As shown in the figure (A), scores for heartburn ($P = 0.004$) and postprandial fullness ($P = 0.029$) consistently and significantly increased with disease duration. Student's *t*-tests for paired samples were performed to compare percentages of subjects with a score of 5 or higher, for each GI region, among five durations of diabetes (A), five age ranges (B), four HbA1c levels (C) and four BMI levels (D). [c]$P < 0.05$ *vs* 5-9 years, [d]$P < 0.01$ *vs* 5-9 years, or [e]$P < 0.05$ *vs* 10-14 years when compared with other disease duration periods and [f]$P < 0.05$ *vs* 6.4% or less which indicate significant differences. As shown in the figure (A), percentages of subjects with a score of 5 or higher for all symptoms except constipation in the disease duration period of 15-19 years were significantly higher than those in the 5-9 years and 10-14 years periods; $P = 0.048$ for heartburn, $P = 0.024$ for gastralgia, $P = 0.008$ for fullness and $P = 0.03$ for diarrhea. Likewise, percentages of subjects with a score of 5 or higher for heartburn and fullness in the disease duration period of 20 years or more were significantly higher than those in the 5-9 years and 10-14 years periods; $P = 0.01$ and $P = 0.04$ for heartburn and $P = 0.02$ for fullness. Furthermore, the percentage of subjects with a score of 5 or higher for fullness in the disease duration period of 10-14 yr was significantly higher than that in the 5-9 years period ($P = 0.029$). As shown in the figure (C), the percentage of subjects with a score of 5 or higher for fullness in those with HbA1c levels of 7.5% or above was significantly higher than that in those whose HbA1c was 6.4% or less ($P = 0.030$). BMI: Body mass index; HbA1c: Hemoglobin A1c.

Lower GI tract symptoms were found to be most frequent and cluster analysis demonstrated these symptoms to be related more to the affected region of the GI tract than to its functions, such as acid secretion and motility. In fact, a high concordance between constipation and diarrhea is unusual in general populations[48]. Upper and lower GI symptoms reportedly overlap and early satiety more frequently overlaps with constipation than diarrhea in general populations[49,50].

This study has several limitations. First, it is difficult to determine the causes of symptoms with a cross-sectional study design. There is diagnostic uncertainty regarding the GI disorders studied, because the reporting of symptoms was mainly subjective. Endoscopy can be used to exclude organic diseases with

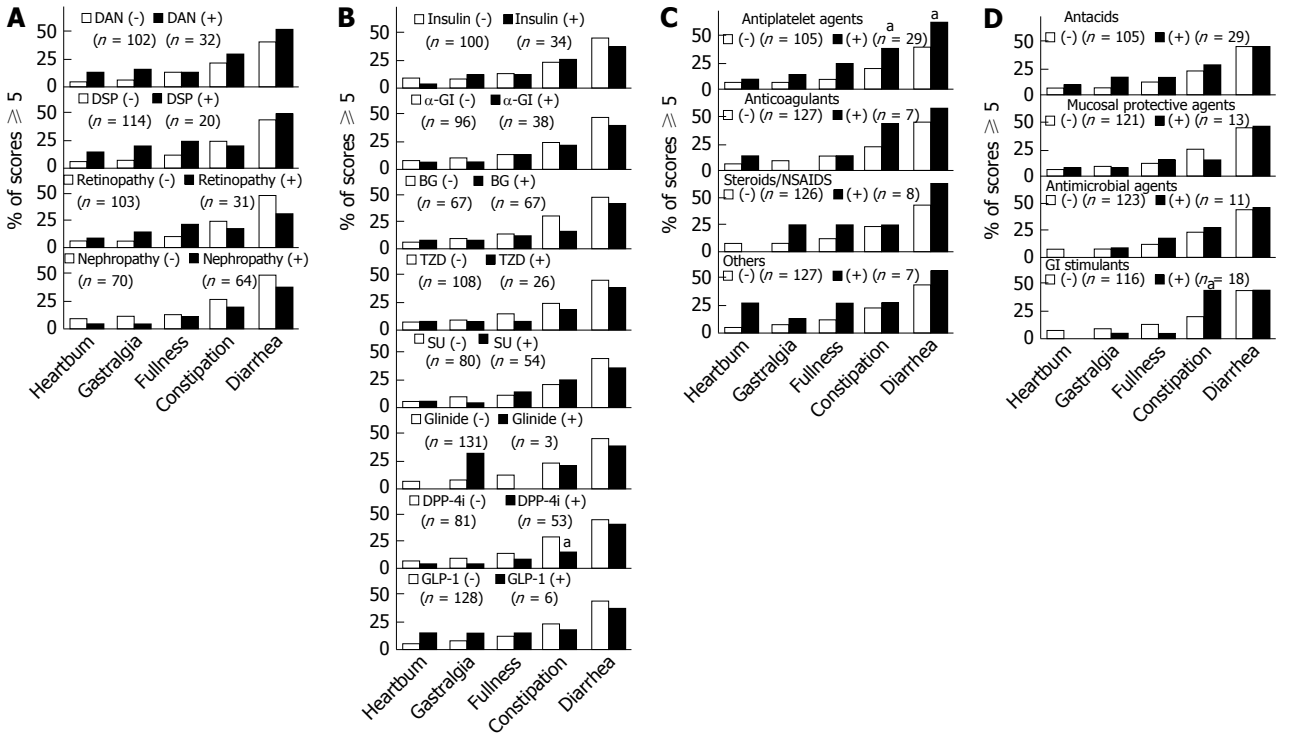


Figure 3 Relationships between various parameters and gastrointestinal symptoms involving each gastrointestinal region ("Heartburn" for esophagus, "Gastralgia" and "Postprandial Fullness" for upper abdomen, and "Constipation" and "Diarrhea" for lower abdomen). Bars: White indicates the absence and black the presence of each parameter. The percentages of subjects with a score of 5 or higher, for each GI region, considered to be sufficiently symptomatic to cause impaired quality of life, are shown. [a]$P < 0.05$, significant difference between presence and absence of each parameter, as analyzed by Fisher's exact test. A: Comparison between subjects with and without diabetic microangiopathy. The indicated percentages were obtained by dividing the number of subjects with a score of 5 or higher by the total number of patients in the same group (with or without diabetic microangiopathy); B: Comparisons between groups of patients with and without antidiabetic agent administration; C: Comparisons between groups of patients with and without antithrombotic or anti-inflammatory agents. 'Others' includes other antithrombotic agents such as prostaglandin E1 derivatives, prostaglandin I2 derivatives, or ethyl esters of eicosapentaenoic acid; D: Comparisons between groups of patients with and without GI agents. αGI: Alpha-glucosidase inhibitors; DAN: Diabetic autonomic neuropathy; DPP-4i: Dipeptidyl-peptidase 4 inhibitor; DSP: Distal symmetric polyneuropathy; GI: Gastrointestinal; GLP-1: Glucagon-like peptide-1; NSAIDs: Nonsteroidal anti-inflammatory drugs; SU: Sulphonyl urea; TZD: Thiazolidinedione.

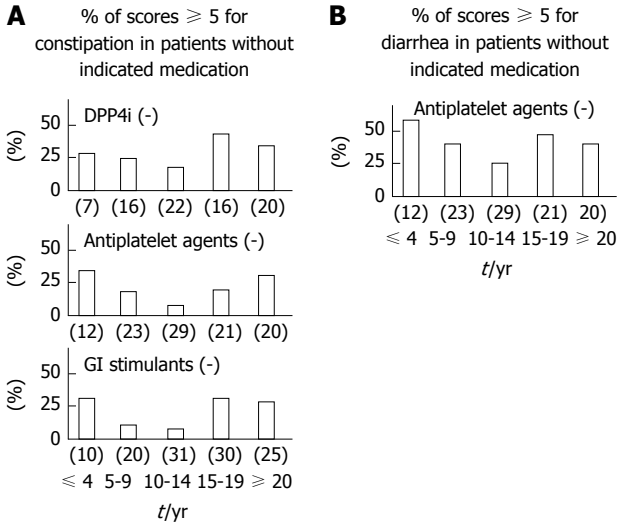


Figure 4 Relationships between various medications, which demonstrated significance as shown in Figure 3, and diabetes duration in terms of the presence of related gastrointestinal symptoms. White bars show the percentages of subjects with a score of 5 or higher for the indicated GI symptoms (A: Constipation, B: Diarrhea) without use of the indicated medications. Numbers under each bar within brackets are the number of patients not using the indicated medications. DPP-4i: Dipeptidyl-peptidase 4 inhibitor; GI: Gastrointestinal.

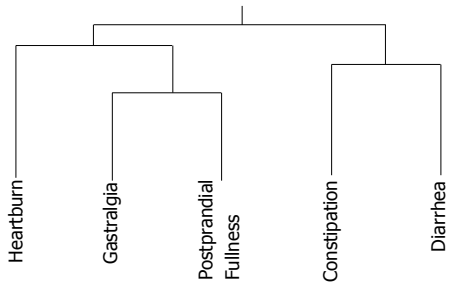


Figure 5 Cluster analysis was conducted for various gastrointestinal symptoms. The treeplot shows relationships among the five symptoms. Lengths of branches between symptoms indicate symptom similarity.

symptoms similar to those of diabetic GI disorders (*e.g.*, inflammatory bowel disease, colonic polyposis, and celiac disease)[51]. Few therapeutic or diagnostic approaches are available to assess the GI disorders possibly associated with diabetes. Organic diseases not requiring surgery and functional disorders could not be precisely distinguished because we did not perform upper and lower GI endoscopy or gastric emptying scintigraphy at the time of this study, since it was retrospective. Our results might have low sensitivity due to the small sample sizes in some groups.

In conclusion, our study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially lower GI symptoms in the early period and both lower and upper GI symptoms in the late period of diabetes. The heterogeneous nature of the underlying pathophysiological mechanisms underlying GI symptoms, including medication usage, should be taken into consideration when managing patients with T2DM.

COMMENTS

Background

Type 2 diabetes mellitus (T2DM) incidence is dramatically increasing, and both micro- and macrovascular complications of diabetes lead to various symptoms and impair quality of life (QOL) in T2DM patients. Among these symptoms, those affecting the gastrointestinal (GI) tract are frequent in T2DM patients. GI disorders in diabetes have been poorly characterized. Hyperglycemia-associated neuropathy was considered to be related to GI symptoms, probably *via* oxidative stress and inflammation. Levels of enteral hormones are altered in patients with DM, affecting both GI motility and neural fiber growth. Furthermore, smooth muscle cells, interstitial cells of Cajal, gut microbiota and intestinal stem cells may also be altered in DM and these changes might be related to GI symptoms. The relevance of these mechanisms in human GI symptoms affecting T2DM patients have yet to be clarified. The Izumo Scale, a validated and useful tool for QOL assessment, is a scale for assessing GI symptoms. Applying this scale, we investigated actual GI symptoms employing a questionnaire and determined whether the symptoms identified are related to the clinical profiles of patients with T2DM.

Research frontiers

This study highlights the importance of not underestimating gastrointestinal symptoms and of questioning patients about QOL impairment due to abdominal symptoms, especially in both the early period and after a diabetes duration of 10 or even 15 years.

Innovations and breakthroughs

The novel finding of this study is that symptom frequencies differed among disease durations and according to affected gastrointestinal regions. Lower abdominal symptoms not only manifested during the late but also in the early stage of diabetes when there were no organ complications related to this disease. This results are apparently inconsistent with the previously suggested mechanisms of GI symptoms related to DM, especially diabetic neuropathy which develops with longstanding diabetes.

Applications

This study highlights the importance of questioning patients about QOL impairment due to abdominal symptoms, especially lower GI symptoms in the early period and both lower and upper GI symptoms in the late period of diabetes, while underscoring the need for systematic and comprehensive research focusing on GI conditions in patients with T2DM.

Terminology

Izumo Scale: This self-administered questionnaire designed to assess the effects of abdominal symptoms on QOL includes 15 items in five domains with three items in each domain: heartburn, gastralgia, postprandial fullness, constipation, and diarrhea. This scale was developed and validated by Furuta *et al*. Each question is rated on a 6-point Likert scale from 0 to 5, with higher values indicating more severe symptoms.

Peer-review

Considering the paucity of data available in the literature on this topic, this study may reinforce the clinical relevance of assessing intestinal disorders in diabetes and increase the interest of the scientific community in this topic. Overall, this report describes relevant novel findings that may be further addressed and confirmed with detailed experimental studies.

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ORIGINAL ARTICLE

Observational Study

Serum angiotensin-converting enzyme level for evaluating significant fibrosis in chronic hepatitis B

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Author contributions: Noguchi R and Kaji K contributed equally to this work; Noguchi R and Kaji K collected and analyzed the data, and drafted the manuscript; Namisaki T, Moriya K, Kitade M, Takeda T, Kawaratani H, Okura Y, Aihara Y, Furukawa M and Mitoro A offered the technical or material support; Yoshiji H designed and supervised the study; all authors have read and approved the final version to be published.

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Informed consent statement: Waiver of informed consent for all study participants was guaranteed by information disclosure on website (http://www.naramed-u.ac.jp/~3int/kenkyu.html).

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Data sharing statement: Informed consent for data sharing was not obtained but the presented data are anonymized and risk of identification is low.

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Abstract

AIM

To evaluate the diagnostic performance of angiotensin-converting enzyme (ACE) on significant liver fibrosis in patients with chronic hepatitis B (CHB).

METHODS

In total, 100 patients with CHB who underwent liver biopsy in our hospital were enrolled, and 70 patients except for 30 patients with hypertension, fatty liver or habitual alcoholic consumption were analyzed. We compared histological liver fibrosis and serum ACE levels and evaluated the predictive potential to diagnose significant liver fibrosis by comparison with several biochemical marker-based indexes such as the aspartate aminotransferase (AST)-to-platelet ratio index (APRI), the fibrosis index based on four factors (FIB-4), the Mac-2 binding protein glycosylation isomer (M2BPGi) level and the number of platelets (Plt).

RESULTS

Serum ACE levels showed moderately positive correlation with liver fibrotic stages (R^2 = 0.181). Patients with significant, advanced fibrosis and cirrhosis (F2-4) had significantly higher serum ACE levels than those with

early-stage fibrosis and cirrhosis (F0-1). For significant fibrosis (≥ F2), the 12.8 U/L cut-off value of ACE showed 91.7% sensitivity and 75.0% specificity. The receiver-operating characteristic (ROC) curves analysis revealed that the area under the curve (AUC) value of ACE was 0.871, which was higher than that of APRI, FIB-4, M2BPGi and Plt.

CONCLUSION

The serum ACE level could be a novel noninvasive, easy, accurate, and inexpensive marker of significant fibrosis stage in patients with CHB.

Key words: Angiotensin-converting enzyme; Hepatitis B virus; Liver fibrosis; Noninvasive fibrosis marker; Aspartate aminotransferase-to-platelet ratio index; Fibrosis index based on four factors; Mac-2 binding protein glycosylation isomer

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Core tip: Liver fibrosis is one of key factors to determine therapeutic intervention for patients with chronic hepatitis B (CHB). However, the noninvasive prediction of CHB-related liver fibrosis is difficult. Angiotensin-converting enzyme (ACE) is reportedly involved in liver fibrogenesis. In this paper, we demonstrate that serum ACE levels are elevated in patients with CHB and show the predictive potential to diagnose significant fibrosis (≥ F2), which is the therapeutically adapted stage, with higher accuracy as compared with other fibrotic markers including APRI, FIB-4, M2BPGi and Plt. The serum ACE level could be a novel noninvasive marker of significant fibrosis stage in CHB.

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INTRODUCTION

Hepatitis B virus (HBV) annually affects 350-400 million people and causes 1 million deaths worldwide[1,2]. Chronic HBV infection results in a risk of progressive liver fibrosis, leading to cirrhosis with decreased liver reserve and hepatocellular carcinoma (HCC)[3]. The annual incidence of HCC is reported to be 10%-17% in HBV-induced liver cirrhosis, and the fibrotic status stepwisely increases the risk of HCC, as in case of chronic infection of hepatitis C virus (HCV)[4]. Therefore, an early assessment of liver fibrosis is required for not only the prevention of disease progression but also the judgment of therapeutic intervention in patients with HBV infection. Liver biopsy is currently the gold standard for estimating fibrosis progression, although frequent application of this procedure is limited because of sampling error, invasiveness, and some other complications[5]. These reasons have prompted many investigators to explore noninvasive predictors for liver fibrosis. Of several noninvasive methods, two tests, namely the aspartate aminotransferase (AST)-to-platelet index (APRI) and the fibrosis index based on four factors (FIB-4), are utilized for relatively accurate detection of liver fibrosis especially induced by HCV infection[6-8]. At present, the accuracy of these models has been externally validated in patients with chronic viral hepatitis, nonalcoholic fatty liver disease (NAFLD), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). With regard to chronic hepatitis B (CHB), a recent meta-analysis suggested that APRI and FIB-4 could identify CHB-related fibrosis with moderate sensitivity and accuracy[9]. However, more methods are required to circumstantially classify liver fibrosis.

The renin-angiotensin-aldosterone system (RAAS) is a key mediator in the regulation of arterial blood pressure and body fluid homeostasis[10]. RAAS also reportedly plays an important role in the hemodynamics of several organs[11,12]. RAAS is frequently activated in patients with chronic liver diseases such as cirrhosis[13,14]. Angiotensin-I-converting enzyme (ACE), a central component of RAAS, converts the inactive decapeptide angiotensin Ⅰ (AT-Ⅰ) into the octapeptide angiotensin Ⅱ (AT-Ⅱ), which shows many physiological activities, including vascular hormonal secretion and tissue growth[15]. AT-Ⅱ has been considered to be a potential mediator of portal hypertension because its plasma level is markedly increased in patients with cirrhosis and its administration induces the elevation of portal pressure[13,16-18]. AT-Ⅱ is pathologically recognized to induce the contractility and proliferation of hepatic stellate cells (HSCs), which play a pivotal role in the progression of liver fibrosis[19-21]. The aim of the present study was to evaluate the behavior of circulating ACE in patients with CHB and assess the relationship between CHB-related liver fibrosis and serum levels of ACE.

MATERIALS AND METHODS

Patients

In total, 100 patients who were diagnosed with serologically and histologically confirmed CHB at Nara Medical University between 2013 and 2015 were enrolled. Chronic HBV infection was diagnosed in patients according to the following criteria: (1) detectable hepatitis B surface antigen (HBsAg) for ≥ 6 mo; and (2) serum HBV-DNA ≥ 1.3 log IU/mL. The status of other HBV markers such as HB envelope antigen (HBeAg), anti-HBe, and anti-HB core (HBc) IgG was not considered as a criterion for the current assessment. All the patients who fulfilled these criteria underwent

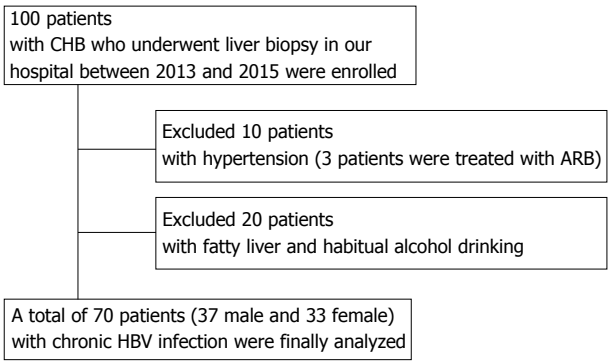


Figure 1 The selection of the study population. 70 patients except for 10 patients with hypertension and 20 patients with fatty liver or habitual alcoholic consumption were finally analyzed.

routine liver biopsies before therapeutic intervention. All pathological specimens were evaluated by at least two experienced pathologists. The degree of hepatic fibrosis was assessed and graded according to the METAVIR score for chronic hepatitis graded 0-4: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis[22,23]. Hepatic steatosis is histologically defined with more than 5% of accumulated lipid droplets in the hepatic parenchyma, and habitual alcohol consumption is defined with a daily ethanol intake of > 20 g (female) or > 30 g (male). Patients were excluded if they had other concomitant chronic liver diseases, including chronic hepatitis C (CHC), autoimmune hepatitis (AIH), PBC, PSC, hemochromatosis, or Wilson's disease as well as cancer, severe cardiopulmonary or renal diseases, diabetes mellitus (DM), sarcoidosis, dysthyroidism, or a previous history of liver transplantation. 10 patients treated with antihypertensive agents were also excluded regardless of the type of drug because high blood pressure was considered to affect serum ACE levels (Figure 1). The study was conducted in accordance with the standards of the Helsinki Declaration, and written informed consents were provided by all the study subjects. The protocols used were approved by the Ethics Committee of Nara Medical University (Nara, Japan; Approval number 1077) and other facilities.

Laboratory analysis and measurement of angiotensin-converting enzyme levels

Serum samples from all patients were collected when liver biopsy was performed and were stored and used for the present study. The following laboratory parameters were routinely measured: complete blood count, AST, alanine aminotransferase (ALT), and albumin (Alb). To assess liver fibrosis, we evaluated four serum fibrotic markers, namely, hyaluronic acid, type 4 collagen 7S, type 3 procollagen-N-peptide (P-Ⅲ-P), and Mac-2-binding protein glycosylation isomer (M2BPGi), as well as the number of platelets (Plt). APRI and FIB-4 were also used as noninvasive tests for the assessment of liver fibrosis. In these tests, evaluation was performed using the following formula: APRI = [(AST of the sample/reference AST) × 100]/platelets; FIB-4 = (age × AST)/[(platelets) × (ALT)$^{1/2}$]. Serum ACE levels were measured using Kasahara's colorimetry-based methods[24]. A serum ACE level between 8.3 and 21.4 IU/L was considered to be within the normal range according to the manufacturer's instruction.

Statistical analysis

Continuous variables of patients with discordance and those without discordance were compared using independent *t*-tests or Mann-Whitney *U* tests, as appropriate. The χ^2 or Fisher's exact tests were used for categorical variables. Area under the receiver operating characteristic (AUROC) curves and other statistical analyses were performed using R software as described previously[25].

RESULTS

Serum ACE levels in patients with fatty liver and alcohol abuse

ACE activity is susceptible to the presence of fatty liver and/or habitual alcoholic consumption (FL/AL), as reported previously[26]. Thus, we initially evaluated the serum ACE activity in patients histologically diagnosed with hepatic steatosis to exclude patients with FL/AL from the current subjects. Histological analysis demonstrated that 20 patients showed hepatic steatosis. Of these patients, 8 patients habitually drank alcohol. Similar to several reports, serum ACE levels were significantly higher in patients with CHB with FL/AL than in those without FL/AL (Figure 2A), although other fibrotic markers, including APRI, FIB-4, and M2BPGi, were not affected by the coexistence of FL/AL (Figure 2B-2D). Interestingly, a fibrosis stage-matched comparison showed that this difference in serum ACE levels was prevalently observed in patients with early-stage (F0 and F1) liver fibrosis (Figure 2E). Consequently, 70 patients with CHB and without hypertension and/or FL/AL were included as subjects in the present study.

Characteristic features of patients

The demographic and clinicopathological characteristics of the patients in the final analysis are presented in Table 1. In total, 70 patients with CHB without FL/AL (37 males and 33 females) with a median age of 48.6 ± 14.1 years were included in the present study. The fibrosis stages were F0, F1, F2, F3 and F4 in nine (12.8%), 25 (35.7%), 17 (24.3%), 13 (18.6%), and six (8.6%) patients, respectively. All the patients in F4 were classified as Child-Pugh A. There were no differences in serum Alb levels and HBV-DNA and HBsAg values among each stage of fibrosis.

Elevated serum ACE level above F2 stage in liver fibrosis

In patients with CHB, the mean serum ACE level was

Table 1 The clinicopathological characteristics of the patients with hepatitis B

Variables	Patients with HB (n = 70)
Sex (males/females)	37/33
Age	48.6 ± 14.1
Fibrosis stage (F0/F1/F2/F3/F4)	9/25/17/13/6
Platelet (× 10^4 μL) (F0/F1/F2/F3/F4)	17.9 ± 5.0 (23.2 ± 4.7/18.4 ± 3.6/18.7 ± 3.8/15.1 ± 5.4/18.9 ± 3.3)
Alb (g/dL)	4.1 ± 0.4
AST (IU/L)	35.8 ± 26.7
ALT (IU/L)	42.6 ± 39.4
HBV DNA (Log IU/mL)	4.04 ± 2.24
HBsAg (IU/mL)	22697.1 ± 52927.9
Hyaluronic acid (ng/mL)	62.4 ± 92.1
Type 4 collagen 7S (ng/mL)	4.1 ± 2.0
Serum ACE (U/L) (F0/F1/F2/F3/F4)	14.1 ± 5.1 (10.8 ± 2.3/11.1 ± 4.7/17.9 ± 4.4/6.4 ± 3.9/15.0 ± 2.9)
APRI (F0/F1/F2/F3/F4)	1.0 ± 1.3 (0.3 ± 0.1/0.7 ± 0.5/0.9 ± 0.6/2.3 ± 2.1/1.0 ± 0.9)
FIB4 (F0/F1/F2/F3/F4)	2.0 ± 1.9 (1.3 ± 0.6/1.6 ± 1.0/1.3 ± 0.8/2.8 ± 1.7/1.7 ± 0.7)
M2BPGi (COI) (F0/F1/F2/F3/F4)	1.2 ± 1.1 (0.9 ± 0.9/0.9 ± 0.6/.1 ± 1.0/2.2 ± 1.8/1.3 ± 0.6)

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; Alb: Albumin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ACE: Angiotensin-I-converting enzyme; APRI: Aspartate aminotransferase-to-platelet index; FIB4: Fibrosis index based on four factors; M2BPGi: Mac-2-binding protein glycosylation isomer.

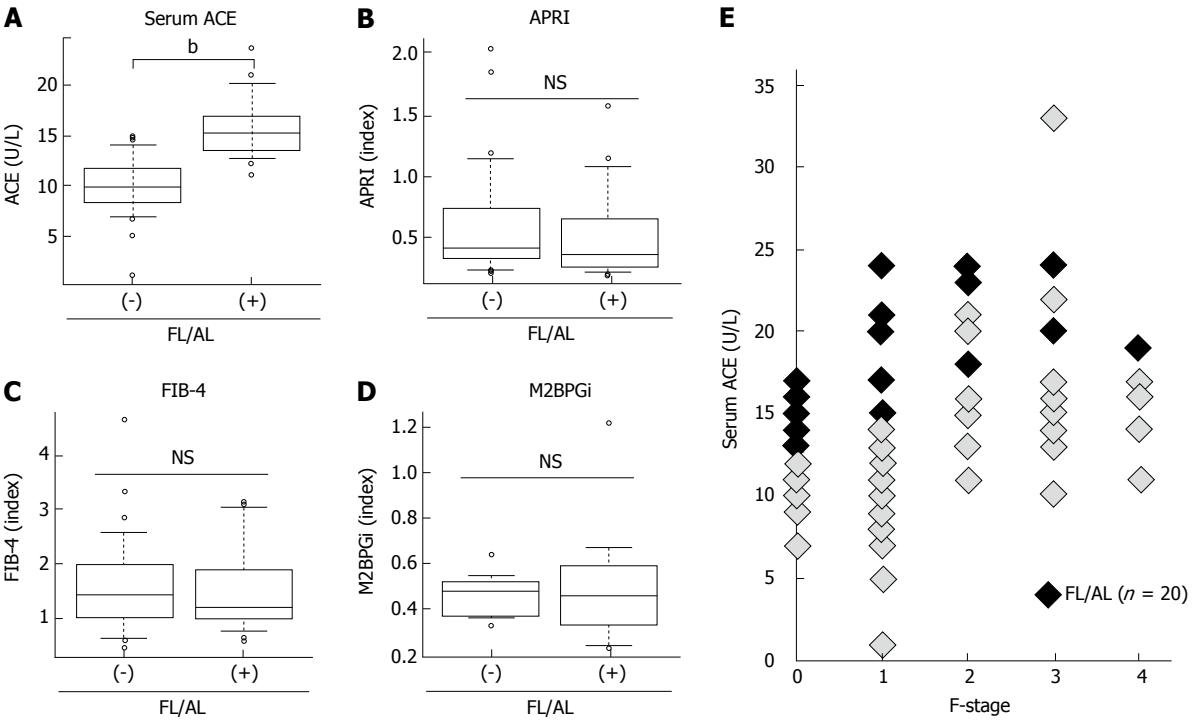


Figure 2 Serum levels of angiotensin-converting enzyme and fibrotic markers in patients with fatty liver and/or habitual alcoholic drinking. A: Serum angiotensin-converting enzyme (ACE) level; B: Aspartate aminotransferase to platelet index (APRI); C: Fibrosis index based on the four factors (FIB-4), D: Serum Mac-2 binding protein glycosylation isomer (M2BPGi) level in chronic hepatitis B patients with and without fatty liver and/or habitual alcoholic drinking (FL/AL). Serum ACE levels were significantly higher in the patients with FL/AL than those without FL/AL; E: Fibrosis stage-matched comparison showed that this difference in serum ACE levels between with and without FL/AL was prevalently observed in early stages (F0 and F1) of liver fibrosis. Data are means ± SD, $^bP < 0.01$.

14.1 ± 5.1 U/L and serum ACE levels at F0, F1, F2, F3, and F4 were 10.8 ± 2.3 U/L, 11.1 ± 4.7 U/L, 17.9 ± 4.4 U/L, 16.4 ± 3.9 U/L, and 15.0 ± 2.9 U/L, respectively. Pearson's correlation coefficient showed moderately positive correlation between serum ACE levels and fibrotic stages (R^2 = 0.181), and Mann-Whitney U tests demonstrated that patients with significant, advanced and cirrhotic stages (F2-4) showed markedly higher serum ACE levels than those with early-stage fibrosis and cirrhosis (F0-1) (Figure 3A). To validate the diagnostic performance of the serum ACE level in predicting significant liver fibrosis, we next performed receiver-operating characteristic (ROC) curve analysis. For significant fibrosis (⩾ F2), ROC curve analysis revealed that the optimal ACE level cut-off point was 12.8 U/L (sensitivity, 91.7%; specificity, 75.0%; PPV, 80.5%; NPV, 89.7%; accuracy, 84.3%), and the area under the curve (AUC) value for significant fibrosis (⩾

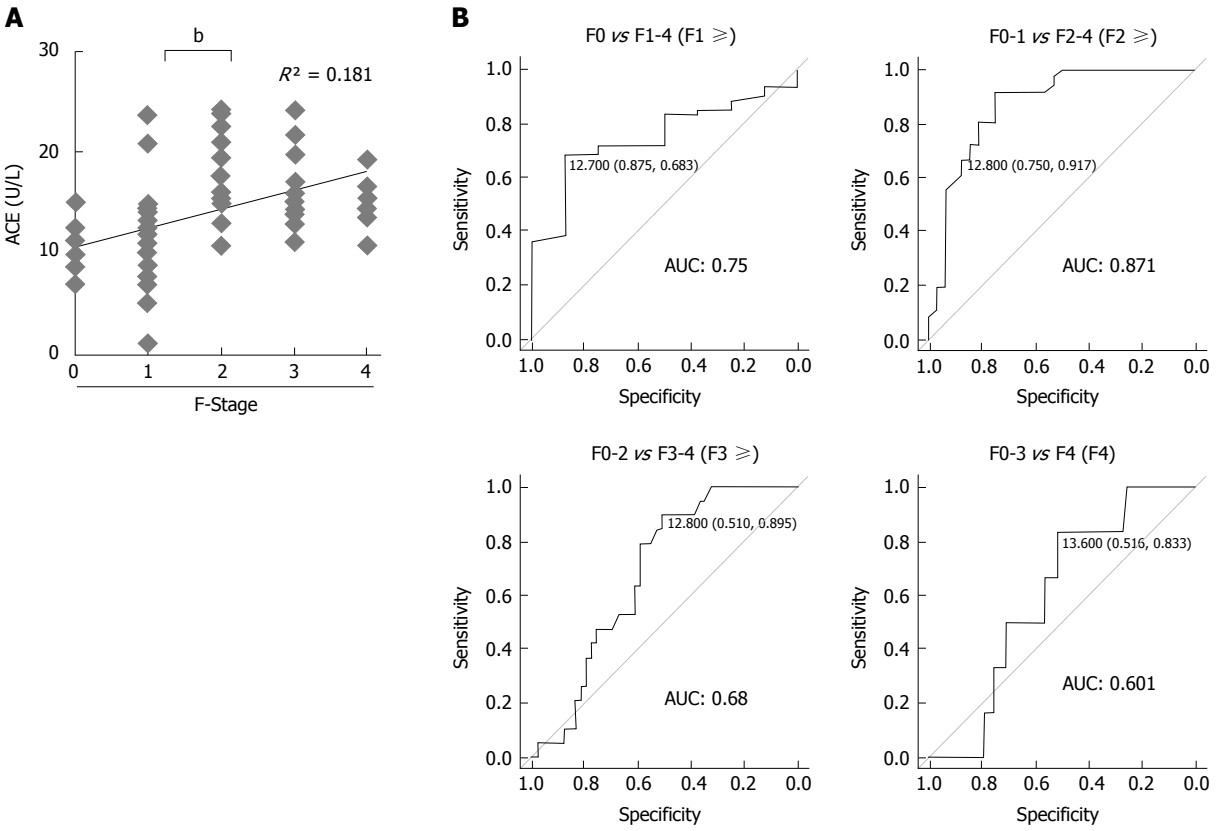


Figure 3 Serum angiotensin-converting enzyme levels and liver fibrosis development in patients with chronic hepatitis B. A: Correlation between serum angiotensin-converting enzyme (ACE) level and liver fibrosis stage (F-Stage) (R = 0.42, R^2 = 0.181); B: Receiver operating characteristic curve analysis and area under curve (AUC) value for diagnostic performance of serum ACE level for predicting each stage of liver fibrosis. The optimal ACE level cut-off point for significant fibrosis was 12.8 U/L. Data are means ± SD, $^bP < 0.01$.

F2) was 0.871 (Figure 3B). Meanwhile, the AUC values to predict mild (≥ F1), advanced (≥ F3) and cirrhotic (F4) stage of fibrosis were 0.75 (sensitivity, 68.3%; specificity, 87.5%), 0.68 (sensitivity, 89.5%; specificity, 51.0%) and 0.601 (sensitivity, 83.3%; specificity, 51.6%), respectively which were remarkably lower than significant fibrosis (Figure 3B). These findings suggest that serum ACE level manifests its efficient performance in diagnosing significant fibrosis (≥ F2).

Diagnostic performance in other markers to predict significant stage liver fibrosis

Next, to evaluate predictive potential for significant fibrosis in other markers, we performed similar analysis for well-known fibrotic parameters, including APRI, FIB-4, M2BPGi and Plt. Unlike the ACE level, there was a significant difference below and above the F3 stage in these parameters, APRI (F2, 0.86 ± 0.6 *vs* F3, 2.27 ± 2.0; $P < 0.05$), FIB-4 (F2, 1.34 ± 0.6 *vs* F3, 2.76 ± 1.7; $P < 0.05$), M2BPGi (F2, 1.1 ± 1.0 *vs* F3, 2.2 ± 1.8; $P < 0.05$) and Plt (F2, 18.7 ± 3.8 *vs* F3, 15.1 ± 5.4 × 10^4/μL; $P = 0.0616$) (Figure 4A-D). Similarly, the established fibrosis markers such as hyaluronic acid, type 4 collagen 7S, and P-Ⅲ-P indicated an advanced fibrosis stage (≥ F3) (Figure 4E-G). Furthermore, we assessed the diagnostic performance of APRI, FIB-4, M2BPGi and Plt to predict significant fibrosis (≥ F2) by ROC curve analysis. For significant fibrosis (≥ F2), the APRI cut-off point was 0.57 (sensitivity, 83.3%; specificity, 69.7%; PPV, 76.0%; NPV, 71.1%; accuracy, 73%), FIB-4 cut-off point was 2.23 (sensitivity, 40.0%; specificity, 84.8%; PPV, 70.6%; NPV, 39.1%; accuracy, 47.6%), M2BPGi cut-off point was 0.81 (sensitivity, 50.0%; specificity, 48.9%; PPV, 69.7%; NPV, 29.4%; accuracy, 49.3%), and Plt cut-off point was 17.8 × 10^4/μL (sensitivity, 68.8%; specificity, 71.9%; PPV, 33.3%; NPV, 72.4%; accuracy, 50.8%) (Supplementary Figure 1). The AUC value of serum ACE (0.871) was higher than those of APRI (0.83, $P = 0.224$), FIB-4 (0.641, $P = 0.0012$), M2BPGi (0.717, $P = 0.0239$), and Plt (0.70, $P = 0.016$) (Figure 5A-D), indicating that compared with other noninvasive markers, the serum ACE level is distinctively capable of the enclosure of significant liver fibrosis (≥ F2) in patients with CHB.

APRI is a beneficial marker in predicting advanced liver fibrosis with the highest accuracy

As the next step to closely diagnose significant fibrosis in patients with CHB, patients with > F2 fibrosis need to be further classified into significant (F2) and advanced (F3) fibrosis. Therefore, we compared the AUC values to predict advanced fibrosis stages (≥ F3) among APRI, FIB-4, M2BPGi, and Plt to evaluate diagnostic

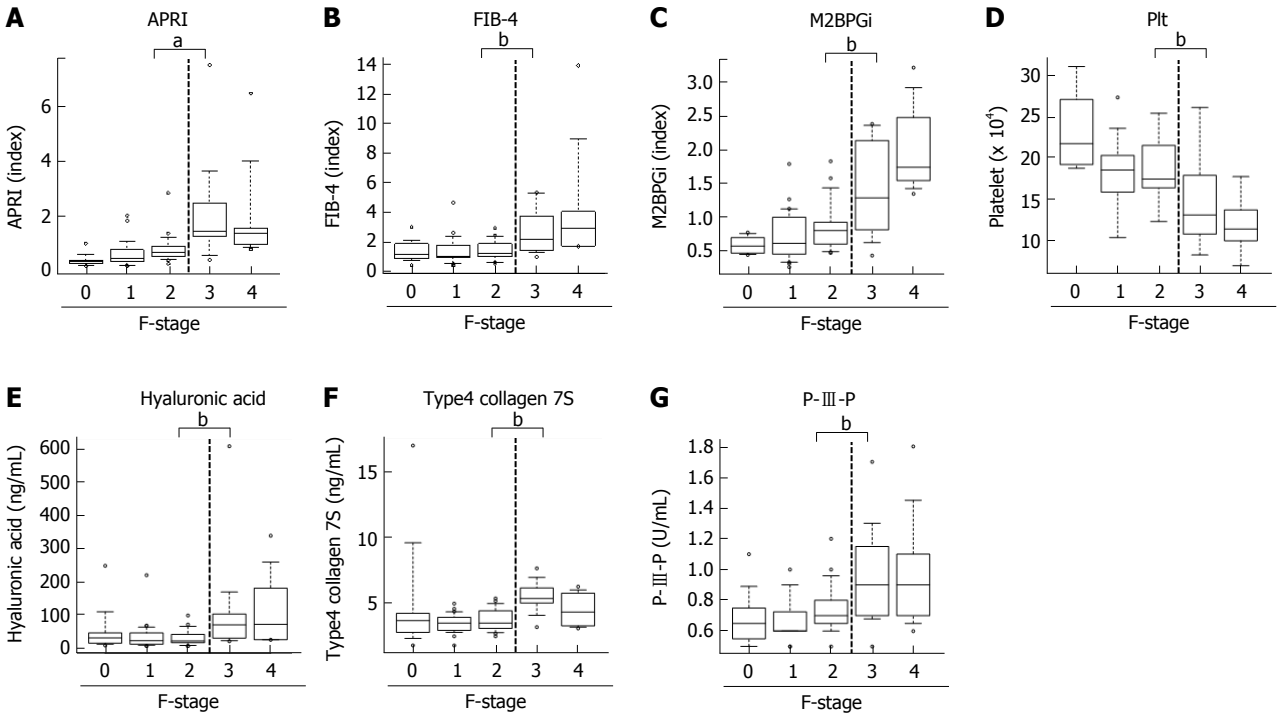


Figure 4 Serum levels of other markers and liver fibrosis in patients with chronic hepatitis B. A: Aspartate aminotransferase to platelet index (APRI); B: Fibrosis index based on the four factors (FIB-4); C: Serum levels of Mac-2 binding protein glycosylation isomer (M2BPGi); D: The number of platelets (Plt); E: Serum hyaluronic acid level; F: Type4 collagen 7S; and G: P-Ⅲ-P levels in patients with each liver fibrotic stage (F-Stage). Data are means ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$.

performance of these parameters for advanced fibrosis. The ROC curve analysis demonstrated that the optimum cut-off points for advanced fibrosis (≥ F3) are APRI; 1.27 (sensitivity, 75%; specificity, 91.5%), FIB-4; 2.51 (sensitivity, 56.2%; specificity, 93.6%), M2BPGi; 0.82 (sensitivity, 87.5%; specificity, 64.0%), and Plt; 15.0 × 10^4/μL (sensitivity, 70.6%; specificity, 87.2%) (Supplementary Figure 1). The AUC value is the highest in APRI (0.879) as compared with FIB-4 (0.803), M2BPGi (0.791), and Plt (0.813).

DISCUSSION

Identifying the degree of liver fibrosis is a clinical requisite for the treatment of chronic liver diseases regardless of the etiology because it plays a key role in predicting therapy responses and long-term outcomes of patients. In the case of CHB, nucleos(t)ide analogs (NAs) inhibit HBV-DNA replication and reduce the serum HBV level to achieve therapeutic improvement. Meanwhile, NAs do not play any role in the complete elimination of HBV and do not provide HBsAg clearance or persistent HBeAg seroconversion. Moreover, NAs frequently cause drug resistance and relapse after the termination of therapy. These pharmacological properties of NAs require long-term administration for patients to avoid the risk of liver decompensation due to cirrhosis and HCC progression, suggesting that the ideal time to start the therapy should be carefully decided considering the fibrotic stage as well as HBV-DNA and ALT levels which are extensively recognized as guideline for treatment. At present, APRI and FIB-4 indexes are widely known as noninvasive predictors to evaluate liver fibrosis[7,8]. Xiao *et al*[9] systematically reviewed the performance of two indexes in HBV-associated liver fibrosis. The total AUC values of APRI/FIB-4 for the diagnosis of significant fibrosis, advanced fibrosis, and cirrhosis were 0.7407, 0.7844 and 0.7347/0.8165, and 0.7268 and 0.8448, respectively. These data indicate that APRI and FIB-4 can identify CHB-related fibrosis with moderate sensitivity and accuracy. However, additional markers are required for diagnosis with higher accuracy and sensitivity. Recent evidences demonstrated that several serum markers are possibly beneficial for prediction of earlier fibrosis. Oztas *et al*[27] reported that soluble ST2, a receptor for the Th2 cytokine IL-33, could be used for differentiating significant fibrosis from mild fibrosis in CHB patients, and Deng *et al*[27] showed that serum complement 5a concentration significantly decreased in severe HBV fibrosis stages and earlier cirrhosis[28].

Our results show that serum ACE levels were markedly higher in patients with CHB who were histologically diagnosed with significant, advanced fibrosis and cirrhosis than in those with early fibrosis, while other predictors distinguished advanced fibrosis and cirrhosis (≥ F3) but not significant fibrosis (F2). These results indicate that the serum ACE level has a distinctive potential to enclose significant fibrosis (≥ F2), which is the therapeutically adapted stage for NAs[23]. As confirmed by AUC, the diagnostic value of serum ACE for detecting significant fibrosis above F2 was 87.1%,

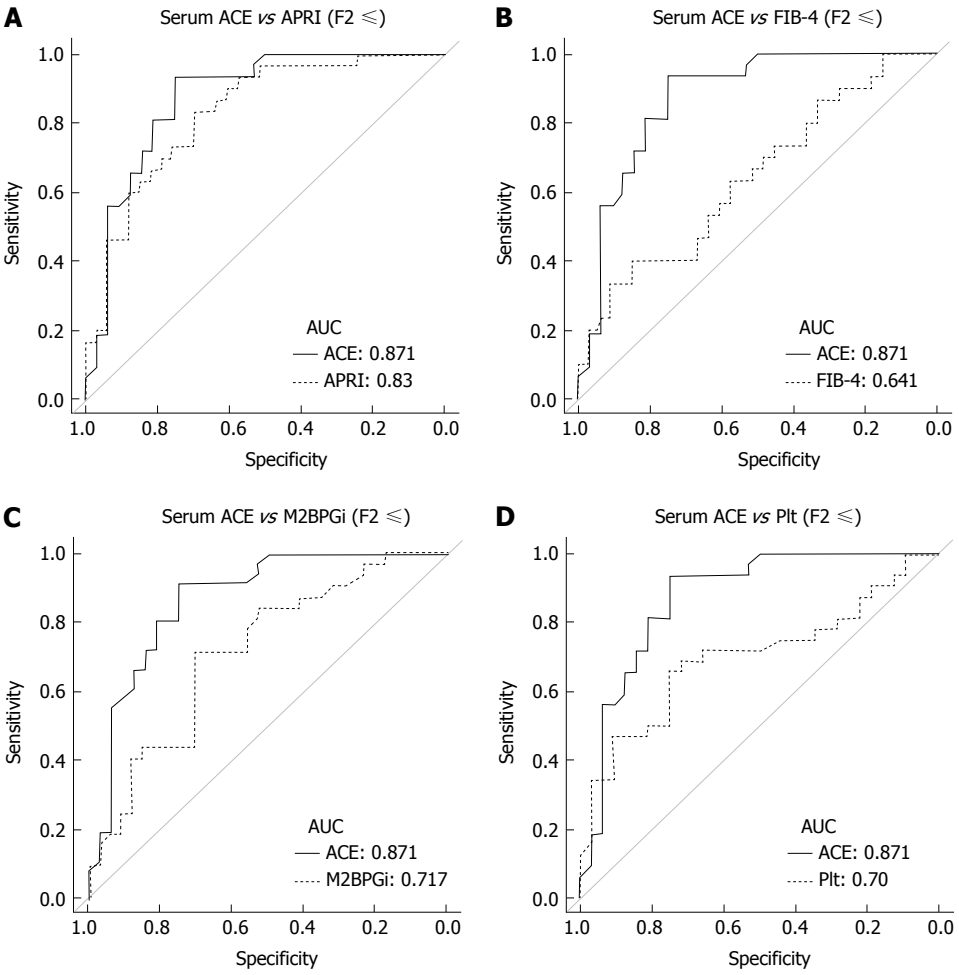


Figure 5 Receiver operating characteristic curve analysis for diagnostic performance for predicting significant liver fibrosis. Compared to other markers, the area under curve (AUC) value in serum angiotensin-converting enzyme (ACE) level, 0.871, was higher than that in A: Aspartate aminotransferase to platelet index (APRI), 0.83 (P = 0.224); B: Fibrosis index based on the four factors (FIB-4), 0.641 (P = 0.0012); C: Serum levels of Mac-2 binding protein glycosylation isomer (M2BPGi), 0.717 (P = 0.0239); D: The number of platelets (Plt), 0.70 (P = 0.016).

which was higher than other that of fibrotic markers, including APRI, FIB-4, M2BPGi and Plt.

As described in previous reports, AT-Ⅱ, which is produced by ACE converted from AT-Ⅰ, plays an important role in liver fibrosis development[21,29,30]. AT-Ⅱ induces HSC proliferation, upregulates transforming growth factor-β and collagen-Ⅰ gene expression, and promotes extracellular matrix synthesis[19,20]. Additionally, previous animal study demonstrated that ACE gene was up-regulated in the bile duct ligation-induced fibrotic liver[31]. Recent evidence also suggested that upregulation of hepatic ACE is accelerated particularly during fibrogenesis and is dampened in the developed fibrosis state[32]. Correspondingly, our results showed that serum ACE level was increased with moderately positive correlation with liver fibrosis development, and reached a threshold in the significant fibrosis (F2) (Figure 3A). These findings suggest that the suppression of RAAS is an inevitable strategy for preventing liver fibrosis progression. Our recent animal studies have revealed that ACE inhibitor (ACE-I) and AT receptor blocker (ARB) show significant antifibrotic effects on experimental liver fibrosis along with the suppression of activated HSC[33-36]. A clinical study also demonstrated that the group of patients with HCV treated using angiotensin-blocking agents exhibited lesser fibrosis than those without hypertension[37]. Another report showed that RAAS blocker-treated hypertensive patients with NAFLD prevalently had a mild degree of liver fibrosis[38]. Liver fibrogenesis progresses through multiple processes that are dependent on the etiology. Therefore, the clinical findings in patients with HCV and NAFLD may provide a basis for the assessment of the antifibrotic properties of RAAS blockers in HBV. A previous report actually showed that circulating ACE levels were elevated in patients with chronic HBV and more prominent elevation was observed in patients with advanced fibrosis[39].

Other reports have validated whether the serum ACE level has the clinical potential to predict liver fibrosis induced by other chronic liver diseases. Efe *et al*[40] suggested that ACE sustains hepatic fibrogenesis in AIH. They demonstrated that serum ACE levels increased for each fibrosis score in 73 patients with AIH, and AUC values of serum ACE for the diagnosis of significant fibrosis (≥ F2), advanced fibrosis

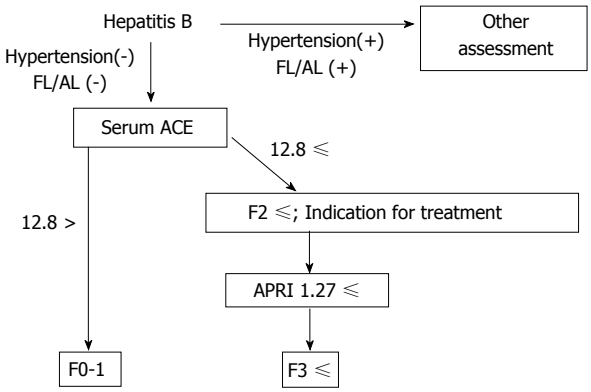


Figure 6 Schematic algorithm for noninvasive diagnosis of chronic hepatitis B. ACE: Angiotensin-converting enzyme; FL: Fatty liver; AL: Habitual alcoholic drinking; APRI: Aspartate aminotransferase to platelet index.

(≥ F3), and cirrhosis (F4) were 0.89, 0.91, and 0.95, respectively. The diagnostic performance for HCV-related fibrosis is controversial. Raslan *et al*[41] showed that ACE polymorphism was associated with the progression of hepatic fibrosis in chronic HCV infection. In contrast, Forrest *et al*[42] reported that no association was identified between four RAAS polymorphisms and fibrosis in chronic HCV infection. Our other assessment also suggested that serum ACE levels in patients with CHC did not correlate with the fibrosis stage (data not shown). This discrepancy may be explained by the interaction between HCV infection and steatosis. HCV infection induces metabolic abnormalities such as insulin resistance, leading to hepatic steatosis[43,44]. Interestingly, our data show that serum ACE levels were elevated in patients with hepatic steatosis (Figure 2). The evidence indicates that hepatic steatosis induced by HCV infection may destabilize serum ACE levels.

In our results, we also found that APRI, FIB-4, M2BPGi and Plt might be good predictive markers for advanced fibrosis in patients with CHB, and APRI showed a potential with the highest accuracy in these parameters. Based on these data, we can propose a strategy to noninvasively evaluate CHB-related liver fibrosis in combination with serum ACE and APRI (Figure 6). This algorithm may be utilized to enclose significant fibrosis, particularly in patients with CHB without fatty liver and/or habitual alcoholic consumption.

Our data have some limitations such as the lack of clinicopathological and prognostic data and a small sample size. Moreover, our study was not performed prospectively. Sequential measurements of serum ACE are required in longitudinal studies to assess the change in the serum ACE level associated with the degree of fibrosis. In addition, the optimum cut-off value for significant fibrosis of 12.8 U/L deviates from the value of 52.5 U/L in the previous study because a different method is used to measure the serum ACE level[39,40]. Therefore, comparison with data from other reports to validate the integration is difficult.

In conclusion, we indicated that the serum ACE level is a beneficial noninvasive marker to evaluate significant fibrosis and to further determine whether therapeutic intervention with NAs is necessary for patients with CHB. Higher accuracy will be expected by combination with other markers, including APRI, FIB4, M2BPGi and Plt. Moreover, our results suggest that blockade of RAAS is an effective new therapeutic strategy against CHB-related fibrosis.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection results in a risk of progressive liver fibrosis, leading to cirrhosis with decreased liver reserve and hepatocellular carcinoma. Therefore, an early assessment of liver fibrosis is required for not only the prevention of disease progression but also the judgment of therapeutic intervention. Currently, several noninvasive markers are explored for estimating early stage of HBV-related fibrosis alternatively to liver biopsy. However, it is required to identify both easier and more beneficial methods.

Research frontiers

Angiotensin-I-converting enzyme (ACE), a central component of renin-angiotensin-aldosterone system, plays a key role in the progression of liver fibrosis, however, its diagnostic performance to evaluate HBV-related earlier fibrosis.

Innovations and breakthroughs

The present study indicates that the patients with significant, advanced fibrosis and cirrhosis (F2-4) have significantly higher serum ACE levels than those with early-stage fibrosis and cirrhosis (F0-1), and serum ACE shows higher accuracy than other markers including APRI, FIB-4, M2BPGi and Plt in diagnostic performance to differentiate significant fibrosis (F2) from mild fibrosis (F1).

Applications

The serum ACE level is a beneficial noninvasive marker to evaluate significant fibrosis and to further determine whether therapeutic intervention is necessary for patients with chronic HBV.

Terminology

ACE converts the inactive decapeptide angiotensin Ⅰ (AT-Ⅰ) into the octapeptide angiotensin Ⅱ (AT-Ⅱ), which shows many physiological activities, including vascular hormonal secretion and tissue growth. AT-Ⅱ has been pathologically recognized to induce the contractility and proliferation of hepatic stellate cells, which play a pivotal role in the progression of liver fibrosis.

Peer-review

The authors report an interesting approach about the diagnostic value evaluation of serum ACE for the detection of earlier HBV-related fibrosis. The sensitivity of the test discriminating F0-F1 from F2-F4 proved to be higher than that of other tests. Overall, the study is well planned and results are presented clearly.

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ORIGINAL ARTICLE

Observational Study

Wilson's disease in Lebanon and regional countries: Homozygosity and hepatic phenotype predominance

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Abstract

AIM

To determine the phenotypes and predominant disease-causing mutations in Lebanese patients with Wilson's disease, as compared to regional non-European data.

METHODS

The clinical profile of 36 patients diagnosed in Lebanon was studied and their mutations were determined by molecular testing. All patients underwent full physical exam, including ophthalmologic slit-lamp examination ultrasound imaging of the liver, as well as measurement of serum ceruloplasmin and 24-h urinary-Cu levels. In addition, genetic screening using PCR followed by sequencing to determine disease-causing mutations and polymorphisms in the *ATP7B* gene was carried on extracted DNA from patients and immediate family members. Our phenotypic-genotypic findings were then compared to reported mutations in Wilson's disease patients from regional Arab and non-European countries.

RESULTS

Patients belonged to extended consanguineous families. The majority were homozygous for the disease-causing mutation, with no predominant mutation identified.

The most common mutation, detected in 4 out of 13 families, involved the ATP hinge region and was present in patients from Lebanon, Egypt, Iran and Turkey. Otherwise, mutations in Lebanese patients and those of the region were scattered over 17 exons of *ATP7B*. While the homozygous exon 12 mutation Trp939Cys was only detected in patients from Lebanon but none from the regional countries, the worldwide common mutation H1069Q was not present in the Lebanese and was rare in the region. Pure hepatic phenotype was predominant in patients from both Lebanon and the region (25%-65%). Furthermore, the majority of patients, including those who were asymptomatic, had evidence of some hepatic dysfunction. Pure neurologic phenotype was rare.

CONCLUSION

Findings do not support presence of a founder effect. Clinical and genetic screening is recommended for family members with index patients and unexplained hepatic dysfunction.

Key words: Wilson Disease; Cu-metabolism; Phenotype; Genotype; ATP7B; Hepatic manifestations

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Core tip: We report on the genotype-phenotype of 36 Lebanese patients with Wilson's disease from 13 different families. The majority were homozygous for disease-causing mutations. The most common mutation worldwide, His1069Trp, was absent in our patients. The ATP hinge region may comprise a hot spot for mutations, as it was detected in 4 families. Hepatic phenotypes were predominant in both symptomatic and asymptomatic patients. Neurologic phenotypes were rare. Compared to findings reported in regional Arab and non-European countries, our results do not support a founder effect. Mutations are scattered over 17 exons, with no common or frequent mutation characterizing the region.

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INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism, resulting from defects in the *ATP7B* gene protein. It is characterized by failure of Cu incorporation into ceruloplasmin (Cp) and decreased biliary Cu excretion. As a consequence, Cu accumulates in various organs, primarily liver and brain. The clinical presentations of WD are characterized by substantial diversity. Patients can present at any age in variable combinations of liver impairment, neurologic dysfunction and/or osseomuscular symptom. Hepatic manifestations include asymptomatic transaminitis, acute or chronic hepatitis, fulminant hepatic failure and/or cirrhosis, while neurologic symptoms vary from mild tremors, articulating problems, dysarthria, Parkinson-like features, seizures and cognitive dysfunction. Some patients have mixed hepato-neurologic presentation[1]. Ophthalmologic involvement with Kaiser-Fleischer (KF) rings is common.

Traditionally, the diagnosis of WD is based on low serum-Cp (< 20 mg/dL), high 24-h urinary Cu and high hepatic Cu content (250 μg/g dry tissue)[2,3]. Recent guidelines for the diagnosis of WD were published by the European Association for the Study of the Liver (EASL)[4]. Nonetheless, the diagnosis of WD may be difficult based on clinical and laboratory criteria, and in some patients it is delayed, leading to detrimental consequences[5]. This is why molecular testing and genotypic analysis may be warranted for confirming and/or supporting a diagnosis of WD, particularly in asymptomatic patients[3].

More than 500 mutations have been identified in WD with a very high allelic heterogeneity. Most patients are compound heterozygous, rendering it difficult to ascribe a phenotype to a specific genotype[6]. Furthermore, a large number of mutations are rare, making it impractical to screen populations for all WD-causing mutations[7]. Some mutations, however, are relatively frequent and population-specific, like the p.His1069Gln on exon 14 in Northern and Eastern European patients[8], the p.Arg778Leu and the p.Arg778Gly mutations on exon 8 among Chinese and Taiwanese patients respectively[9], and the deletion in the 5' regulatory region in Sardinian patients[10]. These findings facilitate molecular diagnosis based on patients' ethnic background. In the Arab World, consanguinity and marriage among individuals belonging to the same ethnic background is very common, thereby increasing the prevalence of genetic disorders, including WD[11]. However, it is not known whether there is a predominant WD mutation in the Arab world, and if so what its phenotypic associations are.

In a cohort of Egyptian patients, genotypic and phenotypic profiles were described, but no prevalent mutation was identified[12]. Moreover, previous reports from Lebanon on a limited number of families suggested an association of liver presentation with homozygous missense mutations: Gly691Arg and non-His1069Trp in exons 7 and 14 of the *ATP7B* gene respectively[13,14]. Whether a specific WD mutation prevails in Lebanon is not known.

In this study, we described the spectrum and frequency of mutations and phenotypes in 36 Lebanese WD patients. We also conducted a comprehensive

literature search for regional studies on WD in Arab and non-European countries in the Middle East. In order to determine whether there is a frequent mutation characterizing the region, a comparative study was undertaken to identify common mutations in the region, and to compare them to our data. We also determined if common mutations in the region were associated with similar clinical phenotypes.

MATERIALS AND METHODS

A total of 36 patients (P_1-P_{36}) from 13 unrelated Lebanese families (U, Or, S, Ah, T, B, H, Ha, Is, Z, Ri, Sc and Gh) were enrolled in the study. Most patients were diagnosed at the American University of Beirut (AUB) Medical Center, a major tertiary referral center in Lebanon. All participating subjects were asked to sign a written consent form (Protocol No. BioCh.JU.01) that was approved by both the Institutional Review Board and Research Committee at AUB.

Clinical testing

Patients' data for evaluation included: history, date of birth, age of onset of symptoms, age at diagnosis, and findings from full physical exam, ophthalmologic slit-lamp examination, and biochemical tests, including liver function tests, serum-Cp and 24-h urinary-Cu levels. Abdominal ultrasound imaging of the liver was performed on all patients and, when necessary, brain magnetic resonance imaging was done. Phenotypic classifications were designated following Ferenci's classification as hepatic, neurologic, mixed or asymptomatic[3]. Diagnosis was further established by computing total WD score developed at the 8th international meeting[15]. Family members (siblings, parents) of all WD-confirmed index patients were also subjected to physical, biochemical and genotypic testing.

Genotypic screening

DNA screening for disease-causing mutations and single nucleotide polymorphisms was performed on all recruited subjects and their immediate family members. Extraction of DNA from blood samples followed by amplification (using PCR) of the 21 *ATP7B* exons was carried out as described[6,14]. Amplified PCR products were purified, sequenced and compared to published normal sequences in the following databanks: Blat at University of California Santa-Cruz, Genome Bio-informatics (http://www.Genome.ucsc.edu/cgi-bin/hgBlat) or Blast at National Center for Biotechnology Information (http://www.ncbi.nih.gov/blast).

WD in regional countries

After identifying the disease-causing mutations in our patients, we compared them to reported mutations in WD patients from regional Arab and Non-European countries. A comprehensive literature search of PubMed and Medline, as well as of the University of Alberta database (http://www.wilsondisease.med.ualberta.ca/database.asp), was conducted for articles published from the regional Arab and non-European countries. Index terms used were Wilson Disease, genotype, phenotype, and each of the following countries: Lebanon, Syria, Jordan, Egypt, Iraq, Saudi Arabia, Kuwait, Bahrain, Qatar, UAE, Yemen, Tunisia, Morocco, Libya, Mauritania, Turkey, Iran and Oman. We included studies in which both the genotype and phenotype were identified. In some studies, it was not clearly indicated whether patients presenting to one medical center with a certain mutation belonged to the same family or to different ethnic groups[16,17]. This made it difficult to estimate the most frequent genotype. We, therefore, opted to identify common mutations between Lebanon and the region, and to determine the frequent regional mutations as indicated by the authors of the various reports.

RESULTS

Clinical presentation

In this study, 36 Lebanese WD patients, including 15 females and 21 males, were recruited from different regions in Lebanon. Patients belonged to 13 unrelated families, referred to as: U (P_1-P_9); Or (P_{10}); S (P_{11}-P_{19}); Ah (P_{20}); T (P_{21}-P_{23}); B (P_{24}-P_{25}); H (P_{26}-P_{28}); Ha (P_{29}-P_{30}); Is (P_{31}); Z (P_{32}-P_{33}); Ri (P_{34}); Sc (P_{35}); and Gh (P_{36}) families. Consanguinity was present in the parents of 27 of the patients (75%), who belonged to the U, S, B, H, Ha, and Z families (Table 1). WD scores computed following EASL guidelines ranged between 4 and 12 (Table 2), confirming the diagnosis.

The clinical profiles of affected subjects are summarized in Table 2. Age at diagnosis ranged between 1 and 39 years. All patients had low Cp level (< 0.2 g/L), except for P_4 and P_{14}. Out of 31 patients, 18 (58%) had KF rings (5/36 were NAV). Out of the 36 WD patients, 24 were symptomatic (67%; 16 males and 8 females) and presented clinically at an average age of 14.5 years. Data on P_6-P_9 were not available. Twelve patients were asymptomatic (33%), diagnosed by genetic screening of family members of index patients. Their average age was 7.6 years.

Pure hepatic phenotype was the most common in our symptomatic patients [9/32: P_1, P_{16}-P_{17}, P_{18}, P_{20}, P_{25}, P_{27}-P_{28} and P_{32}]. Neurologic presentation was noted in 12.5% of patients (4/32: P_{12}, P_{14}, P_{34} and P_{36}). Mixed presentation was observed in 25% of patients (7/32: P_2, P_{10}, P_{24}, P_{26}, P_{29}, P_{31} and P_{35}), two of whom had suicidal attempts/disposition (P_{10} and P_{26}). Notably, liver cirrhosis was present in 12 symptomatic (38%) patients (P_1, P_2, P_{10}, P_{16}-P_{18}, P_{20}, P_{24}, P_{26}-P_{28} and P_{32}), including 4 patients with mixed presentation.

Of the asymptomatic subjects who were diagnosed by screening, 10/12 patients had evidence of liver disease, ranging from transaminitis (P_3-P_5, P_{11}, P_{13},

Table 1 Spectrum of mutations in the *ATP7B* gene of Lebanese patients with Wilson's disease

Family	ID	Sex	Birth date	AD	Exon	Mutation(s)	Region of protein
U	P_1	M	1966	7	7	Gly691Arg	TM2
	P_2	M	1985	9	7	Gly691Arg	TM2
	P_3	F	1986	13[1]	7	Gly691Arg	TM2
	P_4	F	1990	9[1]	7	Gly691Arg	TM2
	P_5	M	1996	3[1]	7	Gly691Arg	TM2
	P_6	M	NAV	3[2]	7	Gly691Arg	TM2
	P_7	M	NAV	7[2]	7	Gly691Arg	TM2
	P_8	M	NAV	12[2]	7	Gly691Arg	TM2
	P_9	F	NAV	NAV	7	Gly691Arg	TM2
Or	P_{10}	F	1986	21	7/10	Gly691Arg/Val845Ser	TM2/Td
S	P_{11}	F	1993	5[1]	8	2299insC/2299insC	TM4
	P_{12}	M	1973	12	8	2299insC/2299insC	TM4
	P_{13}	F	1997	10[1]	8	2299insC/2299insC	TM4
	P_{14}	M	1980	16	8	2299insC/2299insC	TM4
	P_{15}	M	2007	1[1]	8	2299insC/2299insC	TM4
	P_{16}	M	1981	16	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{17}	F	1983	14	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{18}	F	1993	12	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
	P_{19}	F	1989	15[1]	8/13	2299insC/p.Ala1003Thr	TM4/Ch-TM6
Ah	P_{20}	M	1992	15	8	2299insC/2299insC	TM4
T	P_{21}	F	1998	7	12	Trp939Cys	Td
	P_{22}	M	2001	8[1]	12	Trp939Cys	Td
	P_{23}	M	2006	3[1]	12	Trp939Cys	Td
B	P_{24}	M	1992	13	12	Trp939Cys	Td
	P_{25}	M	2002	5[3]	12	Trp939Cys	Td
H	P_{26}	F	1985	18	18	Asn1270Ser	ATP hinge
	P_{27}	F	1987	18[1]	18	Asn1270Ser	ATP hinge
	P_{28}	F	1991	8	18	Asn1270Ser	ATP hinge
Ha	P_{29}	F	1998	14	18	Asn1270Ser	ATP hinge
	P_{30}	F	2002	11	18	Asn1270Ser	ATP hinge
Is	P_{31}	M	1995	13	18	Asn1270Ser	ATP hinge
Z	P_{32}	M	1990	15	18	Pro1273Leu	ATP hinge
	P_{33}	M	2000	6[1]	18	Pro1273Leu	ATP hinge
Ri	P_{34}	M	2009	3	19	Arg1319stop	TM7
Sc	P_{35}	M	1979	22	15/19	Thr1092Met/Arg1319stop	ATP loop/TM7
Gh	P_{36}	M	1970	39	-	None identified	-

[1]Screening; [2]Died at age; [3]Deceased. AD: Age at diagnosis; NAV: Not available.

P_{21}-P_{23}, P_{30} and P_{33}) and hepatomegaly detected by abdominal ultrasound (P_{11}, P_{13} and P_{21}) to full blown cirrhosis (P_{22}-P_{23}). Overall, 27/32 patients (84%) on whom we had clinical information presented with some form of hepatic dysfunction.

Patients with the neurologic phenotype presented at an average age of 22.3 years, while those with hepatic and mixed phenotypes presented at 12.2 and 14 years, respectively. KF rings were present in 17 symptomatic patients (5 symptomatic were NAV) and absent in two (P_{25}, P_{34}). They were not identified in the asymptomatic patients, except for patient P_{19} who had KF rings with no evidence of hepatic or neurologic dysfunctions.

Mutation analysis

Sequencing of the *ATP7B* gene revealed (Table 1) 9 different disease-causing mutations in 70 chromosomes (35 patients), which were distributed as: 7 missense (exons: 7, 12, 10, 13, 15 and 18), 1 non-sense (exon 19), and 1 frame-shift (exon 8). Out of 70 chromosomes, missense/frameshift and/non-sense mutation(s) were detected in 51:16:3 chromosomes at 72.8%:22.8%:4.3% frequency respectively. No mutation was identified in P_{36}, who had been diagnosed based on KF rings, and clinical and biochemical testing.

Out of 35 patients, 29 were homozygous (82.8%) for a disease-causing mutation and 6 were compound heterozygous (17.1%). Parents of our index patients were carriers for the disease-causing mutations. Mutations were most frequent in the exon 18 motif encoding the conserved ATP hinge region of WD gene product. Four out of the 13 unrelated families (H, Ha, Is and Z) had, in this motif, missense mutations in the homozygous state; these were Asn1270Ser in 6 patients (P_{26}-P_{31}) and Pro1273Leu in 2 patients (P_{32}-P_{33}), accounting for 17% and 5.7% of chromosomes respectively. Other identified mutations (Table 1) included missense mutations in exon 7 (Gly691Arg; 10 patients: U and Or) and exon 12 (Trp939Cys; 5 patients: T and B), frameshift in exon 8 (2299insC; 10 patients: S and Ah), and nonsense mutation in exon 19 (Arg1319stop; 2 patients: Ri and Sc), accounting for a chromosome frequency of 27%, 14%, 23% and

Table 2 Phenotypic and genotypic profiles of Lebanese patients with Wilson's disease

ID	Mutation(s)	GI manifestation(s)	Neurological manifestations	KF rings	Cp	Urinary Cu	Score
P_1	Gly691Arg	Liver cirrhosis	Absent	Present	NAV	718.8	8
P_2	Gly691Arg	Liver cirrhosis	Change in school performance	Present	0.11	1998	10
P_3	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.03	148.5	8
P_4	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.22	304	6
P_5	Gly691Arg	Asymptomatic[1]	Absent	Absent	0.02	65.9	7
P_6	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_7	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_8	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_9	Gly691Arg	NAV	NAV	NAV	NAV	NAV	4
P_{10}	Gly691Arg/ Val845Ser	Liver cirrhosis	Suicidal attempts	Present	0.08	2184	12
P_{11}	2299insC	Asymptomatic	Absent	Absent	0.04	99	7
P_{12}	2299insC	Absent	Slurred speech, ataxia, tremors	Present	0.072	512	12
P_{13}	2299insC	Asymptomatic	Absent	Absent	0.03	152.8	8
P_{14}	2299insC	Absent	Choreoathetosis, tremors, rigidity	Present	0.423	2300	10
P_{15}	2299insC	Asymptomatic	Absent	Absent	0.019	10	6
P_{16}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.096	775	10
P_{17}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.096	590	10
P_{18}	2299insC/ p.Ala1003Thr	Liver cirrhosis	Absent	Present	0.17	645	9
P_{19}	2299insC/ p.Ala1003Thr	Absent	Absent	Present	0.12	487	9
P_{20}	2299insC	Liver cirrhosis	Absent	NAV	0.023	651	8
P_{21}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	77.6	7
P_{22}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	20	6
P_{23}	Trp939Cys	Asymptomatic	Absent	Absent	0.02	41.5	6
P_{24}	Trp939Cys	Liver cirrhosis, ascites	Jaw drooping, hypersalivation, slurred speech, narrow based gait, intention tremors	Present	0.021	744	12
P_{25}	Trp939Cys	Liver cirrhosis, Hepatic encephalopathy, Hepatomegaly, Mild to moderate ascites	Absent	Absent	0.04	NAV	6
P_{26}	Asn1270Ser	Liver cirrhosis	Psychiatric symptoms and suicidal attempts	Present	0.03	27.6	10
P_{27}	Asn1270Ser	Liver cirrhosis	Absent	Present	0.03	65.1	9
P_{28}	Asn1270Ser	Ascites, liver cirrhosis	Absent	Present	0.04	55	9
P_{29}	Asn1270Ser	Transaminitis	Neurodevelopmental	Present	0.078	171	11
P_{30}	Asn1270Ser	Asymptomatic	Absent	Absent	0.03	116	8
P_{31}	Asn1270Ser	Chronic liver parenchymal disease	Dysarthria and left-sided dystonia	Present	0.029	402.3	12
P_{32}	Pro1273Leu	Ascites, Liver cirrhosis, Hepatic encephalopathy	Absent	Present	0.17	1041.1	9
P_{33}	Pro1273Leu	Asymptomatic	Absent	Absent	0.19	89.7	6
P_{34}	Arg1319stop	Asymptomatic	Delay in speech	Absent	0.02	92	8
P_{35}	Thr1092Met/ Arg1319stop	Chronic liver disease and early portal hypertension	Clenching of mandible, left side dystonia, sialorrhea, dysarthia, head tremors	Present	0.025	199	12
P_{36}	None identified	Absent	Drooling, dysathria, difficulty concentrating, dysphagia	Present	0.085	NAV	6

[1]Developed later. Normal range: Serum ceruloplasmin: 0.2 to 0.6 g/L; Urine copper: 15 to 50 μg/24 h. Score = Ferrenci Score of diagnosis. 2 or less: Very unlikely; 3: Possible, more tests needed; 4 or more: Established[4]. Cp: Ceruloplasmin (g/L); KF: Keiser-Fleischer; NAV: Not available; Urinary Cu: 24-h urine copper (μg/24 h).

4.3% respectively. Compound heterozygous mutations were identified in exons 10 (Or: P_{10}), 13 (S: P_{16}-P_{19}) and 15 (P_{35}).

Eight polymorphisms were detected in exons 2, 3, 10, 12, 13 and 16 (Table 3) in patients and normal chromosomes obtained from related and unrelated individuals. Three polymorphisms (Lys832Arg, Arg952Lys and Val1140Ala) were present in the homozygous state in 94% (34/36) of patients and in the heterozygous state in 5% (P_{11} and P_{19}), in addition to others in exons 2, 3 and 13 (Table 3).

WD patients: Lebanon vs regional countries

A search of the literature for population studies on the spectrum of mutations in WD patients in the region, including Arab and non-European countries,

Table 3 Identified polymorphisms in the *ATP7B* gene of Lebanese patients with Wilson's disease

Polymorphism	Asp96Gly	Ser406Ala	Val456Leu	Lys832Arg	Arg952Lys	Ala1003 Ala	Val1140Ala	Ser1166Ser
Exon	2	2	3	10	12	13	16	16
Base change	GAC → GGC	TCT → GCT	GTG → CTG	AAG → AGG	AGA → AAA	GCG → GCA	GTC → GCC	AGC → AGT
Domain	Cu1-4	Cu4 binding	Cu4/Cu5	Td	Tm5	ATP binding/Tm6	ATP loop	ATP loop
Family								
U				HM	HM		HM	
Or		HM	HM	HM	HM		HM	
S								
P1, P2, P31, P41, P59				HM	HM		HM	
P7, P8				HT	HT		HT	
P3, P4					HM		HM	
AH			HM	HM	HM		HM	
TF				HM	HM	HM	HM	HM
B				HM	HM	HM	HM	
H		HM	HM	HM	HM		HM	
Ha								HM
Is			HM	HM	HM		HM	
Z		HM	HM	HM	HM		HM	
Ri	HM			HM			HM	
Sc			HT	HM	HM		HM	
Gh		HM	HM	HM	HM		HM	
Ah			HM	HM	HM		HM	

was conducted. A total of 77 articles on WD patients were initially identified, but only those reporting the genotypes and/or the phenotypes were considered. Consanguinity, homozygosity and frequency of mutation were also noted when indicated.

Seventeen articles were included and distributed as follows: Saudi Arabia[18-21], Egypt[12,22-24], Turkey[25,26], Iran[27,28], Oman[29] and Lebanon[6,13,14,30]. Two reports on WD from Iraq were not included, as they had no genotypic information. There were no reports on WD from Jordan, Libya, Tunisia, Morocco or Syria.

Homozygosity was highly prevalent in Lebanese WD patients (83%), and ranged between 68%-85.7% and 50%-53% in Egyptian and Saudi Arabian patients respectively. This finding is attributed to high consanguinity (Table 4) that is common in our societies, or the high prevalence of the same mutation in carriers. Frequency of asymptomatic cases was relatively similar in Lebanon, Egypt and Saudi Arabia. Similar to Lebanese patients, many of asymptomatic patients had evidence of hepatic dysfunction on laboratory and/or imaging studies. Hepatic phenotype was more common than neurologic phenotype in patients from Lebanon, Egypt, Turkey, Iran and Saudi Arabia. Taking into account patients who are asymptomatic and those with mixed phenotype, the vast majority of patients in those countries have some form of hepatic dysfunction. A minority of patients had pure neurologic phenotype. Also, the frequency of patients having KF rings was high and was similar in the 5 countries (Table 4). In a report on a single family from Oman, 78% of patients were asymptomatic and 21% had neurologic phenotype. No patients had a hepatic phenotype in that study.

In conducting our analysis of genotypes, we considered a mutation to be frequent if it was present in multiple unrelated families. We compared genotypic changes in the *ATP7B* gene of Lebanese patients with those from regional Arab and non-European patients. In our patients, the conserved ATP hinge region (exon 18) was the most frequently mutated region identified in 4 unrelated families (Table 1).

Table 3 shows that Lebanese patients share in common with: (1) Egypt, Iran and Turkey, the Val845Ser and Asp1270Ser mutations in exons 10 and 18 respectively; (2) Egypt, the Pro1273Leu mutation in exon 18; (3) Egypt and Turkey, the Arg1319X mutation in exon 19; and (4) Turkey, the Ala1003Thr mutation in exon 13 and the exon 7 mutation (Gly691Arg) reported in one Turkish patient[26]. More interestingly, the mutation in exon 12 (Trp939Cys) was only detected in Lebanese patients and in none of the searched/listed countries. Whereas the worldwide exon 14 mutation (His1069Gln) was detected in some patients from Egypt, Iran and Turkey, it was not identified in Lebanese or in Saudi Arabian patients.

DISCUSSION

The diagnosis of WD based on clinical grounds alone is often difficult. Thus, it may be necessary to resort to genetic testing. In this study, involving more than 500 patients from Lebanon and the region, we found a great deal of genetic heterogeneity with no common or population specific mutation. This reflects the extensive ethnic diversity of people in this part of the world and argues against the presence of a founder gene, even in highly consanguineous populations. It also implies that patients suspected to have WD without a family history, *i.e.*, without a known mutation

Table 4 Lebanese *vs* regional Arab and non-European Wilson's disease patients: Genotype-phenotype

	Lebanon	Egypt	Iran	Turkey	Saudi Arabia	Oman
Number of patients	36	198	88	46	152[1]	14
Number of families	13	135	-	46	53	1
% Homozygosity	83%	68.4% - 85.7%	NAV	NAV	50%-53%	NAV
% Consanguinity	75%	39.5% - 78.9%	NAV	NAV	36.6%-88.8%	NAV
% Hepatic manifestation	28%	45.5% - 84.2%	65.20%	43.50%	25%-54.9%	0%
% Neurologic manifestation	12.50%	4.2%-15.8%	4.30%	34.80%	0%-25%	21.40%
% Mixed manifestation	21.80%	0%-20.9%	21.70%	21.70%	19.6%-55.6%	0%
% Asymptomatic	37%	0%-35.1%	-	0%	30.35%	78.60%
% KF rings	58%	26.3%-69.2%	65.20%	67.40%	50.7%-59%	NAV
Mutation						
E2		Glu396stop				
E3				Gly457stop		
E4-6			No common mutations identified			
E7	Gly691Arg			Gly691Arg		
E8	2299insC	c. 2304-5insC	Trp779Gly	Gly710Ser	Ser744Pro	
		Cys703Tyr		Pro767Arg		
E9			No common or frequent mutations identified			
E10	Val845Ser	Val845Ser	Val845Ser	Val845Ser		
E11			No common or frequent mutations identified			
E12	Trp939Cys					
E13	Ala1003Thr		3061-1G>A sp	Ala1003Thr		Deletion of E13
E14		Thr1076Ile				
		His1069Gln	His1069Gln	His1069Gln		
E15	Thr1092Met	His1126fs	Ile1102Thr			
E16-17			No common or frequent mutations identified			
E18	Asn1270Ser	Asn1270Ser	Asn1270Ser	Asn1270Ser		
	Pro1273Leu	Pro1273Leu				
		IVS18-2A>G				
E19	Arg1319stop	Arg1319stop		Arg1319stop		
E20					Gly1341Ser	
E21					Gln1399Arg	
Ref.	Barada *et al*[13,30]	Abdelghaffar *et al*[12,22]	Dastsooz *et al*[27]	Simsek Papur *et al*[25]	Al Jumah *et al*[18]	Al-Tobi *et al*[29]
					Al Fadda *et al*[19]	
	Usta *et al*[6,14]	El-Karaksy *et al*[23]	Zali *et al*[28]	Loudianos *et al*[26]	Majumdar *et al*[20,21]	
		El-Mougy *et al*[24]				

[1]152 patients described in articles from Saudi Arabia, of which 5 patients were from yemen and syria.

in their family, may need to be screened for mutations in all exons of the *ATP7B* gene. In view of clustering of WD patients within families, their members should be screened for mutations identified in index patients. This is important as it could prevent the silent progression of WD, which may occur as early as 1 year of age, and facilitate management.

Based on the recently published EASL criteria for diagnosis, all our symptomatic and asymptomatic patients had a composite score > 4 (range: 6-12), confirming

the diagnosis beyond doubt. In many of our patients, confirmation of the diagnosis required mutation analysis. Traditionally WD was diagnosed on the basis of low Cp level, KF ring presence and increased 24-h urine Cu level in the context of hepatic and/or neurologic manifestations[31]. In our experience, many patients with WD do not satisfy all these criteria. For example, patients P_4 and P_{14} had normal Cp, 13 did not have KF rings and 4 had normal 24-h urinary Cu. This highlights the difficulties and challenges of making a diagnosis of WD based on clinical grounds alone, particularly in asymptomatic patients.

Worldwide, the majority of WD patients are compound heterozygous[32]. In contrast, in our community, the high rate of consanguinity increases the chance of homozygosity, which is present in 83% of our patients. Only 17% of our patients were compound heterozygous. Missense mutations were the most predominant in Lebanese patients, as worldwide[33]. These occurred in 8 exons of *ATP7B*. One possible hot spot of the WD gene in our patients is that of the conserved ATP hinge region in exon 18. Two mutations in the homozygous state, Pro1273Leu and Asn1270Ser, were the most frequent, being identified in 8 patients from 4 unrelated families. None of the possible hot spot mutations in Lebanon were shared with those of Asia, Latin America or Europe.

One of our WD patients had no identifiable mutations in the coding region of the *ATP7B* gene. Mutations may be present in the promoter or the transcription factor regions which control protein translation and function. In such cases, detailed clinical testing and family history may be of help in diagnosis, such as P_{36}, in whom the diagnosis was based on clinical assessment showing low Cp and presence of KF rings in the context of neurologic manifestation. Finally, all our patients had multiple genetic polymorphisms that may influence the final folded conformation, affinity and/or the function of the Wilson protein and possibly the phenotype of WD patients[34].

Remarkable differences in phenotypes and age at diagnosis were noted among patients and even among family members carrying the same genotype. The age of onset of the disease varied between 1-22 years, with one (P_{36}) diagnosed at 39 years. In the B family, patient P_{25} was diagnosed at 5 and passed away before the onset of his brother's symptoms at the age of 13 (P_{24}). Variation in age at diagnosis was also observed in asymptomatic cases. During a checkup at the age of 7, the female index patient in family T (P_{21}) was found to have transaminitis and hepatomegaly. She was confirmed to have WD and was homozygous for a mutation in exon 12. Genetic screening of her 2 brothers, P_{22} (8 years) and P_{23} (3 years), confirmed WD. Though they were asymptomatic, it was surprising to find that both already had evidence of liver cirrhosis on liver imaging. This raises the question as to whether sex plays a role in the clinical manifestations of disease[15]. Verification of this, however, requires a cohort study with a larger number of patients. Such phenotypic diversity has been reported even among monozygotic twins[35], suggesting a role for epigenetic and/or environmental factors in the expression of WD[36-38].

Diversity in clinical presentation introduces yet another obstacle in the diagnosis of WD, regardless of whether the patient is symptomatic or asymptomatic. A patient, at age of diagnosis, may have mild to severe hepatic and/or neurologic symptoms with or without KF rings. This emphasizes, again, the limitations of pure clinical evaluation and argues for genetic testing of all family members of an affected sibling. In our patients, 28% had pure hepatic manifestations ranging from transaminitis and hepatomegaly to clinically unapparent or overt cirrhosis and portal hypertension. On the other hand, only 9% of our patients had pure neurologic symptoms ranging from weak school performance, slurred speech, tremors, drooling, dysarthria, dysphagia and ataxia to suicidal attempts in some (P_{10} and P_{26}).

Our asymptomatic patients (36%) were found to have liver involvement (transaminitis, fatty liver and cirrhosis) with no KF rings, except for P_{19}. Changes such as fatty liver were detected at the age of 1 year in P_{15}, diagnosed by genetic screening. Therefore, early diagnosis is important in families with index patient(s), to mitigate against progression of the disease. This is in line with the EASL recommendations to perform genetic testing for WD, in individuals with liver disease or neurologic movement disorders of unclear etiology. Whether genetic testing for WD in patients with unexplained hepatic dysfunction will turn out to be cost effective or not in this part of the world is unclear.

Few studies from the Arab world on WD from Lebanon, Egypt, Saudi Arabia and Oman have been published. Similar to Lebanon, the majority of patients from Egypt and Saudi Arabia have consistently shown a high prevalence of consanguinity and homozygosity, with a great deal of genetic heterogeneity, and no mutation characteristic of the region identified. The predominant phenotype of WD in the region was also hepatic, suggesting the benefits of screening for WD in patients with unexplained hepatic dysfunction.

Lebanese and Egyptian patients share missense mutations in exons 8, 10, 18 and 19 (Table 4). However, mutations Gly691Arg and Trp939Cys were identified in Lebanese patients but not in Saudi Arabian or Egyptian ones. There were also common mutations with Turkish WD patients, including exon 7 (Gly691Arg), exon 10 (Val845Ser), exon 13 (Ala1003Thr), exon 18 (Asp1270Ser) and exon 19 (Arg1319stop). Only exon 10 (Val845Ser), and exon 18 (Asp1270Ser) were shared with Iranian patients. Interestingly, the exon 12 mutation of Trp939Cys was detected in Lebanon but not in any regional country. We reported this mutation in the homozygous state in 5 Lebanese patients, while worldwide it was only detected in 1 Hungarian patient in the heterozygous state[39]. This extensive genotypic diversity argues for testing patients suspected to have WD for mutations in all exons of ATP7B. The shared

mutations with the region may be attributed to common ancestors (Turkey and Egypt) who ruled Lebanon in the past. The origin of the Trp939Cys mutation, however, remains undetermined.

To our surprise, the His1069Gln mutation which is common in diverse populations in North America, Europe and several Mediterranean countries[40] was not present in Lebanese patients, but was reported in a minority of patients from Egypt, Iran and Turkey. We did not identify a predominant mutation in Lebanon or the region. Whether mutations in the ATP hinge region in exon 18 may turn out to be a hot spot in this part of the world requires further studies on larger numbers of WD patients.

One major strength of our study is that it involves more than 500 patients from Lebanon and the region. It includes a comprehensive clinical and genetic assessment of WD patients in Lebanon, as well as studies from the region clearly stating the genotype and phenotype. Our patients belonged to extended consanguineous families having similar environmental exposures and dietary habits, which helped in reducing the effects of compounding factors on the genotype and phenotype of patients. In addition, our study has some limitations including the absence of true population studies and the lack of long-term follow-up to determine reliably the true phenotype of patients. It is possible that many WD patients in Lebanon and the region remain undiagnosed or unreported, hence missing new mutations and other genotype-phenotype associations.

In conclusion, WD in Lebanon and the region is characterized by extensive genotypic and phenotypic diversity, and by high rates of consanguinity and homozygosity. No predominant mutation has been identified in the region, while the predominant phenotype seems to be hepatic. Clinical and/or genetic testing of all family members for WD, as well as those with unexplained hepatic dysfunction may increase the detection rate of the disease. This could facilitate early institution of therapy and reduce the mortality and morbidity of this condition.

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COMMENTS

Background

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism, characterized by extensive phenotypic diversity. Most of the patients are compound heterozygotes, having different mutation on each of the *ATP7B* alleles. Attempts to establish genotype-phenotype correlations was hampered by the large number of mutations in the *ATP7B* gene and difficulty in ascribing a phenotype to one allele. This, however, may be overcome by examining WD in homozygous patients. In Lebanon, consanguinity is quite prevalent, increasing the probability of homozygosity and possibility of establishing a phenotype-genotype correlation. They hereby report the spectrum of mutations and phenotypes of 36 Lebanese patients diagnosed with WD. In addition, we examine if a frequent mutation characterizing the region exists by comparing our findings with those reported from regional studies on WD in Arab and non-European countries.

Research frontiers

This manuscript examines whether genotype-phenotype correlation exists in Lebanese patients diagnosed with WD. It also determines if a frequent mutation characteristic of the Lebanese patients and/or the region occurs.

Innovations and breakthroughs

This is the first comparative study that attempts to identify a frequent mutation characterizing WD patients from Lebanon and regional Arab and non-European countries. Although this region is characterized by high rates of consanguinity and homozygosity, no frequent mutation has been identified in the region but predominance of the hepatic phenotype was noted.

Applications

This study improves our understanding of WD pathogenesis and the genetic determinants of patients' phenotype. It emphasizes the importance of genetic screening for WD in family members with index patients, as well in patients with unexplained hepatic dysfunction. This would surely facilitate diagnosis and early management prior to onset of symptoms, thereby preventing the progressive clinical deterioration of the patient.

Terminology

WD is a rare disease of copper homeostasis that results from a defect in the *ATP7B* gene encoding a copper transporter. Ceruloplasmin is the major copper carrying protein in blood with ferroxidase activity. Kaiser-Fleischer rings refer to copper deposition circumscribing the iris of the eye, diagnostic of WD.

Peer-review

It is a very interesting manuscript.

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ORIGINAL ARTICLE

Observational Study

Short-term outcomes of overlapped delta-shaped anastomosis, an innovative intracorporeal anastomosis technique, in totally laparoscopic colectomy for colon cancer

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Abstract

AIM

To introduce an innovative intracorporeal anastomosis technique named overlapped delta-shaped anastomosis (ODA) for colon cancer cases undergoing totally laparoscopic colectomy (TLC) and to assess its feasibility and safety.

METHODS

From January 2016 to March 2017, a total of 20 consecutive patients with colon cancer accepted TLC and the ODA technique at our medical center. Patient demographics, operative outcomes, perioperative complications, and pathological results were collected and analyzed.

RESULTS

We successfully completed TLC and the ODA procedure in all 20 cases, including 6 (30%) males and 14 (70%) females. In total, 11 (55%), 2 (10%), and 7 (35%) cases accepted right hemicolectomy, transverse hemicolectomy, and left hemicolectomy, respectively. None of the surgeries were converted to an open operation. Mean operative time was 178.5 min, and mean estimated blood loss was 58.5 mL. Mean time to first flatus was 2.5 d, and mean postoperative hospitalization duration was 6.8 d. No severe complications occurred, such as anastomotic leakage, snastomotic stenosis, anastomotic bleeding, and wound infection, except for one case who suffered from an abdominal infection and another case who suffered from gastric paralysis syndrome. Tumor recurrence was not observed in any patient during the follow-up period.

CONCLUSION

The ODA technique for colon cancer cases undergoing TLC appears to be safe and feasible, although our current results need to be verified in further studies.

Key words: Overlapped delta-shaped anastomosis; Safety; Totally laparoscopic colectomy; Intracorporeal anastomosis; Colon cancer

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Core tip: Intracorporeal anastomosis technique is one of the biggest difficulties encountered by surgeons during the totally laparoscopic colectomy procedure. In this paper, we introduce an innovative intracorporeal anastomosis technique named overlapped delta-shaped anastomosis and assess its feasibility and safety.

Zhou HT, Wang P, Liang JW, Su H, Zhou ZX. Short-term outcomes of overlapped delta-shaped anastomosis, an innovative intracorporeal anastomosis technique, in totally laparoscopic colectomy for colon cancer. *World J Gastroenterol* 2017; 23(36): 6726-6732 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6726.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6726

INTRODUCTION

With the improvement of living standards and the extension of life expectancy, the incidence of colon cancer is increasingly rising and will continue to rise in China[1,2]. Surgery is the mainstay of treatment for colon cancer. Over the past three decades, minimally invasive surgery for colon cancer has drawn more and more attention[3]. Nowadays, laparoscopic assisted colectomy (LAC) is widely used for cases with colon cancer and the advantages of this procedure have been widely verified[4-6].

With the advances in surgical devices and the improvements in surgical performance, totally laparoscopic colectomy (TLC) has gradually been adopted by experienced surgeons[7-8]. In theory, the TLC procedure conforms more to the concept of minimally invasive surgery and the principle of the tumor-free technique, while the intracorporeal anastomosis (IA) technique still represents one of the biggest difficulties for surgeons during TLC.

As a new technique, delta-shaped anastomosis was first presented by a Japanese scholar named Kanaya in 2002 after he completed the first case of totally laparoscopic gastroenterostomy (TLG)[9]. This procedure has been widely adopted for TLG and has proven to be both safe and feasible[10-12]. However, delta-shaped anastomosis for TLC is rarely reported. Since January 2016, an innovative technique named "overlapped delta-shaped anastomosis (ODA)" has been applied to colon cancer cases undergoing TLC at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. Herein, we introduce this surgical innovation and assess its feasibility and safety.

MATERIALS AND METHODS

Methods

Prior to surgery, the advantages and disadvantages of TLC and ODA were explained to all patients in detail, and then preoperative informed content was acquired. All patients underwent the same preoperative examinations including routine blood tests, chest X-ray, electrocardiogram examination, and computerized tomography (CT) of the abdominal and pelvic cavity to exclude cases with surgical contraindications and distant organ metastasis. Patients with intestinal obstruction were excluded from our study. The American Joint Committee on Cancer (AJCC) staging system (the seventh edition) was used for tumor staging. Postoperative pain was evaluated by the patients on a subjective visual pain scale ranging from 0 to 10, with 0 representing no pain and 10 representing the worst pain imaginable. The ethics committee at our institution approved this study, and this retrospective study conformed to the ethical standards of the World Medical Association Declaration of Helsinki.

Patients

From January 2016 to March 2017, a total of 20 consecutive patients with colon cancer underwent TLC and the ODA procedure. All of these cases accepted colonoscopy examination, and colon cancer was diagnosed by pathology. Preoperative mechanical bowel preparation was performed using polyethylene glycol electrolyte powder. Preventative antibiotics were administered by intravenous drip 30 min preoperatively and continued for 24 h after the operation.



Figure 1 Five-port technique.

Surgical procedure (taking right hemicolectomy as an example)

Step 1: Positioning the patient and placing trocars: The patient was placed in the modified lithotomy position. A five-port technique was used: a 12 mm sub-umbilical port as the observation port, a 12 mm port located in the left upper quadrant as the primary operating port, and three 5 mm ports located in the left lower quadrant, the right lower quadrant and the right upper quadrant, respectively, as the secondary operating ports (Figure 1). Abdominal pressure was maintained at approximately 15 mmHg, and then the patient was placed in the Trendelenburg position and left tilt applied in order to expose the mesenteric root, ileocolic vessels, and superior mesenteric vessels.

Step 2: Clearing lymph nodes and tailoring the mesentery: Along the surface of the superior mesenteric vessels, the mesocolon was dissected in a bottom-up fashion, and then the ileocolic vessels, ascending colon vessels, and the right branch of the transverse colon vessels were exposed and carefully severed (Figure 2A). During this process, attention should be paid to the gastrocolic trunk, pancreatic head, duodenum, and right gastroepiploic vessels, such that these structures are protected. Next, adhesions between the right abdominal wall and the ascending colon were dissected in a similar bottom-up fashion. Finally, the mesentery of the terminal ileum approximately 15 cm away from the ileocecal region, and the right half of the transverse colon, was tailored, respectively.

Step 3: Transecting and anastomosing the bowel: The terminal ileum and the right half of the transverse colon were transected using two endoscopic linear cutter staplers (Johnson and Johnson, PSE60A and ECR60B) (Figure 2B). The broken ends were sterilized using alcohol gauze swabs. Then, the proximal ileum and the distal transverse colon were fixed using a piece of absorbable suture; this was performed in an overlapped fashion in order to facilitate anastomosis (Figure 2C). Two small openings located at the ileum and the transverse colon were created using an ultrasound scalpel. After imbedding the lumens with another endoscopic linear cutter stapler, intestinal walls with no mesentery were got through (Figure 2D). Finally, the common opening was closed using an endoscopic linear cutter stapler (Figure 2E). The specimen was removed from the abdominal cavity using a transverse incision above the symphysis pubis (Figure 2F).

The ways the transverse and left colectomy was performed were similar to the method above mentioned in the right hemicolectomy, including the number of trocars, type of staplers, and same anisoperistaltic anastomosis.

Follow-up

The first day after surgery represented the beginning of the follow-up period. Patients were asked to visit doctors every three months after leaving hospital until two years after surgery, biannually for the next three years and then annually, and the deadline of follow-up period was March 31, 2017.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software (21.0 version for Windows; SPSS Inc. Chicago, IL, United States) was used for data analyses. Quantitative data following a normal distribution are provided as mean and its range. Qualitative data are provided as number and its percentage. The statistical methods of this study were reviewed by Wang M from our institution.

RESULTS

We successfully completed TLC and the ODA procedure in all 20 cases, including 6 (30%) males and 14 (70%) females. None of the surgeries were converted to an open operation. Mean patient age was 52.6 years (range: 38-67 years), and mean body mass index (BMI) was 22.9 kg/m^2 (range: 20.2-25.5 kg/m^2). Our study included 10 (50%) cases with ascending colon cancer, 4 (20%) with transverse colon cancer, and 6 (30%) with descending colon cancer. In total, 11 (55%), 2 (10%), and 7 (35%) cases accepted right hemicolectomy, transverse hemicolectomy, and left hemicolectomy, respectively. Four (20%) out of the 20 cases accepted preoperative chemotherapy (Table 1).

The mean operative time was 178.5 min (range: 155-225 min), and mean estimated blood loss was 58.5 mL (range: 30-100 mL). For all 20 cases, the mean time to first flatus was 2.5 d (range: 1-3 d), and mean first time to oral intake was 3.0 d (range: 2-4 d). Mean postoperative hospitalization period was 6.8 d (range 5-8 d). Patients only reported slight pain (scoring 2.8 on average) on the first day after the operation, and there was almost no pain (scoring 0.7 on average)

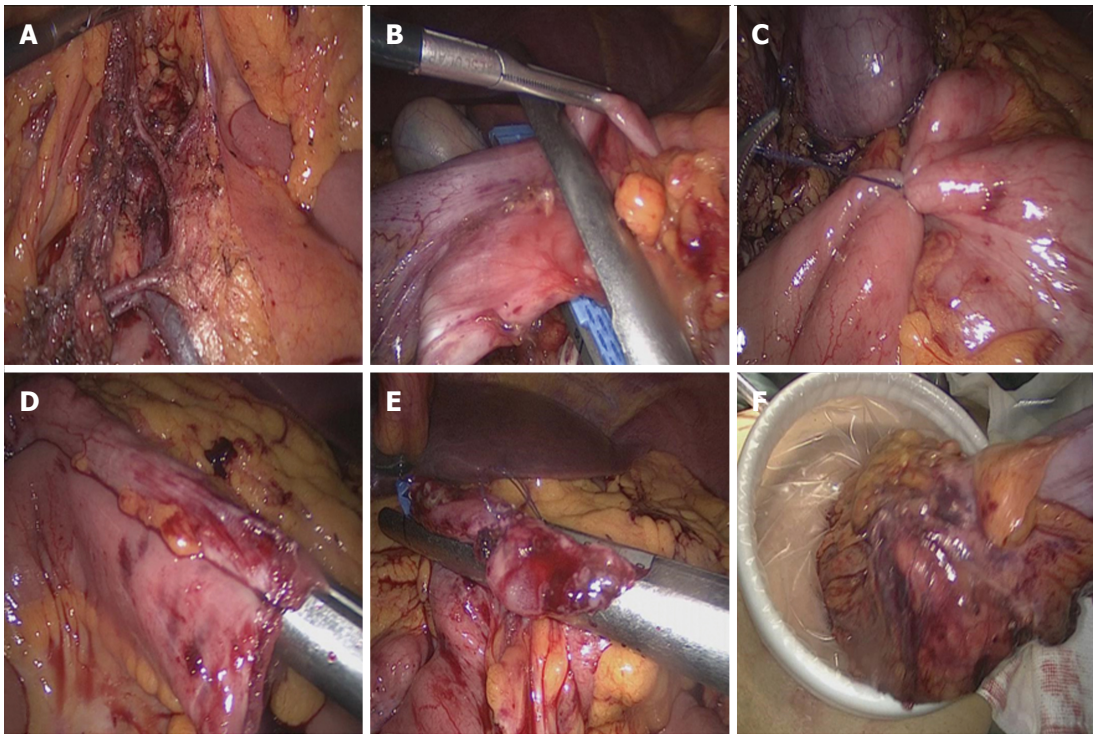


Figure 2 Surgical procedure. A: The ileocolic vessels, ascending colon vessels, and the right branch of the transverse colon vessels were exposed; B: The right half of the transverse colon was transected using endoscopic linear cutter staplers; C: The proximal ileum and the distal transverse colon were fixed in an overlapped fashion using a piece of absorbable suture to facilitate anastomosis; D: After imbedding the lumens with an endoscopic linear cutter stapler, intestinal walls with no mesentery were got through; E: The common opening was then closed using an endoscopic linear cutter stapler; F: Finally, the specimen was removed from the abdominal cavity using a transverse incision above the symphysis pubis.

Table 1 Patient demographics *n* (%)

Parameter	
Gender	
Male	6 (30)
Female	14 (70)
Age, yr, mean (range)	52.6 (38-67)
BMI, kg/m^2, mean (range)	22.9 (20.2-25.5)
ASA score	
1	10 (50)
2	7 (35)
3	3 (15)
Tumor site	
Ascending colon	10 (50)
Transverse colon	4 (20)
Descending colon	6 (30)
Surgical procedure	
Right hemicolectomy	11 (55)
Transverse hemicolectomy	2 (10)
Left hemicolectomy	7 (35)
Preoperative chemotherapy	4 (20)

BMI: Body mass index; ASA: American Society of Anesthesiologists.

by the third day after the operation. No severe complications were encountered, such as anastomotic leakage, anastomotic stenosis, anastomotic bleeding, deep vein thrombosis, or intestinal obstruction, except one case who suffered from abdominal infection due to chylous fistula, and another case who suffered from gastric paralysis syndrome. However, both of these patients made a heathy recovery with conservative treatments. There were no deaths during the perioperative period (Table 2).

Pathological results are shown in Table 3. Mean tumor size was 4.2 cm (range: 2.8-6.3 cm) and mean proximal and distal resection margins were 19.5 cm (range: 13.8-23.5 cm) and 17.8 cm (range: 12.2-21.6 cm), respectively. The mean number of lymph nodes harvested was 32.4 (range: 23-45). Among these cases, there were five (25%) cases of stage Ⅰ, eight (40%) cases of stage Ⅱ, and seven (35%) cases of stage Ⅲ disease. A typical specimen and the transverse incision above the symphysis pubis are shown in Figure 3. The mean follow-up time was 8.5 mo (range: 1-15 mo). No patient was lost to follow-up and no cases experienced recurrence during the follow-up period.

DISCUSSION

Surgery plays an important role in the treatment of colon cancer and the goal of every surgeon is to create as little trauma as necessary and encourage a quick recovery. With recent advances in surgical devices and improvements in surgical performance, surgeons have achieved a significant breakthrough in the treatment of colon cancer by changing from open surgery to laparoscopic surgery[3]. Minimally invasive surgery and quick recovery following laparoscopic surgery have

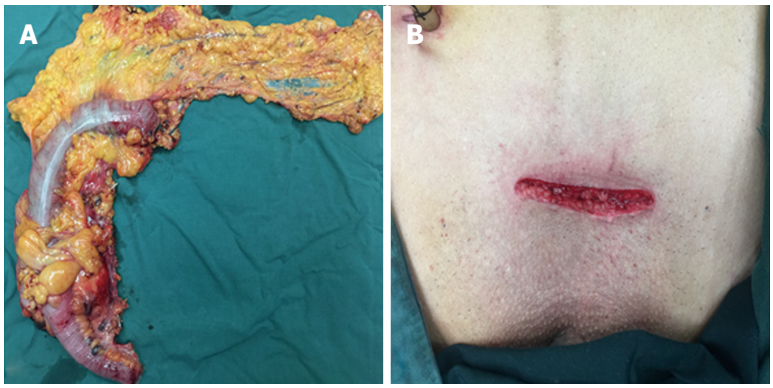


Figure 3 Typical specimen (A) and a transverse incision above the symphysis pubis (B).

Table 2 Operative outcomes and perioperative complications

Parameter	
Operative outcomes	
Operative time, min, mean (range)	178.5 (155-225)
Estimated blood loss, mL, mean (range)	58.5 (30-100)
Time to first flatus, d, mean (range)	2.5 (1-3)
Time to first oral intake, d, mean (range)	3.0 (2-4)
Postoperative hospitalization, d, mean (range)	6.8 (5-8)
Length of transverse incision, cm, mean (range)	4.8 (4-6)
Postoperative pain score	
The first day, mean (range)	2.8 (2-4)
The second day, mean (range)	1.5 (1-3)
The third day, mean (range)	0.7 (0-1)
Perioperative complications (%)	
Anastomotic leakage	0 (0)
Anastomotic stenosis	0 (0)
Anastomotic bleeding	0 (0)
Abdominal infection	1 (5)
Deep-vein thrombosis	0 (0)
Wound infection	0 (0)
Intestinal obstruction	0 (0)
Gastric paralysis syndrome	1 (5)
Reoperation (%)	0 (0)

Table 3 Pathological results

Parameter	
Tumor size, cm, mean (range)	4.2 (2.8-6.3)
Proximal resection margin, cm, mean (range)	19.5 (13.8-23.5)
Distal resection margin, cm, mean (range)	17.8 (12.2-21.6)
No. of lymph nodes harvested, mean (range)	32.4 (23-45)
pTNM stage (%)	
Ⅰ	5 (25)
Ⅱ	8 (40)
Ⅲ	7 (35)

pTNM: Pathological tumor node metastasis.

been well verified and documented. As a consequence, laparoscopic surgery has become incredibly popular over the last three decades and several new laparoscopic techniques, including robotic-assisted colectomy, single incision laparoscopic surgery, natural orifice specimen extraction surgery, and natural orifice transluminal endoscopic surgery, have also been reported[3,13-17]. Although, totally laparoscopic surgery has been carried out by experienced surgeons[7-8], IA technique still represents a significant difficulty during TLC. Therefore, an easy and feasible anastomosis technique is very important.

In 2002, the Japanese scholar Kanaya first reported the delta-shaped anastomosis of TLG[9]. In 2011, he summarized 100 cases of delta-shaped anastomosis performed by eight surgeons, and the results showed that, on average, only 13 min were needed to complete anastomosis, and that all eight surgeons experienced a short learning curve. In addition, this earlier study reported that patients had an early time of first oral intake[18]. Subsequently, more and more studies confirmed the advantages of this procedure[10-12].

In recent years, several studies have described IA for patients with colon cancer. Wang *et al*[17] reported that 11 patients with sigmoid cancer undergoing totally laparoscopic sigmoid colectomy with delta-shaped anastomosis and transvaginal specimen extraction, and these procedures were successful without serious complications. Jian-Cheng *et al*[19] reported 56 cases with colon cancer, who had accepted total laparoscopic right hemicolectomy (LRH) with 3-step stapled intracorporeal isoperistaltic ileocolicanastomosis (TLG group). Compared to cases in the extracorporeal anastomosis group (LG group), cases in the TLG group had shorter IA time (9.9-15.5 min *vs* 13.5-18.2 min, $P < 0.001$), lower mean intraoperative blood loss (83.2 mL *vs* 93.3 mL, $P < 0.001$), faster recovery of bowel function ($P < 0.001$), and lower postoperative pain score ($P < 0.001$). van Oostendorp *et al*[20] conducted a meta-analysis including 12 non-randomized comparative studies and the results showed that IA in the LRH group was associated with reduced short-term morbidity and decreased length of hospital stay suggesting faster recovery. In addition, Wu *et al*[21] also reported that the IA for LRH could improve cosmetic effect and result in better postoperative recovery outcomes without increasing intraoperative and postoperative complications. However, the difficulties of IA procedure behind the satisfactory results were all reported by above-mentioned studies.

In the present study, we performed TLC in 20

colon cancer cases using a new IA method named the ODA procedure; all surgeries were carried out smoothly and none of the cases were converted to open operation. Patients had slight pain after surgery and showed early time of first flatus and oral intake. The results also showed that the ODA procedure did not increase postoperative complications and that radical resection was also guaranteed. In particular, the transverse incision above the symphysis pubis was only 4.8 cm in length, much shorter and more covert than that required for LAC. Collectively, these observations provide promising results with regard to its feasibility and safety. It thus appears that TLC and the ODA procedure are more in line with the concept of minimally invasive surgery and enhance recovery following surgery.

This was a retrospective study and only 20 patients were included; these factors may, therefore, represent potential limitations. However, all operations were successfully accomplished and no severe complications occurred. Prospective randomized controlled trials with larger sample sizes and longer follow-up periods are now needed to confirm our results.

The ODA technique for cases with TLC appears to be safe and feasible for suitable patients when performed by experienced surgeons. This promising result now allows us to cater for colon cancer patients who require medical cosmetology.

COMMENTS

Background

With the advances in surgical devices and the improvements in surgical performance, totally laparoscopic colectomy (TLC) has gradually been adopted by experienced surgeons in large medical centers. However, the intracorporeal anastomosis (IA) technique is one of the biggest difficulties encountered by surgeons during the TLC procedure, and the currently adopted delta-shaped anastomotis technique is difficult to operate.

Research frontiers

Now, the mainstream of colectomy for colon cancer is still laparoscopic assisted colectomy. TLC is only adopted by experienced surgeons in large medical centers for the difficulties of IA technique.

Innovations and breakthroughs

In this study, a modified delta-shaped anastomotis technique named overlapped delta-shaped anastomosis (ODA) has been applied to colon cancer cases undergoing TLC in this medical institution. The results show that the ODA technique is a feasible and safe procedure.

Applications

In the present study, we have reported, for the first time, the ODA technique adopted during the TLC procedure. The perfect result has proven its feasibility and safety. Therefore, the ODA procedure can be applied to colon cancer patients who have no contraindications for laparoscopic surgery.

Terminology

The ODA procedure is currently used for the anastomosis between the proximal and distal intestinal canals for colon cancer cases who accepted TLC.

Peer-review

IA technique is one of the biggest difficulties during the TLC procedure. The authors report an innovative IA technique named the "ODA technique". The short-term outcomes are exciting and it deserves further promotion.

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ORIGINAL ARTICLE

Prospective Study

Effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy

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Author contributions: Sun JX collected the data and drafted the manuscript; Liu YF, Fu ZH, Zhang H, Yang JH and Wang XY contributed to data collection and statistical analysis; Du G and Wang B helped revise the manuscript; Jin B and Bai KY contributed to the study design and revised the manuscript; all authors read and approved the final manuscript.

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Abstract

AIM

To prospectively evaluate the effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy.

METHODS

A total of 56 patients undergoing open hepatectomy

were randomly divided into two groups: a ropivacaine group (wound infiltration with ropivacaine solution) and a control group (infiltration with isotonic saline solution). A visual analog scale (VAS) at rest and on movement was used to measure postoperative pain for the first 48 h after surgery. Mean arterial pressure (MAP), heart rate (HR), time to bowel recovery, length of hospitalization after surgery, cumulative sufentanil consumption, and incidence of nausea and vomiting were compared between the two groups. Surgical stress hormones (epinephrine, norepinephrine, and cortisol) were detected using enzyme-linked immunosorbent assay, and the results were compared.

RESULTS

VAS scores both at rest and on movement at 24 h and 48 h were similar between the two groups. Significantly lower VAS scores were detected at 0, 6, and 12 h in the ropivacaine group compared with the control group ($P < 0.05$ for all). MAP was significantly lower at 6, 12, and 24 h ($P < 0.05$ for all); HR was significantly lower at 0, 6, 12, and 24 h ($P < 0.05$ for all); time to bowel recovery and length of hospitalization after surgery ($P < 0.05$ for both) were significantly shortened; and cumulative sufentanil consumption was significantly lower at 6, 12, 24, and 36 h ($P < 0.05$ for all) in the ropivacaine group than in the control group, although the incidence of nausea and vomiting showed no significant difference between the two groups. The levels of epinephrine, norepinephrine, and cortisol were significantly lower in the ropivacaine group than in the control group at 24 and 48 h ($P < 0.01$ for all).

CONCLUSION

Local wound infiltration with ropivacaine after open hepatectomy can improve postoperative pain relief, reduce surgical stress response, and accelerate postoperative recovery.

Key words: Local wound infiltration; Ropivacaine; Open hepatectomy; Postoperative pain; Surgical stress

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Core tip: This study prospectively evaluated the effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy. Wound infiltration with ropivacaine could provide more effective analgesia both at rest and on movement in the first 48 h after surgery, with lower mean arterial pressure, heart rate and sufentanil consumption, accelerated postoperative recovery, and reduced stress response. These results suggest that local wound infiltration with ropivacaine is a simple, convenient and effective analgesic method that can provide postoperative analgesia and short-term benefits after open hepatectomy.

Sun JX, Bai KY, Liu YF, Du G, Fu ZH, Zhang H, Yang JH, Wang B, Wang XY, Jin B. Effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy. *World J Gastroenterol* 2017; 23(36): 6733-6740 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6733.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6733

INTRODUCTION

Postoperative pain is an important issue for surgeons, anesthetists, patients, and other related personnel. The intensive pain caused by upper abdominal laparotomy may influence postoperative recovery, prolong hospitalization, and cause stress response and complications, including respiratory and cardiovascular depression and gastrointestinal and neuroendocrine dysfunction[1,2]. Currently, intravenous analgesia or epidural analgesia (EA) with a patient-controlled analgesia pump is the most common analgesic approach for controlling postoperative pain after laparotomy[3]. Although favored after major laparotomy, patient-controlled intravenous analgesia (PCIA) can delay postoperative recovery because of nausea and vomiting, excessive sedation, and dizziness; moreover, this analgesic mode involves the risk of addiction with large opioid dosages over long periods[4,5]. As to EA, which provides better analgesia than PCIA, it is restricted by contraindications, epidural puncture failure, and side effects[6,7]. Therefore, finding other analgesic strategies with fewer potentially serious adverse effects will be beneficial for patients suffering from postoperative pain.

Postoperative analgesia is a crucial section of perioperative management, and local anesthetic methods are more effective than systemic analgesia regardless of the operation type[8]. Currently, wound infiltration with local anesthetics, which is a simple, effective, and inexpensive method, is performed in various surgical procedures and provides satisfactory analgesia without major side effects[9]. Ropivacaine and bupivacaine, as long-acting local anesthetics, are commonly used for local anesthesia and pain management in China[10]. Ropivacaine has the same analgesic effects as bupivacaine and levobupivacaine, but it is associated with a low incidence of motor block[11]. Thus, ropivacaine appears to be an important component for local anesthesia and postoperative analgesia. Meanwhile, surgical pain frequently increases the systemic stress response during the perioperative period, which can induce the excessive release of catecholamines (epinephrine and norepinephrine) and cortisol. Optimistically, local anesthesia provides considerable advantages over general anesthesia by suppressing catecholamines and cortisol levels[12].

In this study, we aimed to assess the effect of local wound infiltration with ropivacaine on postoperative pain control, mean arterial pressure (MAP), heart rate

(HR), cumulative sufentanil consumption, incidence of nausea and vomiting, time to bowel recovery, and length of hospitalization after open hepatectomy. The changes of three stress hormones, namely, epinephrine, norepinephrine, and cortisol, were evaluated in patients undergoing wound infiltration with and without ropivacaine.

MATERIALS AND METHODS

A total of 56 patients undergoing open hepatectomy, which was performed by the same experienced surgical team at the Department of Hepatobiliary Surgery of Qilu Hospital of Shandong University from January 2016 to March 2017, participated in this study. The study was approved by the Medical Ethics Committee of Qilu Hospital of Shandong University (No. 2017052), and written informed consent was obtained from all patients. The inclusion criteria included adult patients (aged 18-75 years) who would undergo open hepatectomy and were classified as grades Ⅰ-Ⅲ according to the American Society of Anesthesiologists (ASA) Physical Status Classification System. Patients with a history of known allergy to local anesthetics, chronic preoperative opioid consumption, or any other analgesic treatment for chronic pain before surgery, psychiatric or neurological diseases, or acquired or genetic hemostatic abnormality were excluded from the study.

On the day of surgery, the patients were randomly divided into two groups with a table of random numbers. Surgeons and patients were kept blinded to the assigned treatment groups throughout the study. Wound infiltration was performed with a 7.5 mg/mL ropivacaine solution in the ropivacaine group and with an isotonic saline solution in the control group. Solutions were prepared and provided by the anesthetist, and surgeons were blinded to patient allocation. When closing the abdomen at the end of the surgical procedure, 20 mL of the prearranged solution was used to infiltrate the subcutaneous tissues, deep muscular fascia, and parietal peritoneum. Moreover, one or two drainage tubes were routinely placed near the cutting surface of the liver and then pulled out and fixed on the abdominal skin. In the presence of tube incision or pulling of the tube during movement or when turning over, the surrounding tissues of the tube were also infiltrated with the solution. Infiltration was performed under direct vision by the surgeon. All patients were given unrestricted access to sufentanil through a 100 mL disposable patient-controlled analgesic (PCA) device containing 1 μg/mL sufentanil that was delivered at a rate of 2 μg/h and a bolus of 0.5 μg with a 15 min lockout time. When the skin was closed, the PCA pump was connected to the venous catheter and routinely removed 36 h after the operation.

The intensity of postoperative pain at rest was measured on a visual analogue scale (VAS) graded from 0 (no pain) to 10 (very severe pain) for the first 48 h after surgery. Movement pain was scored using VAS when coughing or turning over. Pain scores were recorded both by nurses and surgeons blinded to patient allocation. Pain measurements were performed at 0, 6, 12, 24, and 48 h after the surgery. Other variables were recorded, including time to bowel recovery, length of hospitalization after surgery, hemodynamic data represented by MAP and HR, cumulative sufentanil consumption, and postoperative nausea and vomiting (PONV). The three surgical stress hormones, namely, epinephrine, norepinephrine, and cortisol, were detected using commercial enzyme-linked immunosorbent assay kits (Cusabio Biotech Co., Ltd., Wuhan, China). Time to bowel recovery was defined as the time to first anal exhaust. PONV was recorded with a three-point rating scale: 1, no nausea and vomiting; 2, nausea without vomiting; 3, nausea with vomiting.

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, United States). All data were checked for normal distribution and the results are expressed as mean ± SD for continuous variables. The *t* test, χ^2 test, Fisher's exact test, or analysis of variance was carried out where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

All patients successfully received the surgical procedure, including the wound infiltration with a prearranged solution. However, three patients (two in the ropivacaine group and one in the control group) were dropped from the study for postoperative bleeding and bile leakage; finally, 26 patients were enrolled in the ropivacaine group and 27 enrolled in the control group. The demographic characteristics of the patients assigned to the two groups were comparable in terms of age, gender, weight, ASA grade, incision length, and postoperative pathology, except for operation type, which showed a statistical difference but had no clinical significance (Table 1).

The VAS scores both at rest and on movement were similar between the two groups at 24 h and 48 h after open hepatectomy (Figure 1A and B). Significant differences in VAS scores at rest were detected at 0 h ($P = 0.0106$), 6 h ($P = 0.0032$), and 12 h ($P = 0.0002$). Moreover, significant differences in VAS scores on movement were observed at 0 h ($P = 0.0208$), 6 h ($P = 0.0043$), and 12 h ($P = 0.0089$). The details are shown in Table 2.

Hemodynamic data are presented in Figure 1C and D and Table 2. MAP was significantly lower in the ropivacaine group than in the control group at 6 h ($P = 0.0241$), 12 h ($P = 0.0001$), and 24 h ($P = 0.002$). In the ropivacaine group, HR was significantly lower at 0 h ($P = 0.0103$), 6 h ($P = 0.0087$), 12 h ($P < 0.0001$), and

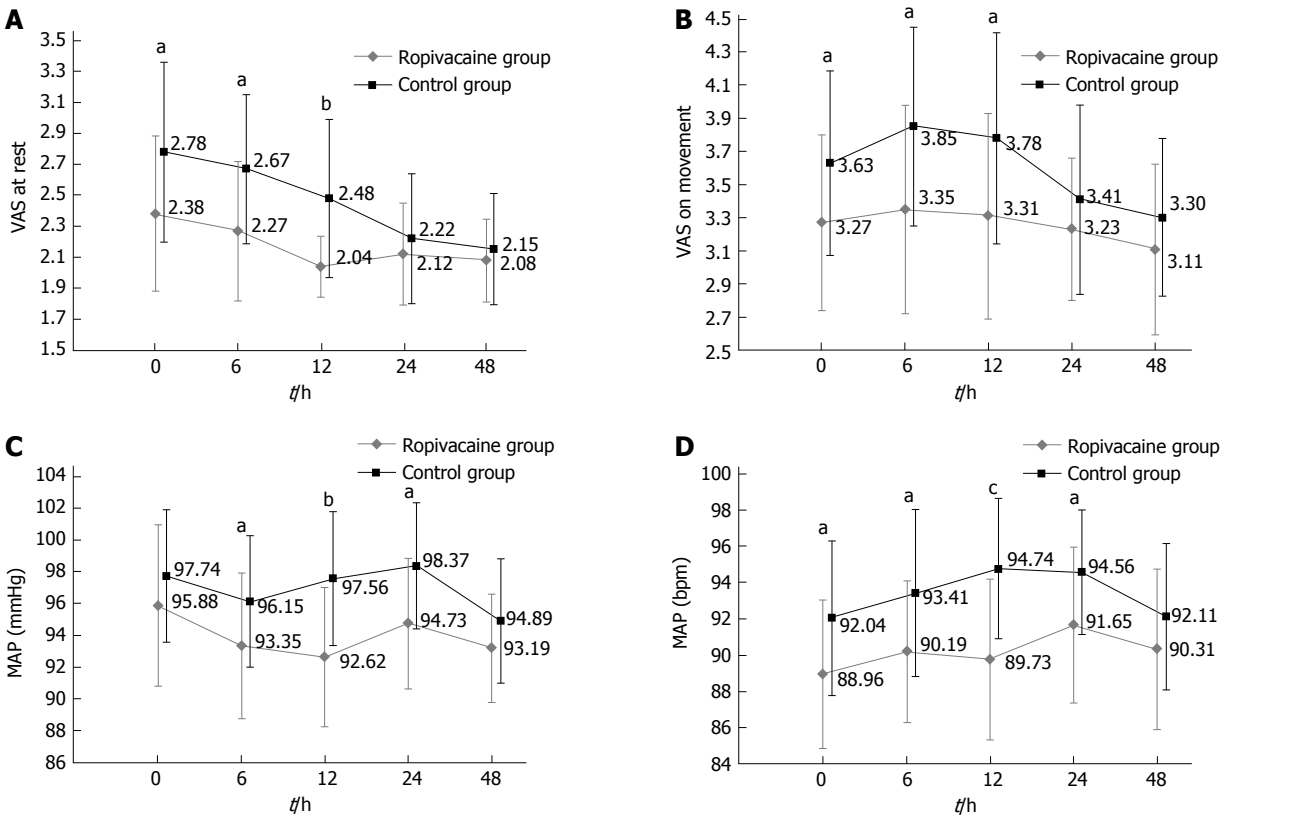


Figure 1 Visual analogue scale scores at rest and on movement, mean arterial pressure, and heart rate during the first 48 h after surgery. A: VAS scores at rest; B: VAS scores on movement; C: MAP; D: HR. [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$. VAS: Visual analogue scale; MAP: Mean arterial pressure; HR: Heart rate.

Table 1 Demographic characteristics of the patients studied (mean ± SD)

Characteristic	Ropivacaine group	Control group	t/χ^2	*P* value
Age (yr)	48.38 ± 11.74	49.59 ± 12.42	-0.36	0.7176
Gender				
Male/female	18/8	18/9	0.04	0.8415
Weight (kg)	63.04 ± 9.21	66.04 ± 9.86	-1.14	0.2583
ASA grade				
I/II/III	4/17/5	7/15/5	0.92	0.6298
Incision length (cm)	24.65 ± 1.83	24.22 ± 2.76	0.67	0.5075
Operation type				
Left hepatectomy	8	5		0.0086
Right hepatectomy	13	8		
Mesohepatectomy	1	0		
Caudate lobectomy	2	1		
Irregular hepatectomy	2	13		
Postoperative pathology				
Hepatocellular carcinoma	22	18		0.3292
Intra-and extrahepatic cholangiolithiasis	3	7		
Hepatic focal nodular hyperplasia	1	1		
Hepatocellular adenoma	0	1		

ASA: American Society of Anesthesiologists.

24 h ($P = 0.0089$).

No statistically significant difference was observed in baseline levels (0 h) of epinephrine, norepinephrine, or cortisol between the two groups. The levels of epinephrine at 24 and 48 h were significantly lower in the ropivacaine group than in the control group ($P = 0.0064$, $P = 0.0078$). Similarly, the values of norepinephrine and cortisol at 24 and 48 h were significantly reduced in the ropivacaine group ($P < 0.0001$ for all), as shown in Figure 2A-C and Table 3.

Cumulative sufentanil consumption at 36 h after surgery is presented in Figure 2D and Table 2. The consumption was significantly lower in the ropivacaine group than in the control group at 6 h ($P = 0.022$),

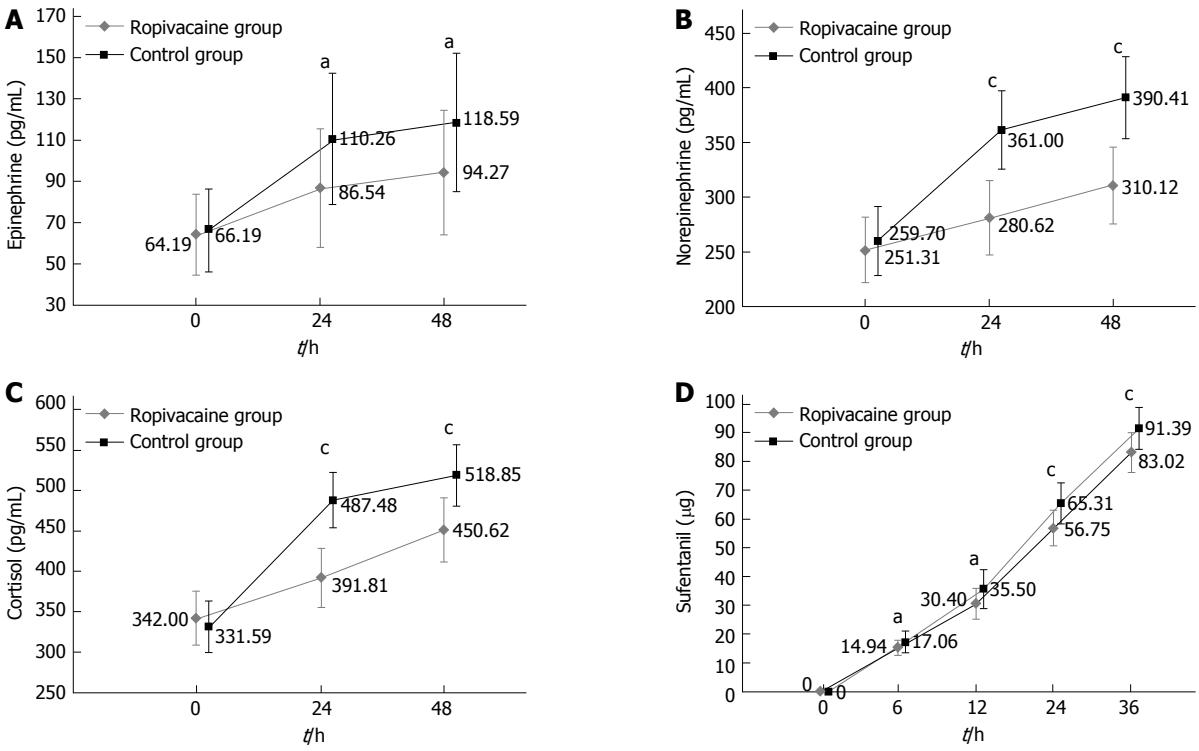


Figure 2 Plasma levels of epinephrine, norepinephrine and cortisol and cumulative sufentanil consumption during the first 48 h after surgery. A: Epinephrine; B: Norepinephrine; C: Cortisol; and D: Cumulative sufentanil consumption. $^{a}P < 0.05$; $^{c}P < 0.0001$.

Table 2 Visual analog scale scores at rest and on movement, mean arterial pressure, heart rate, and cumulative sufentanil consumption

Characteristic	0 h	6 h	12 h	24 h	48 h[1]
VAS at rest					
Ropivacaine group	2.38 ± 0.50	2.27 ± 0.45	2.04 ± 0.20	2.12 ± 0.33	2.08 ± 0.27
Control group	2.78 ± 0.58	2.67 ± 0.48	2.48 ± 0.51	2.22 ± 0.42	2.15 ± 0.36
t	-2.65	-3.17	-4.21	-1.03	-0.81
P value	0.0106	0.0032	0.0002	0.3096	0.4230
VAS on movement					
Ropivacaine group	3.27 ± 0.53	3.35 ± 0.63	3.31 ± 0.62	3.23 ± 0.43	3.11 ± 0.52
Control group	3.63 ± 0.56	3.85 ± 0.60	3.78 ± 0.64	3.41 ± 0.57	3.30 ± 0.47
t	-2.39	-2.99	-2.72	-1.27	-1.34
P value	0.0208	0.0043	0.0089	0.2110	0.1857
MAP (mmHg)					
Ropivacaine group	95.88 ± 5.08	93.35 ± 4.63	92.62 ± 4.43	94.73 ± 4.16	93.19 ± 3.41
Control group	97.74 ± 4.17	96.15 ± 4.14	97.56 ± 4.23	98.37 ± 3.99	94.89 ± 3.90
t	-1.46	-2.32	-4.16	-3.25	-1.69
P value	0.1515	0.0241	0.0001	0.0020	0.0981
HR (bpm)					
Ropivacaine group	88.96 ± 4.12	90.19 ± 3.92	89.73 ± 4.45	91.65 ± 4.30	90.31 ± 4.45
Control group	92.04 ± 4.27	93.41 ± 4.62	94.74 ± 3.88	94.56 ± 3.43	92.11 ± 4.05
t	-2.66	-2.73	-4.37	-2.72	-1.54
P value	0.0103	0.0087	< 0.0001	0.0089	0.1289
Cumulative sufentanil consumption (μg)					
Ropivacaine group	0	14.94 ± 2.56	30.40 ± 5.39	56.75 ± 6.20	83.02 ± 7.05
Control group	0	17.06 ± 3.81	35.50 ± 6.91	65.31 ± 7.09	91.39 ± 7.34
t		-2.36	-2.99	-4.67	-4.23
P value		0.0220	0.0043	< 0.0001	< 0.0001

[1]As to cumulative sufentanil consumption, the time point was set at 36 h. VAS: Visual analog scale; MAP: Mean arterial pressure; HR: Heart rate.

12 h ($P = 0.0043$), 24 h ($P < 0.0001$), and 36 h ($P < 0.0001$). Even so, the incidence of nausea and vomiting, the side effects of sufentanil, between the two groups had no significant difference (Table 4). Moreover, in the ropivacaine group, time to bowel recovery ($P = 0.0133$) and hospitalization after surgery

Table 3 Plasma concentrations of epinephrine, norepinephrine and cortisol (pg/mL)

Stress hormone	0 h	24 h	48 h
Epinephrine			
Ropivacaine group	64.19 ± 19.62	86.54 ± 28.64	94.27 ± 30.10
Control group	66.19 ± 20.30	110.26 ± 31.88	118.59 ± 33.65
t	-0.36	-2.85	-2.77
P value	0.7180	0.0064	0.0078
Norepinephrine			
Ropivacaine group	251.31 ± 30.19	280.62 ± 34.22	310.12 ± 35.15
Control group	259.70 ± 31.72	361.00 ± 36.06	390.41 ± 37.73
t	-0.99	-8.32	-8.01
P value	0.3286	< 0.0001	< 0.0001
Cortisol			
Ropivacaine group	342.00 ± 33.72	391.81 ± 36.53	450.62 ± 39.39
Control group	331.59 ± 31.92	487.48 ± 34.36	518.85 ± 38.21
t	1.15	-9.82	-6.40
P value	0.2537	< 0.0001	< 0.0001

Table 4 Time to bowel recovery, postoperative nausea and vomiting, and hospitalization length in the two groups

Characteristic	Ropivacaine group	Control group	t/χ^2	*P* value
Time to bowel recovery (d)	3.15 ± 1.01	3.93 ± 1.17	-2.56	0.0133
PONA				
No PONA	7	3		0.2729
Nausea without vomiting	16	18		
Nausea with vomiting	3	6		
Hospitalization (d)	8.65 ± 2.43	10.52 ± 3.49	-2.25	0.0289

PONA: Postoperative nausea and vomiting.

(*P* = 0.0289) were significantly shortened (Table 4).

DISCUSSION

Laparoscopic hepatectomy is commonly adopted in clinical settings because of its many advantages, including little trauma, low pain, fast recovery, and short hospitalization; however, open hepatectomy remains irreplaceable, especially in the presence of lesions close to or invading the root of the hepatic veins or the inferior vena cava, history of previous hepatectomy or any previous surgery potentially causing severe adhesion around the liver, and concomitant cardiopulmonary disease[13]. A right subcostal incision or reversed L-shaped incision (> 20 cm) is often made for open hepatectomy, and either of these two incision types is the most important source of postoperative pain. Thus, finding an effective way to reduce postoperative pain is urgent and necessary.

Local anesthetic wound infiltration is a useful and important component of a multimodality approach to postoperative pain control, and it can be applied in many types of surgery, including lumbar spine surgery, breast surgery, and inguinal hernia repair[9,14,15]. Local anesthetics used in the wound can block parietal afferents, reduce the sensitization of spinal dorsal horn neurons, and provide analgesia by inhibiting the transmission of noxious impulses from the incision[16]. Moreover, local anesthetics can suppress local inflammatory responses to incision injury that could sensitize nociceptive receptors and contribute to hyperalgesia[17]. Ropivacaine, a pure levorotatory stereoisomer and long-acting amide local anesthetic agent, has been widely used for local anesthesia and postoperative analgesia, and its reduced lipophilicity is associated with decreased incidence of central nervous system toxicity and cardiotoxicity[18]. Postoperative pain comes from superficial structures and deep muscular-peritoneal components; therefore, ropivacaine infiltrated not only the subcutaneous tissues but also the parietal peritoneum and deep muscular fasciae in our study. Our results showed that in the first 12 h after surgery, the local anesthetic ropivacaine significantly relieved the pain intensity at rest and on movement, demonstrating the potential of local wound infiltration with ropivacaine as a reliable analgesic strategy after open hepatectomy.

Surgical stress could cause a spectrum of changes in the body, involving the neuroendocrine, metabolic, immunological, and hematological systems[12]. The body's surgical stress response is mainly determined by the surgical wound severity, including the length of the incision in the abdominal wall from the skin to the parietal peritoneum[19]. The incision of open hepatectomy often exceeds 20 cm, and the surgical stress is thought to be high. Therefore, using local anesthetics to block surgical stress is feasible. Surgical stress response to injury causes a series of hormone changes; moreover, catecholamines (epinephrine and norepinephrine) and cortisol, as the main and most reliable peripheral hormones, correlate well with the extent of surgical stress. In this study, surgical stress was significantly reduced in the first 48 h after surgery as revealed by the levels of epinephrine, norepinephrine, and cortisol. Changes in MAP and HR were recorded, and the results demonstrated that the indexes of the ropivacaine group were obviously decreased. These results indicate that local wound infiltration with ropivacaine could also reduce surgical stress responses.

Opioids are commonly used for postoperative analgesia *via* venous access. However, they are associated with a potential risk of addiction, especially in large doses over long periods. Moreover, opioids possess potentially serious side effects, such as nausea, vomiting, constipation, respiratory depression, excessive sedation, and liver function impairment; hence, sparing opioids may reduce the incidence of the above side effects[20]. Reducing the dosage and duration of opioid usage is regarded suitable for avoiding

potentially serious adverse effects. Wound infiltration, as part of an opioid-sparing, multimodal analgesic regime, should therefore be recommended. Our current study showed that cumulative sufentanil consumption was significantly reduced in the ropivacaine group. Moreover, the time to bowel recovery was shorter in the ropivacaine group than in the control group. This may be caused by a combination of several reasons. First, sufentanil inhibits gut motility and propulsive activity by combining the μ-2 and κ receptors in the digestive tract[21]. Second, a previous animal study demonstrated that catecholamines reduce gut motility[22] and that the level of catecholamines in the ropivacaine group was reduced. Finally, ropivacaine could accelerate postoperative intestinal motility by reducing the inflammatory response.

The current study may have some limitations. The sample size was relatively small, and thus, more patients are needed in future studies to confirm our results. Compared with a previous study using catheters as a continuous wound infiltration method to deliver ropivacaine into the wound[23], we used single-shot ropivacaine infiltration into the superficial and deep muscular-peritoneal layers to achieve an analgesic effect. In our study, the drainage tube was routinely placed beside the liver resection surface and fixed outside. Movement and turning over could drag the tube and cause intensive pain, and thus, infiltration around the tube was highly effective. Moreover, the catheter under the wound could bring potential risks, such as infection and delayed wound healing, and the delivery rates and volumes of local anesthetics remain unidentified. Thus, single-shot infiltration with ropivacaine is a simple, convenient, and effective analgesic method that can bring short-term benefits for patients who underwent open hepatectomy. A previous study suggested that local anesthesia and stress response reduction could decrease cancer formation and that local anesthesia and analgesia may improve overall patient survival after oncologic surgery[24]. Thus, future research about local anesthesia, tumor recurrence, and patient survival after open hepatectomy is required.

In conclusion, local wound infiltration with ropivacaine after open hepatectomy can decrease acute postoperative pain and surgical stress response. This simple, convenient, and effective analgesic method provides postoperative analgesia and short-term benefits after open hepatectomy.

COMMENTS

Background

The postoperative pain caused by laparotomy delays patients' recovery and incurs stress response. Although commonly used to control pain, intravenous analgesia and epidural analgesia still have their contraindications and side effects. Local wound infiltration is a simple and effective method that can provide satisfactory analgesia without major side effects. The current study was designed to evaluate the effect of local wound infiltration with ropivacaine on postoperative pain and stress response after open hepatectomy.

Research frontiers

Postoperative analgesia is an indispensable component of fast track surgery for surgical patients, especially those who undergo laparotomy. Local anesthetics can effectively provide analgesia by inhibiting the transmission of noxious impulses from the wound and suppress local inflammatory responses to wound injury.

Innovations and breakthroughs

Wound infiltration with ropivacaine could provide effective analgesia in the first 48 h after open hepatectomy, with lower mean arterial pressure, heart rate and sufentanil consumption, accelerated postoperative recovery, and reduced stress response. These results suggest that this method is a simple, convenient and effective analgesic method that can provide postoperative analgesia and short-term benefits after open hepatectomy.

Applications

This study provides additional evidence supporting that local wound infiltration with ropivacaine after open hepatectomy can improve postoperative pain relief, reduce surgical stress response, and accelerate postoperative recovery.

Terminology

The intensity of postoperative pain at rest was measured on a visual analogue scale graded from 0 (no pain) to 10 (very severe pain) after surgery. Postoperative nausea and vomiting was recorded with a three-point rating scale: 1, no nausea and vomiting; 2, nausea without vomiting; 3, nausea with vomiting.

Peer-review

The study was well written and its findings are informative. Local wound infiltration with ropivacaine has good effects for pain relief and stress response reduction after open hepatectomy.

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CASE REPORT

Pedicled omental patch as a bridging procedure for iatrogenic bile duct injury

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Informed consent statement: The patient provided verbal consent authorizing use and disclosure of her protected health information prior to the writing of this manuscript.

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Abstract

Iatrogenic bile duct injuries during cholecystectomy can present as fulminant intra-abdominal sepsis which precludes immediate repair or biliary reconstruction. We report the case of a 29-year-old female patient who sustained a bile duct injury after an open cholecystectomy in a neighboring country. She presented to our institution 22 d after initial surgery with septic shock and multiple intra-abdominal collections. Endoscopic retrograde cholangiography revealed a large common hepatic duct defect corresponding to a Strasberg type D bile duct injury. Definitive reconstruction such as a hepaticojejunostomy cannot be performed due to the presence of dense adhesions with infected and friable tissues. She underwent a combination of endoscopic biliary stenting and pedicled omental patch repair of the bile duct to control bile leak and sepsis as a bridging procedure to definite hepaticojejunostomy three months later.

Key words: Cholecystectomy; Endoscopic retrograde cholangiopancreatography; Case reports; Bile ducts; Abdominal abscess

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Core tip: Iatrogenic bile duct injury is a challenging condition to treat. This case report describes a novel and innovative surgical technique, whereby a pedicled omental patch repair was performed as a bridging procedure to definitive repair such as a hepatico-jejunostomy, in a patient who presented in a delayed fashion with severe intra-abdominal sepsis.

Ng JJ, Kow AWC. Pedicled omental patch as a bridging procedure for iatrogenic bile duct injury. *World J Gastroenterol* 2017; 23(36): 6741-6746 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i36/6741.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i36.6741

INTRODUCTION

Iatrogenic bile duct injuries (BDIs) are uncommon but can lead to significant morbidity and mortality in patients who had undergone cholecystectomy. Although the incidence of BDIs in LC was reported to be almost tenfold that of OC during the 1990s, LC has now become the gold standard in the treatment of gallstone disease, with a low and declining incidence of BDIs[1,2]. Several studies have reported the rate of BDIs during LC to be between 0.4% to 0.7%, but a more recent retrospective review of more than 10000 patients who had undergone LC showed that the incidence of BDIs was 0.2%, which was comparable to that of OC[3-5]. Approximately one-third of BDIs is diagnosed intra-operatively and are amenable to immediate repair or reconstruction. However, the majority of BDIs are recognized only in the post-operative period with varying clinical presentations such as abdominal pain, jaundice, bile peritonitis or shock[6-8]. BDIs can be classified using the Strasberg classification, with most BDIs manifesting as biliary leaks (Strasberg type A) that can be managed using endoscopic techniques[6]. The other large group of BDIs are common bile duct or common hepatic duct transections (Strasberg type E1 to E3) which usually warrants surgical repair[9]. A tension-free Roux-en-Y hepaticojejunostomy is the preferred surgical procedure for reconstitution of bilio-enteric continuity in major common bile duct or common hepatic duct transections, but rarely, in some patients, the presence of severe intra-abdominal sepsis precludes immediate biliary reconstruction[8]. We report a novel method of using a pedicled omental patch, combined with endoscopic biliary stenting, as a bridging procedure to control bile leak, in a patient with severe intra-abdominal sepsis from a large common hepatic duct transection after OC, before attempting definitive hepaticojejunostomy.

CASE REPORT

A 29-year-old female underwent an open cholecystectomy for acute calculous cholecystitis in a hospital from a neighbouring country. Exact intra-operative findings and details of her post-operative recovery were not made available to us. She developed obstructive jaundice on post-operative day three with bilious effluent from her abdominal drain on post-operative day seven. She was initially treated expectantly but due to persistent high bilious output from her abdominal drain, she underwent an exploratory laparotomy on post-operative day 16. The surgeons noted a collapsed common bile duct, but were not able to locate the exact site of bile leak. She was subsequently transferred to our institution on post-operative day 22 for further management for further management of her bile leak.

Upon presentation to our institution, the patient was in septic shock. There was active leakage of bilious fluid from her a right subcostal incision. Her abdomen was distended with generalized tenderness. She was resuscitated and started on broad spectrum antibiotics. Laboratory investigations revealed elevated infective markers with a white cell count of 19 × 10^9/L and a C-reactive protein level of 48 mg/L. Serum bilirubin was normal. Computed tomography scan of the abdomen revealed a 3 cm biloma at the gallbladder fossa (Figure 1A) with multiple rim-enhancing abdominal collections around the upper abdomen (Figure 1B) - the largest being a 9.3 cm × 8.5 cm perisplenic collection (Figure 1C). Endoscopic retrograde cholangiography performed on post-operative day 23 revealed a large common hepatic duct defect just below the bifurcation, corresponding to a Strasberg type D BDI with contrast seen extravasating into the sub-hepatic recess (Figure 2A). After much difficulty, guide-wires were placed across the bile duct defect and plastic biliary stents were deployed into the left and right hepatic ducts respectively (Figure 2B).

In view of persistent sepsis with the presence of multiple intra-abdominal collections, a decision to perform damage control surgery was taken. At the exploratory laparotomy performed on post-operative day 24, a large 2 cm defect at the anterolateral aspect of the common hepatic duct with biliary stents visible from within was found (Figure 3A). There was a large perisplenic abscess with presence of turbid bile and debris in the sub-hepatic recess which was drained and washed out thoroughly. Neither primary repair of the biliary defect nor biliary reconstruction with a hepaticojejunostomy were suitable options due to dense adhesions around the hepatic hilum with infected and friable surrounding tissues. The biliary drains were unable to completely exclude the biliary system entirely due to the size of the biliary defect. Hence, a well-vascularized and healthy pedicled omental patch was harvested and tagged down circumferentially to the biliary defect using absorbable sutures (Figure 3B). An illustration of the pedicled omental patch is as demonstrated (Figure 4). Drains were placed and the abdomen was closed primarily. The patient improved after surgery with resolution of pyrexia and sepsis. Abdominal drains were removed within eight days of surgery. Laboratory infective markers normalized and serum bilirubin levels remained normal. Intra-abdominal cultures grew Pseudomonas aeruginosa and Klebsiella pneumoniae and the patient was sent home to complete four weeks of intravenous antibiotics.

A repeat computed tomography scan of the abdomen three months later revealed complete resolution of the intra-abdominal collections. Although the biliary stents were in-situ, there was mild intrahepatic biliary dilatation suggesting the possibility of a common hepatic duct stricture. The patient subsequently underwent creation of a hepaticojejunostomy one week later. Intra-operatively, there were dense adhesions around the porta hepatis with evidence of early stricture

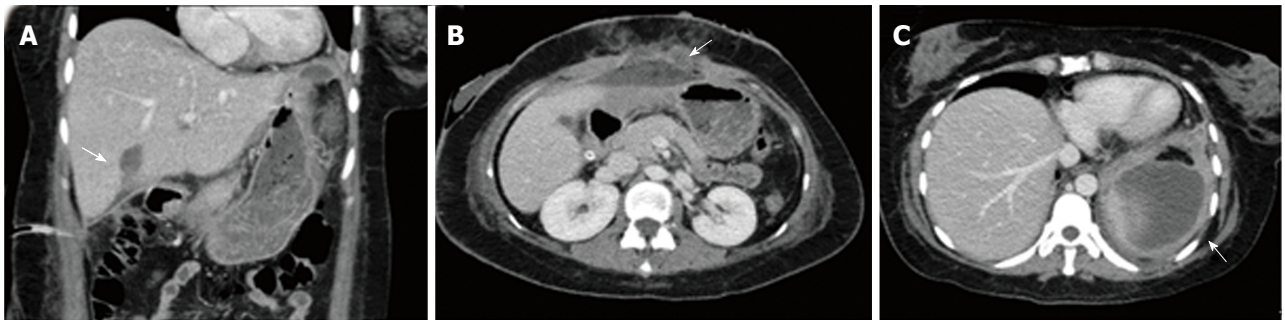


Figure 1 Computed tomography scan. A: Initial computed tomography scan of the abdomen revealing a 3 cm biloma at the gallbladder fossa with bile tracking into the sub-hepatic recess; B: Multiple rim-enhancing smaller intra-abdominal collections were also present in the upper abdomen; C: The largest intra-abdominal collection was a 9.3 cm × 8.5 cm perisplenic collection.

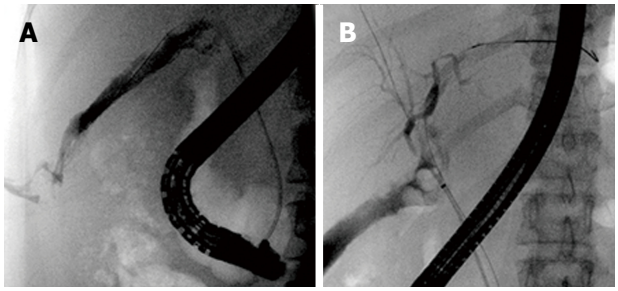


Figure 2 Endoscopic retrograde cholangiography. A: Endoscopic retrograde cholangiography revealed a large Strasberg Type D common hepatic duct defect with contrast seen immediately in the sub-hepatic recess; B: Plastic biliary stents inserted across the biliary defect into the left and right hepatic ducts.

formation at the previous site of BDI. The patient had an uneventful recovery after surgery and was discharged on post-operative day five.

DISCUSSION

BDIs rarely present in such a delayed fashion with fulminant intra-abdominal sepsis. The initial management of BDIs especially for patients that present late in the post-operative period is directed at delineating the type and extent of BDI, controlling sepsis and ongoing bile leak[8]. We describe a technique that can be utilized as a bridge to definitive bilioenteric reconstruction such as a hepaticojejunostomy when prevailing conditions such as severe intra-abdominal sepsis renders immediate or early reconstruction unfavourable and not ideal. Our technique fulfils the above-mentioned principles of BDI management - first by using endoscopic retrograde cholangiogram to characterize the BDI with placement of biliary stents endoscopically to help control the bile leak. The stents will negate the function of the sphincter of Oddi as well as direct bile into the duodenum. Subsequent laparotomy will allow source control of sepsis *via* drainage of intra-abdominal abscesses whilst the placement of a pedicled omental patch will ensure proper sealing of the biliary defect and exclusion of the biliary system from the peritoneal cavity. This restores continuity of bile flow, allows for resolution of intra-abdominal sepsis, inflammation and fibrosis, after which definitive bilioenteric reconstruction can be performed at an elective setting as the presence of peritonitis has been reported to confer a poorer outcome in patients undergoing biliary reconstruction[10].

The use of omentum as an autologous graft to seal, patch or reinforce tissues during surgery is widely practiced, and its ability to support and adhere to local tissue is due to its abundant blood supply, angiogenic activity, innate immune function and high concentration of tissue factor[11,12]. Moreover, animal studies have shown that the use of a pedicled omental flap in tissue reconstruction confers an anti-inflammatory effect, which can lead to an improved healing response[13].

Omentum has been commonly used as a patch to close perforations in the gastro-intestinal tract such as a perforated duodenal ulcer. There have been several reports describing the use of omentum in biliary reconstruction. Meissner described his technique of "T-tube stented omentoplasty" used to successfully treat a common hepatic duct stricture. After incising the strictured segment of bile duct resulting in a 4 cm non-circumferential biliary defect, a large bore T-tube was placed within and a pedicled omental flap was then sutured down over the horizontal limb of the T-tube to bridge any remnant biliary defect as well as to envelop the vertical limb. The T-tube was removed after 6 mo with no sequelae and repeat ERC 19 years later showed normal biliary anatomy[14]. Ebata *et al*[15] also described a similar technique of "hilar cholangioplasty" where he used a pedicled omental flap with a 16F T-tube to seal a 2 cm × 3 cm ductal defect due to a biliobiliary fistula. T-tube cholangioscopy 1 mo after surgery revealed a yellowish polypoid mass at the previous ductal defect and a biopsy taken revealed normal biliary epithelium lining the surface of the grafted omentum. The patient was followed up for 3 years without evidence of cholangitis or abnormal liver function. Another similar technique was described

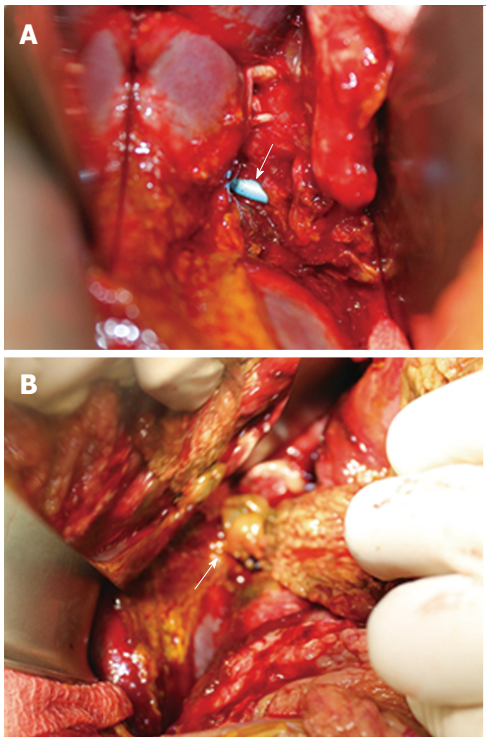


Figure 3 Damage control surgery. A: At the time of exploratory laparotomy, endoscopically placed biliary stents were visible within a large 2 cm anterolateral common hepatic duct defect; B: A pedicled omental patch was harvested and secured to the biliary defect using absorbable sutures.

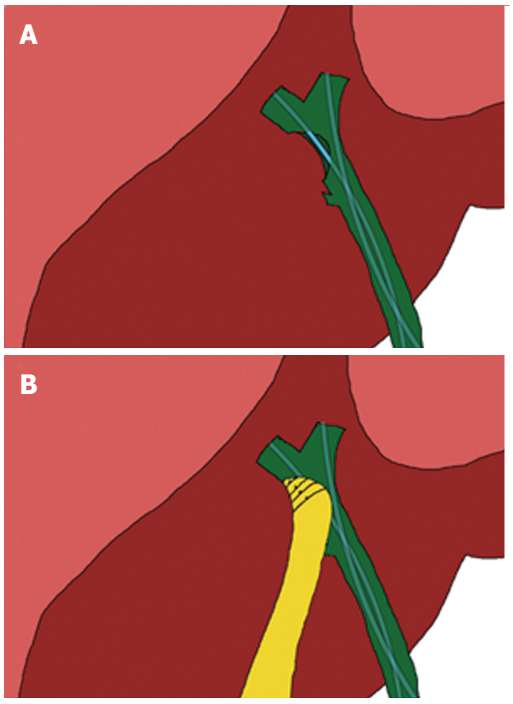


Figure 4 An illustration of the pedicled omental patch. A: An illustration showing the location of the common hepatic duct defect and endoscopic biliary stents placed across; B: The harvested pedicled omental patch was placed over the biliary defect and secured using absorbable sutures that run through the anterior and posterior margins of the biliary defect.

by Chang but he used the falciform ligament instead of the omentum to patch a large bile duct defect due clip necrosis after laparoscopic cholecystectomy[16]. Some authors have also used pedicled omental flaps to reinforce high risk biliary anastomoses in liver transplantation with good results[17].These examples, although limited to individual case reports reaffirms the ideal properties of omentum that make it a suitable autologous graft to aid in biliary reconstruction.

We postulate that our technique could also have been used as a stand-alone procedure without the need of a subsequent hepaticojejunostomy. The biliary stents could then be retrieved endoscopically after 10 to 12 wk. The above-mentioned case reports have shown good long-term patency of the reconstructed bile duct but established data is lacking. A hepaticojejunostomy would still confer the best long-term outcomes, especially in our patient who is young[18]. Using our technique as a stand-alone procedure remains experimental and may be suitable in patients who cannot tolerate longer periods of general anaesthesia needed in a hepaticojejunostomy. Intensive follow-up would be necessary for such patients due to the risk of biliary strictures. We performed a definitive hepaticojejunostomy for our patient as she was due to return to a rural area with no access to regular follow-up for the development of biliary strictures.

Alternative management strategies that could be deployed in our patient would be the placement of percutaneous trans-hepatic biliary drainage tubes or endoscopic biliary stents, combined with either percutaneous or surgically inserted intra-abdominal drains. This might be feasible if the ductal defect was small. However, if the ductal defect is large, the bile leak not be controlled leading to persistent biliary peritonitis and the development of intra-abdominal collections. Moreover, the drains would have to be placed on active suction for a significant period of time to be able to successfully divert and control the bile leak. More often than not, these drains would have to be kept for weeks to months until the patient undergoes definitive bilioenteric reconstruction. In a patient like ours who present with uncontrolled bile leak, intra-abdominal collections and sepsis, the utilization of our technique would allow for control of bile leak without any other additional percutaneous procedures or prolonged drain placement. Another management strategy that could be considered would be use of a T-tube in combination with an omental pedicled flap similar to techniques described above. We were, however, with the assistance of an experienced operator, able to cannulate the left and right hepatic ducts individually and place biliary stents across the biliary defect obviating the need of a T-tube.

Lastly, and most importantly, our case highlights the

need for early referral to an institution with experienced hepatobiliary surgeons, endoscopists, or interventional radiologists once a BDI is diagnosed. It is irrefutable evidence that best outcomes are obtained when first repair is performed in experienced tertiary centres[18,19].

This case highlights the need for early referral of BDIs to specialist tertiary centres for further management once detected. Immediate repair or biliary reconstruction is contraindicated in patients with BDIs who present in a delayed fashion with severe intra-abdominal sepsis. We describe an alternative technique which combines endoscopic biliary stenting with a pedicled omental patch as a bridging procedure to definitive hepaticojejunostomy in this group of patients.

COMMENTS

Case characteristics

A 29-year-old female presented with septic shock and generalized abdominal pain following an open cholecystectomy performed three weeks ago in another hospital.

Clinical diagnosis

Physical examination revealed generalized abdominal tenderness and leakage of bile stained fluid from a right subcostal incision.

Differential diagnosis

Bile duct injury, duodenal injury, small bowel injury.

Laboratory diagnosis

Initial laboratory investigations revealed an elevated total white cell count and C-reactive protein levels.

Imaging diagnosis

Computed tomography scan of the abdomen revealed multiple large intra-abdominal collections. Endoscopic retrograde cholangiography revealed a large common hepatic duct defect (Strasberg type D bile duct injury).

Treatment

An exploratory laparotomy with a pedicled omental patch repair of the common hepatic duct was performed initially, followed by a definitive hepaticojejunostomy three months later.

Related reports

This is the first report describing the use of a pedicled omental patch combined with biliary stenting for treatment of a large bile duct injury. Two other case reports have described the use of a pedicled omental patch in conjunction with a T tube for similar bile duct injuries.

Experiences and lessons

In patients who present with severe intra-abdominal sepsis after bile duct injury, up-front creation of a definitive hepaticojejunostomy may not be possible. Instead, a pedicled omental patch repair of the biliary defect may be performed as a bridging procedure to a hepaticojejunostomy, or even as a stand-alone procedure.

Peer-review

The authors presented a novel and alternative technique by using a pedicled omental patch repair as a bridge to a definitive procedure in bile duct injuries.

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EDITORIAL

Microbial dysbiosis in spouses of ulcerative colitis patients: Any clues to disease pathogenesis?

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Abstract

A number of alterations have been found within the gut microbial profile of patients with inflammatory bowel diseases when compared with the healthy population; however, it is unclear whether such dysbiosis is the cause or simply the consequence of the disease state. In ulcerative colitis, the environment seems to play a crucial role in disease etiology since monozygotic twins show a concordance rate of only 8%-10% - though it is unclear whether it does so by acting through the microbiome. In this study, the authors investigated the influence of cohabitation on the gut microbial community in healthy partners of ulcerative colitis patients - with the intent of clarifying some of these issues. As expected, ulcerative colitis patients had a significant dysbiosis and alterations in microbial metabolism. Interestingly, these abnormal fecal microbial communities were relatively similar amongst patients and their spouses. Thus, this study shows that the microbial profile might be partially transferred from ulcerative colitis patients to healthy individuals. Whether this finding impacts on disease development or has any implication for the role of the microbiome in inflammatory bowel disease etiology remains to be determined.

Key words: Ulcerative colitis; Cohabitation; Spouses; Gut microbiome

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Core tip: Dysbiosis in inflammatory bowel diseases is common. However, microbial dysbiosis could be a consequence, rather than cause of the disease state. In this study the authors detected dysbiosis and altered microbial metabolism not only in ulcerative colitis patients but also in their healthy cohabiting partners. Therefore, the microbiome might be partially transferred from ulcerative colitis patients to healthy individuals. Since spouses of ulcerative colitis patients do not have an increased risk of developing the disease this study suggests that dysbiosis might be an effect rather than the cause of the disease. Overall, the precise role of the microbiome in inflammatory bowel

disease etiology remains unknown.

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Man has coexisted with microbes since the beginning of time. We bear trillions of microbes in our body. Indeed, this symbiotic microbial cell population (or microbiome) has been reported to outnumber that of the human host by 10:1 - though more recent estimates appear to validate such ratio only when considering human nucleate cells[1]. The largest portion of the microbiome is located in the gastrointestinal tract and functions as a digestive organ, a training tool for the immune system, and a guardian of harmful infections[2]. A number of factors can affect the microbial community in the gut--both in healthy and diseased states-- and include diet, age, medical therapy, smoking, and genetics[3]. Many human diseases have been linked to an altered gut microbiota. Among those, the most studied are the inflammatory bowel diseases (IBD) - both Ulcerative colitis (UC) and Crohn's disease (CD). Several alterations (dysbiosis) have been found in the gut microbial profile of IBD patients including a decrease in diversity, *Bacteroides, Firmicutes, Clostridia, Ruminococcaceae, Bifidobacterium, Lactobacillus, and F prausnitzii*; an increase in *Gammaproteobacteria*; and the presence of adherent invasive *E. coli* and *Fusobacterium*[2]. Altered microbial function has also been reported in IBD patients-specifically, butyrate, butanoate and propanoate metabolism are decreased while the oxidative stress, the type II secretion system, secretion of toxins, the transport of both amino acids and sulfates are increased[2].

Despite these impressive and reproducible alterations in the gut microbiome of IBD patients, it is unclear whether such dysbiosis is the cause or simply the consequence (*i.e.*, bacterial adaptation for survival) of the disease state. This is a major mechanistic issue with a potential crucial impact on development of new medications and on IBD management. Current evidence supports both views. For example, disease activity seems to affect the microbial profile in IBD while microbial alterations similar to those described in IBD have been described in patients with nonspecific intestinal injury[4]. These observations suggest that the microbial profile in IBD might be the result of local changes in the intestinal mucosa following the disease onset. On the other hand, dysbiosis also seems to be associated with genetic polymorphisms. In addition, immune mediated colitis can be elicited in genetically susceptible mice with single commensal bacterial species[4]. Thus, these findings highlight the potential role of the microbiome in IBD etiology.

As for the individual IBD's, studies in monozygotic twins show a concordance rate of 44%-55% in CD and only 8%-10% in UC[5]. Thus, the environment seems to play a crucial role in disease etiology - especially in UC - though it is unclear whether it does so by acting through the microbiome.

In the paper published in the 25th issue of the Journal, Chen *et al*[6] studied the influence of cohabitation on the gut microbial community in healthy partners of UC patients using 16S rRNA amplicon sequencing. Fecal samples were collected from 8 UC patients and their healthy partners at Lishui People's Hospital in China. Fecal microbial communities showed a higher similarity among UC patients than in their healthy partners (*i.e.*, the partners' group was more heterogeneous). As a whole, UC patients had a lower relative abundance of bacteria belonging to the *Firmicutes* - especially *Blautia*, *Clostridium*, *Coprococcus* and *Roseburia*. In addition, the microbiome of UC patients showed a greater lipid and nucleotide metabolism. Microbiota dysbiosis and altered microbial metabolism were detected in both UC patients and their healthy partners, with the most relevant genera in the latter group being *Akkermansia*, *Bacteroides*, *Escherichia*, *Lactobacillales*, *Klebsiella,* and *Parabacteroides*. Importantly, using 3 different types of analysis the authors showed that the entire microbiota of UC patients was not significantly divergent from those of their partners. Healthy partners of UC patients also showed an increased microbial membrane transport and metabolism of cofactors and vitamins.

The authors concluded that microbial composition and metabolism in healthy partners of UC patients may be impacted by cohabitation.

This is a small study without follow up which would have possibly clarified if the altered microbiome in spouses of UC patients may eventually impact on their health and bear any clinical meaning. No information is given regarding the number of samples per study subject and whether samples were collected the same day(s) by patients and their spouses. Furthermore, there were differences in age and sex distribution between the UC group and the spouses' group. This difference might bear implications for microbial profile heterogeneity[3]. Also, based on C-reactive protein levels, the disease activity was not uniformly distributed among UC patients - another potential source of heterogeneity in the microbial profile results. The methodology used by the authors in this study - 16S rRNA gene sequencing - is sound and more accurate than culture dependent methods used in many similar studies[7]. The authors' conclusions are reasonable and apparently support an important role of the environment in changing the gut microbiome - in this case, the UC microbiome modifying the bacterial profile of their healthy cohabiting partners. The study does not clarify whether the transfer of the microbiome might occur by direct contact or by other means.

To lend more weight to the authors' conclusion

in this and similar types of studies, IBD and any unrelated upper GI condition should be duly excluded in cohabiting spouses. While disease is unlikely in the absence of symptoms, the hypothetical concomitant presence of IBD in the spouses could clearly explain the similarities between their microbial profile and that of their patient partners. Furthermore, in the Chen *et al*[6] study only the stool bacteria were tested; however, the stool microbiome might be different from the intestinal tissue microbiome - which more closely represents the intestinal mucosa flora[2]. Finally, the microbial profile of individuals cohabiting with IBD patients should also be compared with that of healthy controls living in the same geographical area and socio-economic conditions to minimize the impact of unrelated environmental factors. The microbial profile of the Asian population might differ from that of the Western population[8] - an important factor to keep in mind in comparative studies.

So, does this study suggest that the microbiome dysbiosis in IBD might be a primary event rather than the result of the disease? The answer is no. This study shows that the environment might influence the microbial profile in healthy individuals; however, the impact of such change on disease development remains unclear. Likely, dysbiosis in and by itself is insufficient to cause disease. Indeed, there is no evidence that cohabitation with IBD patients might increase the risk of disease in the spouses, so the results of this study only indicate that the microbiome can be partially transmitted from individual to individual. The final microbial profile in each individual - whether healthy or not - might be the result of the very complex nature of the microbial development process[9], which evolves over time and is affected by a number of factors[3].

CONCLUSION

The findings of this study support the well-known observation that cohabitation is indeed a way to share our microbial cell population with family members, or even with our dogs[10]. Whether this impacts disease development or points to a specific role of the microbiome on IBD etiology remains to be determined.

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REVIEW

Road to stemness in hepatocellular carcinoma

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Abstract

Carcinogenic process has been proposed to relay on the capacity to induce local tissue damage and proliferative repair. Liver has a great regeneration capacity and currently, most studies point towards the dominant role of hepatocytes in regeneration at all levels of liver damage. The most frequent liver cancer is hepatocellular carcinoma (HCC). Historical findings originally led to the idea that the cell of origin of HCC might be a progenitor cell. However, current linage tracing studies put the progenitor hypothesis of HCC origin into question. In agreement with their dominant role in liver regeneration, mature hepatocytes are emerging as the cell of origin of HCC, although, the specific hepatocyte subpopulation of origin is yet to be determined. The relationship between the cancer cell of origin (CCO) and cancer-propagating cells, known as hepatic cancer stem cell (HCSC) is unknown. It has been challenging to identify the definitive phenotypic marker of HCSC, probably due to the existence of different cancer stem cells (CSC) subpopulations with different functions within HCC. There is a dynamic interconversion among different CSCs, and between CSC and non-CSCs. Because of that, CSC-state is currently defined as a description of a highly adaptable and dynamic intrinsic property of tumor cells, instead of a static subpopulation of a tumor. Altered conditions could trigger the gain of stemness, some of them include: EMT-MET, epigenetics, microenvironment and selective stimulus such as chemotherapy. This CSC heterogeneity and dynamism makes them out reach from therapeutic protocols directed to a single target. A further avenue of research in this line will be to uncover mechanisms that trigger this interconversion of cell populations within tumors and target it.

Key words: Cancer stem cell; Liver progenitor cell; Liver stem progenitor cell; Stemness; Plasticity; Cancer cell of origin; Cancer stem cells subpopulations

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Core tip: In agreement with their dominant role in liver regeneration, mature hepatocytes are emerging

as the cell of origin of hepatocellular carcinoma. The relationship between the cancer cell of origin (CCO) and hepatic cancer stem cell is unknown. There is a dynamic interconversion among different cancer stem cells (CSCs), and between CSC and non-CSCs. Because of that, CSC-state is currently defined as a description of a highly adaptable and dynamic intrinsic property of tumor cells, instead of a static subpopulation of a tumor. This CSC heterogeneity and dynamism makes them out reach from therapeutic protocols directed to a single target.

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INTRODUCTION

The problem of multiple propositions for the cell of origin in liver cancer

The hepatocellular carcinoma (HCC) liver cancer subtype is more common (approximately 78%) than others, such as bile-duct cancer, hepatoblastoma, liver sarcoma and other carcinomas (approximately 22%)[1]. This carcinoma is one of the most important cancers; it ranks sixth in incidence and is the second leading cause of cancer-related death worldwide, with a high probability of increasing in upcoming years and an estimated 1 million cases anticipated by 2030[2]. In addition, HCC is a cancer that has a high number of risk factors, such as infection by hepatitis B or C virus, alcohol, and prolonged aflatoxin exposure[3].

Despite new data and increasing information about the origin of liver tumor cells, there is still controversy due to inconclusive evidence. Many studies support the hierarchical hypothesis, which suggests that liver tumors have specialized cells that begin and maintain the tumor, give anti-cancer drug resistance; these cells are denoted as cancer stem cells (CSCs). The limit of many studies is that cells are suggested to function similar to CSCs based on only some of their functional capacities or immunophenotypic characteristics. Some considerations must be taken because different models provide controversial results; CSCs are generally evaluated in late tumor stages, which means that the evaluation is limited to only one late period of tumor development and that some results are only clinical observations. Many questions are still unresolved: Are CSCs really the origin of liver tumors? Which is the cell of origin for these tumors? How are the selected markers considered to be adequate and representative of all subpopulations of CSCs in tumor masses? Are the subpopulations of cancer stem cells (SCs) similar in all kinds of tumors, independent of their etiology? In Supplemental Box 1, we summarize some current problems that are important in the identification of HCSCs in tumor. Hence, there is need for a better and more precise definition to help us clarify confusion about the concept of CSCs.

Since the evidence of CSC was consolidated during pioneer work on leukemia mouse models, subsequent studies have reported the participation of CSCs in various solid tumors, including liver tumors[4-6]. A divergent position is the case of hepatocellular carcinoma (HCC); a few years ago, it was believed that tumor formation was exclusively due to the main parenchymal cell, the hepatocyte[7]. Later findings change the idea of the cell of origin, proposing as a candidate the progenitor cell[8]. Additionally, the existence of SCs in the adult liver is still heavily debated. Nevertheless, some evidence suggests the participation of CSCs in HCC, for example, when tumors have high recurrence after anticancer treatments, such as surgery, radiation or chemotherapy. Additionally, CSCs are considered responsible for tumor metastasis, and it has been reported that a distinct subpopulation of cells exhibit properties that are consistent with stemness, such as self-renewal, cell proliferation and cell survival. Examples of distinct subpopulations include a side population (SP) of the human HCC cell lines MHCC97-H, MHCC97-L, Huh7 and HCCLM3 that give rise to tumors when transplanted into immunodeficient mice[9]. In addition some pluripotency transcription factors are expressed in these SP cells[10]. Moreover, in 2006, Durnez and coworkers used HCC immunophenotyping in HCC and found that 28% of HCCs express liver progenitor cells (LPC) markers. These authors proposed that HCCs may have a progenitor origin[11]. Finally, one study proposed that 40% of HCCs are probably clonal and could potentially arise from stem SCs[12].

Based on the recent literature, the present review has the following aims: to summarize the current knowledge of HCSCs; discuss many pertinent, current questions about CSCs; analyze the significance and limitations of CSC studies; determine the cellular origin of HCCs and discuss other controversial concepts about HCSCs. Finally, we suggest future research perspectives that pertain to not only tumor biology but also clinical development or specific therapies that effectively target HCSCs.

Similarities and differences between two hypotheses of cancer composition

Thus far, there have been two hypotheses, or major schools of thought, related to the cellular composition of cancers. The first hypothesis, denoted as the "stochastic theory" or the clonal-evolution model of carcinogenesis, suggests that tumor development is

initiated by any cell that has suffered mutations by a random process. This hypothesis supports the idea that the accumulation of mutations and epigenetic changes that trigger cancer development may occur in any somatic cell. At some point during tumor progression, a clonal selection of cells may exist that will eventually form cancer. This hypothesis supports the fact that any cancer cell can participate in tumor development, resistance, and recurrence because the tumor could be considered relatively homogeneous; therefore, all cells can function as tumor-supporting cells and are then targets for treatment. Nevertheless, some studies contradict this hypothesis. For example, an autotransplant assay performed in the 1960s demonstrated a low frequency of tumor formation from ovarian and cervical cancer cells. When the cells were injected subcutaneously, the researchers obtained only positive data when they used a high number of tumor cells ($> 1 \times 10^6$)[13]. Another injecting cell assay supported the hypothesis of a small tumor cell subpopulation is responsible for the initiation and maintenance of cancer[14]. Considering the variability of different markers in the tumor, specifically SC markers, some authors support the idea that tumoral heteroigeneity is a consequence of continuous degeneration following sequential genomic insults and epigenetic changes that trigger different signature patterns.

The second hypothesis postulates that there is a tumor mass hierarchy, and indeed, this hypothesis proposes that different populations form the tumor. Similar to the stochastic model, this model proposes that any single mutated cell (the cell of origin) can gain the ability to proliferate but that this situation creates a heterogeneous tumor because most progeny cells will form subpopulations that do not contribute to tumor growth to the same degree and that cannot form secondary tumors. The CSC hypothesis fits with the inefficient and ineffective treatment of cancer because anti-cancer drugs do not discriminate between all tumor cells. If indeed these drugs are not specific, then they could not eliminate the cells that are responsible for sustaining tumors. Evidence from tumorgenicity assays confirms this phenomenon. For example, in pancreatic adenocarcinoma, a cell population enriched with $ALDH^{(high)}$ and CD133+ has an enhanced tumorigenic potential relative to $ALDH^{(low)}$ and CD133+ or $ALDH^{(high)}$/CD133-[15], showing the existence of a distinct cell subpopulation as part of the tumor. In another study, a research group demonstrated that metastasis was established by tumor cells that had characteristics related to quiescence, mesenchymal and stemness in breast cancer [16]. Additionally, it was found that approximately 68% of patients who were subjected to hepatic resection after being diagnosed with HCC showed a pattern of having blood cells expressing the SC markers CD90+, CD45-, and CD44+, indicating that these cells were liberated from the tumor and probably responsible for metastasis[17]. It was reported that a small population in HCC cell lines (HCCLM3, MHCC97H and MHCC97L) have high expression levels of CSC markers, including CD90, EpCAM and CD24; these subpopulations were associated with stronger invasive ability[18]. Moreover, one meta-analysis suggested that CSCs markers were associated with a less differentiation grade in histopathology analysis and decreased survival in HCC patients[19]. This evidence supports the existence and participation of CSCs in solid tumors, including HCC.

Another discrepancy regarding CSCs is the terminology. Some permutations used in this field are varied, such as "stem-like cell", "tumor-initiating cell", "tumor-progenitor cell", "propagating cell" and "CSC". Nevertheless, some authors use the terms "tumor-initiating cell" and "CSC" to describe the same population or considered equal[20]. According to the hierarchical model of cancer biology and the definition proposed by the European Consortium for Stem Cell Research, SC is defined as a cell that can potentially produce unaltered descendants and has the capacity to produce daughter cells with different and restricted progeny. A progenitor cell is defined as any cell that produces progenies composed of transit-amplifying cells that are fated for differentiation or initially uncommitted and that retain self-renewal capabilities, although the existence of progenitor cells is still being debated[21]. CSC is considered a self-renewing cell that is responsible for sustaining cancer and producing differentiated progeny that form the bulk of cancer cells[22].

Cancer cell of origin (CCO) is an interesting term used when any normal somatic cell (progenitor cell, differentiated cell or normal SC; n-SC) that acquired the first cancer-promoting mutation(s) gains self-renewal capacity and can generate the full repertoire of tumor cells (both tumorigenic and non-tumorigenic cells, nTC)[23]. Then, one difference between CCO and CSC concerns their functional status as cancer-initiating cells or cancer-propagating cells, respectively. Until now, the relationship between CCO and CSC is not well understood, but some authors propose that the phenotype between them may be different and change in a dynamical way. Despite all the uncertainly about which cell is the CCO, the use of terminology based on the operational functions helps to clarify some questions. It is better to classify a CSC according to its functional definition instead of as a group of markers, and continuous changes in the definitions of CSC-hypotheses that are based on experimental evidence and conclusions will very likely materialize in the upcoming years.

Although current discoveries have provided com-

Table 1 Liver cancer stem cell markers in hepatocellular carcinoma

Marker	Another name	Function	Ref.
CD133	Prom-1	Neurotensin/IL-8/CXCL1 signaling	[33,186]
OV6	Oval cell marker	Unknown until know, but it is considered to be an inducible progenitor cell marker in rodent models.	[8,38]
EpCAM	Epithelial cell adhesion molecule	Activation of Wnt signaling	[187,188]
CD13	Cluster of differentiation 13	ROS-induced DNA damage reduction	[71,72,189]
CD24	Heat-stable antigen	STAT3-mediated NANOG regulation	[18,34,150]
CD90	Thy-1	Glycophosphatidylinositol (GPI) anchor	[18,32]
CD44	Hyaluronic acid receptor	Participation in epithelial mesenchymal transition	[33,69,190]
ALDH	Aldehyde dehydrogenase	Group of intracellular enzymes that oxidize aldehydes (thereby serving a detoxifying role)	[15,39]

pelling evidence that CSC populations in solid cancers are key contributors of tumor maintenance, recurrence and therapy failure, further research is required to understand how these cells participate in HCC. It is important to note that both hypotheses have significant differences, but they cannot be considered mutually exclusive or lacking cooperation in cancer.

One marker is not enough to characterize HCSCs

Evidently, surface markers are not enough to define a CSC. Indeed, is important to note that none of the markers used to isolate normal and cancerous SCs are expressed exclusively by the SC itself. Moreover, markers used to identify SCs from one organ are not useful for identifying SCs in other tissues, and identifying a marker in SCs from one kind of tumor does not mean that it will be present in other tumors; it could be present in the same kind of tumor but in a different context[24]. For example, CD133 is used to successfully enrich SC fractions from various solid tumors, but it is also present in normal brain SCs and is not restricted to only SCs[25]. CD44 is now considered a marker of CSCs, although determining which isoform is representative and dominant is still under debate. However, CD44 has been implicated in many physiologic processes, especially leukocyte homing, activation and migration, because it is considered the major adhesion molecule in the extracellular matrix. Furthermore, aldehyde dehydrogenases (ALDHs) are an enzyme family in charge of catalyzing the oxidation (dehydrogenation of aldehydes) and detoxification of aldehydes. The identification of the isoform responsible for this process and that has high activity in SCs and CSCs was unknown until now. ALDH1A1 is expressed by hepatocytes in the centrilobular region of healthy mice livers[26]. Thus, the conceptualization that CSCs only have one specific pattern of markers needs to be re-evaluated because, as it was mentioned before, the previously considered phenotype is no specific to the population of CSCs and is not exclusive to SCs.

The first thing to consider for a cell to be a CSC is its phenotype. Until now, there have been various accepted markers used to identify HCSCs, such as Hoechst 33342 dye staining, ALDH, CD133, CD90, CD44, EpCAM, and CD13 (Table 1, Figure 1). Briefly, cell sorting after Hoechst 33342 dyeing is used to identify CSCs, including HCSCs. Because CSCs overexpress adenosine triphosphate (ATP)-binding cassette (ABC) transporters, they can efflux Hoechst 33342 dye, which results in low levels of staining with Hoechst. Additionally, Hoechst- cells have been related to stemness gene expression and depict higher tumorigenicity ability. However, one problem with this staining is that Hoechst 33342 dye is toxic[27]. The ALDH family is composed of cytosolic isoenzymes that are responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early SC differentiation. The human ALDH superfamily is composed of cytosolic isoenzymes, which includes 19 putatively functional genes at distinct locations on chromosomes. Their function consists in the oxidation of retinol to retinoic acid in the earliest stages of SC differentiation. Cells that are ALDH+ express CSC markers, are invasive and clonogenic[28]. Recently, it was proposed a system denominated label-retaining cells (LRCs) to identified slow-cycling cells in HCC lines and HeLa tumors. These cells were evaluated *in vitro* and *in vivo* and showed a functional behavior of CSC, unlike no selected cells[29]. It would be very interesting to further study HCC tumoral LRCs in order to determine if they express CSC markers, and their role in HCC. Ma and colleagues identified for first time CD133 in HCSCs, this transmembrane glycoprotein is also named Prominin 1[30]. CD133 was reported to be expressed in approximately 1%-3% of human HCC specimens, however in peripheral tumor tissue the expression was between 0.025%-0.1%. In addition, it was showed that HCC cells expressing CD133 have some survival pathways activated and consequently high resistance to anticancer therapy[31]. CD90, or Thy-1, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein with a role in cell-cell and cell-matrix interactions and is expressed predominantly in leukocytes. Yang and coworkers found that $CD90^+$ HCC cells have greater tumorigenic and metastatic potential than $CD90^-$. CD90+CD45- cells found in tumor and blood samples from HCC patients were evaluated in a model of immunodeficient mice and were able to form tumors. The fact that CSCs circulate in the blood

indicates that HCSCs have a probable role in preparing a new place for colonization, but until now, no reliable data have been reported[32].

EpCAM is a glycoprotein with the three domains, a N-terminal extracellular, a single transmembrane domain and a short C- terminal fragment. This protein is one of the most representative and successful markers of HCSCs, and it is also considered an early biomarker for HCC. Its coexpression with alpha feto-protein (AFP) is indicative of a poor prognosis in patients. EpCAM+ is considered a molecular signature of hepatic progenitor cells and/or stem/progenitor markers, whereas EpCAM- is a characteristic of mature hepatocytes in HCC probes. In HCC EpCAM is a Wnt/β-catenin target gene, and this pathway activation increases the EpCAM+ cell population. Another important marker in HCC is CD44, which adds properties to the CD133+ or CD90+ CSC population. Fewer CD133+ CD44+ cells are required to initiate tumor growth in immunodefficient mice when compared with CD133+ CD44- cells[33]. Similarly CD90+ CD44+ cells are more metastatic than CD90+ CD44- cells in immunodeficient mice[32].

Recently, CD24 was identified as a possible HCSC marker. CD24+ cells possess more CSC properties of tumor initiation, self-renewal, chemoresistance, high metastatic capacity and differentiation than CD24- cells in an HCC mouse model. Indeed, CD24 overexpression showed a significantly correlation with poor survival in one study with HCC clinical samples. Finally, some stemness genes have a good correlation with CD24 in HCC cell lines and clinical samples. These genes were identified as downstream effectors of CD24 signaling, which acts through STAT3 activation to mediate tumor initiation and self-renewal[34].

Another HCSC marker is OV6, or the oval cell marker. The oval cells were first identified by Farber in carcinogen-treated rats[8]. These cells co-express several hepatic lineage markers [*e.g.*, AFP, Albumin, cytokeratin (CK)-7,-8, and -19][35]. Since then, several studies have found OV6+ cells in HCCs and in hepatoblastomas[36]. Additionally, Yang and coworkers demonstrated that $OV6^+$ HCC cells possess greater tumorigenic ability and chemotherapeutic resistance[37]. In addition, the $CD133^+$ population was significantly enriched for OV6+ cells[38].

All of these markers have been tested based on the abilities and qualities that are recognized for CSCs. A considerable problem is that some authors place higher importance on some markers over others based on functional assay results in their respective models of study. For example, in one study where CD133+ was isolated in HCC cell lines, CD44 and CD34 showed higher expression when compared with CD133- cells, but both subpopulations displayed similar expression of CD49f and CD117. This casted doubt as to whether these subpopulations should be considered definitive CSCs[30]. Others authors have demonstrated the participation of only one marker, such as CD133 in human HCC and HCC cell lines, and these cells were evaluated based on other abilities, such as to self-renew, to create differentiated progenies and to form tumors. CD90+CD45- cells present in blood from liver cancer patients were tumorigenic, however CD90-CD45- cells were not evaluated[32]. One study provided evidence of the existence of a hierarchical organization that bears tumorigenic potential in the order of CD133+ALDH+ > CD133+ALDH- > CD133-ALDH- in HCC cells. The authors considered the presence of both markers as a more specific method of characterizing the tumorigenic HCSC population[39]. Another example is the association of coexpression of CD90+ and EpCAM+ with poorly differentiated morphology. This classification might help to determine a clinical outcome and a therapeutic approach in HCC patients, but the authors did not consider other markers in relation to the selected markers[40].

Main signaling pathways that govern the stemness

In addition to surface markers and enzymatic activities, other hallmarks are important in characterizing CSCs. Signaling pathway behavior is considered an important hallmark of every cell. The signature of CSC pathways corresponds to the pattern of pluripotency-associated transcriptional networks. For example, in normal hepatic SCs, the high expression of Sox9, Oct4, Sox2, STAT3 was considered, in addition to some hepatocyte markers, such as CK18, ABCG2, E-cadherin, CD117 and/or CD49f[30]. It is well known that n-SCs and CSCs share pathways for sustaining the property of stemness. Some important signaling pathways related to self-renewal characteristics include Wnt/β-catenin, Notch, sonic hedgehog (Shh), TGF-β, and AKT (Figure 1).

The Wnt family consists of inter-cellular signaling molecules that are involved in the regulation of embryonic development and zonation, which is required for spatial separation of the diverse metabolic functions. These molecules are frequently altered during the processes of epithelial to mesenchymal transition (EMT), postnatal hepatic growth adult liver homeostasis, liver metabolism, and carcinogenesis. Wnt/β-catenin is an important signaling pathway involved in the tumorigenesis, progression, invasion and maintenance of CSCs[41]. This pathway is disrupted by mutational and non-mutational events in approximately one-third of HCCs[42]. Finally, the Wnt pathway involves the translocation of the β-catenin component to the nucleus, which induces the transcription of prominent targets, such as CD44[43], cyclin D1[44], and c-Myc[45], which is also a major target of EpCAM signaling. Current data indicate that the protein c-Myc is important in the

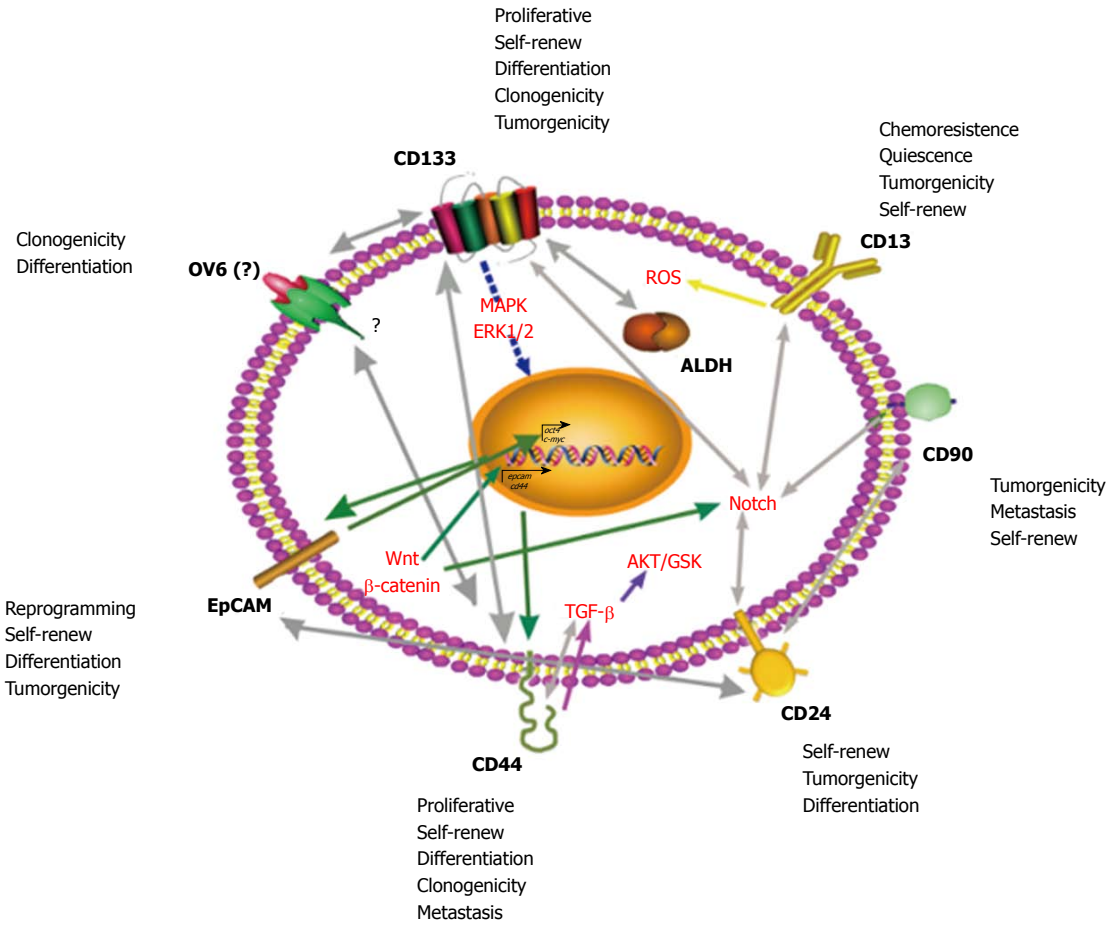


Figure 1 One marker is not enough for designate one cell as cancer stem cell. Some markers are reported as representative of HCSCs (CD133, CD13, CD90, CD24, CD44, EpCAM, OV6 and activity of the ALDH family) nevertheless there are main signaling pathways dictating the stemness state. OV6 structure and function is still unknown. Properties or characteristics related of every HCSC marker are listed besides each marker (self-renew, chemoresistence, quiescence, tumorgenicity, *etc*). Gray lines with double arrows indicate correlation in expression (*i.e.* Notch and CD90, CD133 and CD13 are overexpressed in the same CSCs population). Straight lines indicate that the molecule or pathway affects directly another molecule or pathway (*i.e.* CD44 affects directly TGF-β pathway, and TGF-β pathway affects directly AKT/GSK pathway). Doted line indicates that it affects indirectly the molecule or pathway (*i.e.*, CD133 expression affects IL-8/CXCL1 *via* MAPK/ERK indirect activation).

switch from adult to embryonic SCs[46,47].

The Notch signaling pathway plays an important role in SC self-renewal and differentiation. Notch receptors are single-pass transmembrane proteins. These receptors are processed in the endoplasmic reticulum and in the Golgi, resulting in cleavage and a glycosylated heterodimer. The processed receptors are translocated to the membrane where they could bind to their respective ligands, members of the Delta-like and Jagged family[48]. In breast cancer, for example, the activated intracellular form of Notch3 and the Notch ligand Jagged are highly expressed[49]. Notch signaling plays an essential role in liver embryogenesis and bile duct formation. CSCs CD133+ overexpress Notch1 compared to that of CD133- in HCC. Moreover in HCC, Notch3 and the Notch ligand Jagged are overexpressed[50]. An expansion of Hedgehog-responsive cells in hepatitis B/C virus (HBV/HCV) infection, promotes liver fibrosis and cancer. Increased expression of the Hedgehog pathway was identified in CD133+ HCSCs[51]. Another example of the participation of the Hedgehog pathway in CSCs was described in pancreatic cancer where CSC-like cells were treated with the inhibitor GDC-0499, which resulted in a decreased of CSC-like cells[52].

Another important pathway is the AKT signaling pathway, which is involved in regulating homeostasis and chemoresistance of HCSCs. During hypoxia, the AKT pathway significantly increases the expression of hypoxia-inducible factor-1 alpha (HIF-1α), and in a positive loop, HIF-1α will affect the phosphorylation of AKT. The positive interaction between AKT and HIF-1α leads to overexpression of platelet-derived growth factor (PDGF)-B, vascular endothelial growth factor (VEGF) and to the expression of ABCG2, which are important for homeostasis and chemoresistance of CSCs. In addition, the activated AKT pathway is associated with high levels of cell survival proteins, such as Bcl-2 in $CD133^+$ HCSCs[53].

The TGF-β signaling pathway is prominent at the

interface between liver development and cancer in the liver. Smad signaling is pivotal for embryonic hepatocyte proliferation and cancer proliferation. TGF-β is a cytokine that controls proliferation and differentiation in both normal and cancer SCs. It had been reported that TGF-β regulates the expansion of CD133+ though the induction of EMT. Additionally, this cytokine can activate differentiation programs and inhibit cell-cycle progression during early carcinogenesis through intermediary SMAD proteins. The association and cooperation between TGF-β and oncogenic RAS activates the nuclear β-catenin signaling pathway, which causes neoplastic hepatocyte loss differentiation and directs them to become immature progenitor cells, which facilitates tumor recurrence; this transition supports the idea that CSCs participate in HCC. Moreover, the loss of the TGF-β adaptor b2-Spectrin triggers malignant transformation of liver SCs and LPC by growth-arrest signals and dedifferentiation. Finally, Mishra and colleagues found that there is a functional link between IL-6, a major SC signaling pathway, and the TGF-β signaling pathway in HCC and that suppression of IL-6 signaling, through the generation of mouse knockouts, resulted in a reduction of HCC in mice[54].

Functional assays for the evaluation of different stem cells

CSCs have properties that differ from n-SCs: (1) the ability to develop a malignant tumor; (2) resistance to chemotherapeutic agents used to treat malignant tumors, thus making them responsible for recurrence; and (3) the ability to give rise to distant metastases. These properties can be evaluated by some assays *in vitro* and *in vivo*. While some authors consider one of the *in vitro* assays sufficient to determine that a cell is CSC-like, until now, the best assay has been *in vivo* evaluation.

Some authors consider the *in vivo* tumorgenicity assay the gold standard in identifying CSCs; the tumor cell population of interest is transplanted into animal models, followed by an evaluation of the tumor-propagating capacity of the cells[55]. This assay has been useful because the selection of specific subpopulations of CSCs yields different results, indicating the ability for cells to have different tumor behaviors. A question then arises of whether tumorigenicity assays can be used to search for the potential CCO. The problem is that this assay only suggests that a specific cell population can be transformed into a tumorigenic cell nevertheless may not necessarily be the genuine CCO *in vivo*[56]. Nevertheless, some conflicts exist about this kind of assay. One issue is that this assay uses immunodeficient animals (NOD/SCID); therefore, the original context of tumor development is not similar to the normal immune system of a healthy animal. Additionally, this assay has only been analyzed by the selection of some markers, which hides some probable differential expression of the total population. Finally, it is important to consider that these models do not have the same tissue microenvironment. Xenotransplants differ in architecture and stroma compared with their native niche. Indeed, one study performed by Quintana and colleagues confirmed this affirmation when they showed that the tumor-initiating capacity may be an artificial consequence of the conditions employed in xenograft mouse models. For example, when these authors changed some parameters, such as the extracellular matrix in immune-compromised mice, they obtained better results[57].

The limiting dilution assay, or clonogenicity assay, is an in vitro cell survival assay based on the capacity of a single cell to grow into a colony. This assay tests every cell in the population for its ability to divide an unlimited number of times. Currently, this assay is used to determine responsiveness to cell death; cancer therapies, such as cytotoxic agents or ionizing radiation; and tumor recurrence[58]. In addition, this assay has given information about tumor survival, resistance and some extracellular matrix components, such as fibronectin and laminin[59]. It is possible to distinguish between effects on cell viability and cell proliferation by including colony size as an endpoint in the assay. However, this assay does have limitations. For example, this assay cannot measure the effect of cell-cell communication on cell proliferation because cells are plated at low densities to form colonies; therefore, there is a loss of the three-dimensional environment. Moreover, this assay is not applicable when a substance concentration decreases cell growth without affecting cell cycle progression and/or DNA synthesis. Additionally, this assay is considered inappropriate for some agents. Last, quality control is another problem, especially because it is difficult to distinguish cell clones from cell clumps in this assay[60].

The sphere assay is an *in vitro* method for assessing the self-renewal and multipotency capacity of a cell population in a low adherence system in the absence of serum. The result is the selection of undifferentiated cells that have a high self-renewal capacity[61]. Under these low-adherence and non-differentiating conditions, it is hypothesized that CSCs are able to form three-dimensional spheroids containing a heterogeneous population of progenitor cells with the competence to differentiate into multiple cell types. The self-renewal capacity of a cell population is assessed by the ability of the cells to form tumorspheres upon multiple passages, and this capacity is proportional to the number of spheres formed. Another advantage of the spheroid assay is that it minimizes the solid tumor characteristics in

its inside area during hypoxia and the low pH and interior cell characteristics that may be inaccessible to metabolites and drugs in comparison to exterior cells. Moreover, this assay has been used to evaluate the migration and invasive ability of CSCs, which correlates with their ability to metastasize. Even though the tumorsphere assay has been useful for self-renewal phenotype studies associated with CSCs, several limitations exist for this assay[62]. The first drawback is the inconsistency to form and maintain spheroids of uniform size; it was reported that the size and morphology of spheroids are different in different HCC cell lines. Some cells are more viable during the formation of spheroids, and the formation of spheroids varies according to the methodology used (*e.g.*, spinner flasks, non-adherent surfaces, hanging drop technique and microfluids)[63]. The second disadvantage is the variability in the number of cells that are necessary to form spheroids. It is difficult to extrapolate data from all kind of cells. Finally, analyzing the spheroids as if they were tissue is sometimes not representative for drug testing because spheroids cannot give the same information and do not have the same metabolism as other organs or tissues. Thus, we cannot obtain all the data that would be collected in a complete organism[64].

Determining whether CSCs are heterogeneous

In the 1980s, morphological intratumor heterogeneity became evident through histological analyses. Data from many investigations about CSCs showed evidence for the existence of different subpopulations of CSCs that had a great degree of heterogeneity. CSCs isolated from different stages of the same type of tumors are distinct. For example, some CSCs isolated from the primary tumor differ from those found in metastatic growths[65]. Even though different subpopulations of CSCs share the same niche, they usually coexist and may interconvert. Recently, a new concept was proposed that CSCs are a cell 'state', not a fixed 'category' of cells. CSCs are not an immutable, frozen cell population. Because of cellular plasticity, CSCs and non-CSCs coexist and can interconvert in a dynamic equilibrium[66]. Non-CSCs can acquire CSC properties by reprogramming or dedifferentiating, and they can interconvert in a similar manner as EMT to MET and vice versa.

Intra-tumor heterogeneity has been described in pancreatic cancer with regard to metastatic progression. By next-generation sequencing of the primary tumor and corresponding distant metastatic lesions, it was found that a primary pancreatic tumor is composed of spatially and genetically distinct subclones that give rise to lung or liver metastasis in the same patient[67]. In melanoma cancer, CSC-like populations have been shown to not only be heterogenic but also have differential functional activity depending on their location. CSC located at the invasive tumor front were of mesenchymal phenotype and quiescent (CD24- CD4+), meanwhile the central CSC were highly proliferative and epithelial-like (ALDH+)[68]. For CD44 expression gives distinct cell features to the $CD133^+$ or $CD90^+$ CSC populations. Two functions of CD44 in CSC maintenance have been reported. CD44s, the standard isoform, regulates TGF-β-mediated epithelial-mesenchymal transition (EMT), maintaining the mesenchymal phenotype, and results in resistance to sorafenib in HCC[69]. Meanwhile in gastrointestinal cancer, CD44v protects CSCs against reactive oxygen species (ROS) by stabilizing xCT, which leads to a regulated redox status[70]. Additionally, Kim and colleagues, indicated that the subpopulation of CD13+ in CSCs play a role in resistance to chemotherapy and radiation therapy in HCC by increasing plasticity linked to the cellular "stemness"[71], indeed, the combination of CD13+CD166- population demonstrated higher expression of stemness-related genes than CD13+CD166+[72].

In another study in 2010, CD133+ and CD44+ cells exhibited preferential expression of some SC-associated genes and were more resistant to chemotherapeutic agents due to upregulation of the ATP-binding cassette (ABC) superfamily transporters ABCB1, ABCC1, and ABCG2. Those findings suggested that CD133+ CD44+ cells might represent an important subpopulation of CSCs cells, which allows for a better understanding of HCC initiation and progression and establishes a precise target for implementing more effective therapies[33]. Finally, it has been demonstrated that the patterns of markers have different functions in CSCs. Singh and coworkers indicated that signaling at the IGF-1R-AKT axis is important for functional heterogeneity in ovarian CSC populations, and they concluded that oscillatory IGF-1R-AKT signaling affects chemo-resistance during early and late stages. This result emphasizes the importance of inhibition of a given target during different stages: using only IGF-1R during early time points and only AKT inhibition at late time points will significantly affect ovarian CSC population[73].

The consensus is that CSC subpopulations exist. These subpopulations seem to have important functions, and they are able to inter-convert, depending on the cellular context. These CSC phenotypes can vary according to the different cancer entities. Further research regarding the functions of CSC subpopulations in HCC, which will probably emerge in the coming years, will be necessary for understanding tumor growth.

RELATIONSHIP BETWEEN LIVER REGENERATION AND CELL OF ORIGIN OF CSCs

The carcinogenic properties of some extrinsic factors might rely on their capacity to induce local tissue damage and proliferative repair to create an expanded population of cells that are susceptible to malignant transformation[74]. The liver has a unique ability to regenerate after damage[75]; however, the type of cells that participate in liver repair is a highly debated topic. Recent studies have shed light on this issue, allowing us to have a more comprehensive view of the very complex liver regeneration process. Therefore, to better understand which cells are candidates for the cell of origin of HCC, we will briefly describe the state of the art of liver regeneration in the next section.

The classical model of liver regeneration

The classical model establishes that under most circumstances, liver function is restored through replacement of damaged hepatocytes by self-duplication of the remaining hepatocytes. However, when hepatocyte proliferation is impaired, as it is under chronic or severe injury conditions, other cells may contribute to liver regeneration by giving rise to hepatocytes[76].

Initial historic studies in rats that have severe liver damage and a blockade of hepatocyte proliferation described the emergence of a short-lived, highly proliferating cell type that expresses both cholangiocyte- and hepatocyte-specific markers as well as the embryonic liver marker AFP[77]. These cells were designated oval cells mainly because of their morphology (they have oval nuclei and a high nuclear-to-cytoplasmic ratio) and of their ectopic emergence/expansion in the parenchymal region of injured livers, which often forms a cluster that has duct-like and/or cord-like structures[77]. Oval cells are proposed to originate from a quiescent, facultative stem cell that is anatomically located at the interface between bile ducts and hepatocytes in the Canals of Hering[78]. This phenomenon is named the "ductular reaction"[8,79]. Experiments labeling these transit-amplifying cells with (3H)-thymidine suggested that oval cells were responsible for rat liver repopulation by generating new hepatocytes[80,81], although lineage tracing studies are required to formally prove this finding. Since oval cells are only seen in rodents, we will use the term liver stem progenitor cells (LSPCs) for all species[82].

Histologic/clinical correlation of LSPCs in human diseases

Notably, in human liver diseases, a severe and progressive impairment of hepatocyte proliferative capability is common[79,83-86], and the ductular reaction is correlated with the progression of liver injury. In such scenarios, the LSPC pathway originating from the biliary compartment can theoretically be a mechanism for the repopulation and regeneration of cirrhotic livers in humans. Histological examination of liver buds present in cirrhotic livers with three-dimensional reconstruction revealed that intraseptal hepatocytes were connected mostly to the CK19+ ductules, implying that the hepatocytes are newly derived from putative LSPCs located in the ductules[87]. EpCAM+ hepatocytes in cirrhotic livers are suggested to come from CK19+ ductular cells because they have a telomere length between that of EpCAM- hepatocytes and bile ductular cells[88]. The degree of LSPC activation correlates with survival in severe human liver diseases, such as alcoholic hepatitis[89]. However, the clinical significance of LSPC propagation in patients during late stage cirrhosis is obscured by the lack of restoration of hepatocyte numbers and functional recovery[87,90].

Available murine models and labeling strategies

In murine models of liver injury, existing protocols do not cause as severe blockade of hepatocyte proliferation as that seen in rat or human chronic damage, thus limiting the induction of LSPC activation during parenchymal renewal[75]. Acute acetaminophen or carbon tetrachloride injury does not produce LSPC expansion, while alpha-naphthyl-isothiocyanate (ANIT), dietdiethoxycarbonyl-1,4-dihydro-colldine (DDC)-supplemented diet, a choline-deficient ethionine (CDE)-supplemented diet and a methionine choline-deficient diet supplemented with ethionine (MCDE) do produce LSPC expansion. Differences in the type, duration, and severity of liver injury between experimental models and human diseases may also suggest that the contribution of LSPCs to liver regeneration in human chronic diseases may exceed the extent of what is observed in mouse models. However, mice are the only species that have available the genetic lineage tracing tool[91].

In the absence of specific LSPC markers, researchers have turned to broader lineage markers to perform cell isolation or lineage tracing studies. These markers could be shared by cholangiocytes and putative LSPCs or expressed in an inducible population of LSPCs. The ability to reach strong conclusions about lineage is deeply dependent on the specificity of the tracing tools used[92], and we will discuss the specificity of each tool.

LSPCs role in liver regeneration

Labeling LSPCs inducible populations: In 2011, Furuyama *et al*[93] showed that SRY (sex determining region Y) box 9 (Sox9)+ cells contributed to the majority of new hepatocytes formed during homeostatic maintenance and during different kinds

of regeneration after liver injury. However, later, a new study demonstrated that the Cre driver strain used in the study marked not only the Sox9+ cholangiocytes but also the hepatocytes surrounding the bile duct[92,93], which was partially due to the re-expression of SOX9 after tamoxifen induction (see below)[93,94] and nonendogenous artificial promoter use[95].

Osteopontin (OPN)-marked cholangiocytes and LSPCs did not contribute significantly to hepatocytes under normal homeostatic conditions or after partial hepatectomy, DDC or carbon tetrachloride treatment (acute and chronic). However, in mice fed a CDE diet, 2.45% of hepatocytes were derived from OPN-marked cells after the mice were allowed to recover on normal chow (CDE-stop model)[95]. However, OPN is shown to be expressed in cell types other than cholangiocytes and LSPCs (*e.g.*, hepatic stellate cells (HSC) and inflammatory cells)[96,97]. This could be an important limitation of this study since recent studies suggest that HSC can act as progenitor cells in the injured liver[98,99].

HNF1β is expressed in cells that form the ductular reaction and in expanded LSPCs in human diseases and in animal models of liver injury. Lineage tracing demonstrated that mice fed with a CDE diet showed a small population of hepatocytes derived from HNF1β+ cells that were expanded to 1.86% of the total hepatocyte population after injury recovery[91].

In Cre-based inducible models, the inducer tamoxifen is usually administered as a pulse label before beginning the injury model, so that only pre-existing populations are marked. However, tamoxifen remains active in mouse livers for one to four weeks[78,100,101]. Hepatocytes have a propensity for expressing certain biliary markers, including OPN and Sox9, during stress[102-104]. The Sox9, OPN and HNF1β studies and the GFAP study (discussed below) were conducted by initiating liver injuries one week or less after induction with tamoxifen, which may have resulted in marker gene activation in injured hepatocytes that non-specifically expressed genes whose promoters were used to drive Cre. Therefore, these studies discuss the genes expressed by facultative LSPCs that are induced by liver injury; however, they cannot provide information about the cell of origin of these populations.

Forkhead box protein L1 (Foxl1) is a marker for LSPCs found in injured livers, and Foxl1-Cre-expressing cells can be isolated, expanded, and differentiated into both cholangiocyte and hepatocyte lineages *in vitro*[95,105]. By tracing Foxl1-expressing LSPCs, more than 50% of CK19+ cholangiocytes and 29% of HNF4α+ hepatocytes were marked by YFP in the livers of Foxl1-Cre mice that were fed a CDE diet after the recovery period[106]. However, at the hepatocellular and cholestatic levels, there is not a significant difference in the function of injured livers in which Foxl1 cells were ablated during the recovery phase and those that were not depleted. One caveat to the current model is the fact that it employed Foxl1-Cre mice, which is not an inducible system. Thus, it is definitely possible that pre-existing hepatocytes activated the Foxl1-Cre promoter sometime during the course of the CDE paradigm and became YFP+ by this route[106]. Following liver damage and similar to Foxl1, Lgr5 (leucine-rich-repeat-containing G protein-coupled receptor 5) marks a population of cells that proliferate, and differentiate into hepatocytes and/or ductal cells, upon damage caused by carbon tetrachloride, DDC, or MCDE, as shown by lineage tracing[107]. In both models, lineage tracing was induced after the liver injury and detected Lgr5 cells in hepatocytes and biliary ducts. Lgr5+ cells can differentiate towards cholangiocytes and hepatocytes (bipotentiality) *in vitro* and *in vivo* after transplantation in the FAH-/- mouse model. However, because this marker only appears after damage *in vivo*, the cell of origin from which these Lgr5+ cells arise is still unknown [107].

Labeling LSPCs preexisting populations: In contrast to the mass labeling of SOX9+ cells conducted by Furuyama[93], a study involving clonally traced SOX9+ cells was recently reported. Biliary-derived Sox9+ proliferative ducts insignificantly contributed to regeneration of the hepatocyte pool in several classic mouse oval cell injury models, including the CDE oval cell activation regimen (< 1%)[78]. To address the possibility of this clonal tracing strategy could have failed to mark hypothetical LSPCs, the authors created a chimera lineage experiment. In this model, all hepatocytes are ablated (> 99% in analyzed zones) and replaced by a transplantation of mTomato+ donor hepatocytes. Thus, this model system generally tests whether any non-parenchymal cell (NPC) has the capacity to differentiate into hepatocytes. This model further confirmed the scarce (< 1%) contribution of NPC precursors to the hepatocyte pool after CDE LSPC activation, while hepatocyte-derived progenitors give rise to hepatocytes at a much higher efficiency (> 60%)[78].

As a terminal biliary marker, CK19 appears to be an exception to the marker specificity problem; in contrast to Sox9 and OPN, CK19 is not expressed by hepatocytes upon injury. CK19-CreER mice are likely to represent a more specific and, hence, more reliable tool for assessing the contribution of cholangiocytes and atypical ductal cells to liver regeneration[92]. Under all injury-recovery circumstances (DDC, CDE, CCl_4 or ANIT), all YFP+ cells stained for the biliary marker CK19, but not the hepatocyte marker HNF4α (hepatocyte nuclear factor 4 alpha)[92]. Schuab *et al*[101] also used CK19 tracing but used the CDE-Stop model

that was reported by Español-Suñer *et al*[95], which produced 2.45% Osteopontin+ derived hepatocytes; however, with CK19 tracing, the frequency of fate-traced hepatocytes remained negligible[101].

Cells with slow proliferation kinetics that retain a nuclear label over long periods, called label-retaining cells (LRCs), represent multipotent stem cells in a number of adult tissues. To identify quiescent cells without the bias of using a marker, in 2017, Vill *et al*[108] induced the expression of histone 2B fusion protein (enhanced green fluorescent protein [H2B-EGFP]) in the liver cells of newborn pups and chased the label until the liver matured. The liver label-retaining cells (LLRCs) were clustered in portal areas in biliary ducts and expressed biliary and oval cell markers. In this model, the EGFP mark is diluted among the daughter cells when LLRCs proliferate, and this how proliferation is detected. Moreover, the LLRCs were induced to proliferate under general (CDE) and biliary (total bile duct ligation or DDC) injuries but not upon hepatocyte injury (acute CCl_4 damage and partial hepatectomy), and they formed colonies of cells bearing only biliary but not hepatocyte markers in culture. This study demonstrated for the first time that LLRCs established during normal liver morphogenesis act as unipotent biliary progenitor-like cells[108].

A severe hepatocyte depletion mouse model demonstrates a nonparenchymal source of hepatocytes: A novel mouse model was developed in which hepatocyte-specific inactivation of Mdm2 results in upregulation of p53 and induction of p53-mediated hepatocyte death and senescence in more than 99.96%. The extent of the ductular reaction in this model was significantly greater than that in the CDE diet, resulting in rapid activation of LSPCs throughout the liver, which can proliferate, differentiate into hepatocytes, and completely restore liver architecture and function. Loss of the reporter was seen over 6 months, indicating that there was progressive replacement of Mdm2-negative hepatocytes of parenchyma by LSPC-derived (Mdm2 intact, p53 low) hepatocytes. These data suggest the existence of a facultative and functionally significant LSPC population that does not contribute to homeostatic repair but that is activated in severe liver injury where hepatocyte senescence is widespread[109]. It will be interesting to see what results from lineage tracing experiments with available LSPC markers using this new model.

Hepatocyte role in liver regeneration

Experiments utilizing hepatocyte lineage tracing in mice have shown in various liver injury models that hepatocytes regenerate themselves without any significant contribution from LSPCs. Yanger *et al*[92] used the hepatocyte-specific promoter thyroid hormone-binding globulin (AAV8-TBG-Cre) to label hepatocytes. This transduction was highly specific, as all YFP+ cells were HNF4α+. Under DDC, CDE, CCl_4, or ANIT injury conditions, the percentage of labeled hepatocytes remained unchanged. As a control, a 2/3 partial hepatectomy was performed, which also showed no change in the YFP labeling index. Thus, using this sensitive labeling technique, the study failed to find evidence that hepatocytes arise from non-hepatocytes after recovery from multiple types of LSPC-inducing injuries[92]. The same was confirmed by lineage tracing using a hepatocyte-specific transthyretin promoter (AAV8-Ttr-Cre) that does not label cholangiocytes, HSC, macrophages, or endothelial cells. In this study, a CDE diet did not change the frequency (0.40% ± 0.23%) of non-fate-traced hepatocytes[101].

The key enzymes from various pathways and thus the corresponding metabolic capacities are found to be differentially expressed according to the zone[110]. Wnt signaling is active in perivenous hepatocytes[111] and has been shown to induce metabolic zonation of liver lobules[112]. Additionally, it has been shown that hepatocytes do not have equivalent replicative ability during homeostasis[113]. By lineage tracing of the Wnt-responsive gene Axin2 in mice, Wang *et al*[113] identified a population of proliferating and self-renewing cells adjacent to the central vein in a liver lobule. Axin2+ hepatocytes express the early liver progenitor marker Tbx3, and they are diploid; therefore, they differ from mature hepatocytes, which are mostly polyploid. The descendants of pericentral cells differentiate into Tbx3-negative, polyploid hepatocytes, and they can replace all hepatocytes on the liver lobule during homeostatic renewal. Adjacent central vein endothelial cells provide Wnt signals that maintain pericentral cells, thereby constituting the niche[113]. It has been postulated that these Axin2+ hepatocytes are a "unipotent stem cell-like" hepatocyte subpopulation[82]. Their role in regeneration upon liver damage remains to be determined[82].

Font-Burgada *et al*[114] recently described a pre-existing population of periportal hepatocytes that are found in the limiting plate around the bile duct and the portal vein of normal livers that contact the Canals of Hering. These cells express normal amounts of HNF4α and low amounts of Sox9 and other bile-duct-enriched genes (OPN, EpCAM, HNF1β), but they are negative for the ductal marker CK19. Because of this expression pattern, these cells were called hybrid hepatocytes (HybHPs), and they are a specialized type of hepatocytes that poorly express drug metabolizing genes. This and other characteristics protect HybHPs from toxic injury minimizing the probability that they will originate cancer (see below). These cells have a high regenerative potential because they undergo extensive proliferation and they replenish liver mass after chronic hepatocyte-depleting injuries, such as

repetitive CCl_4, producing fully differentiated and functional hepatocytes. A CDE diet leads to extensive liver damage with high mortality[115], and in these mice, most HybHPs are probably lost, and LSPC expansion is observed, suggesting that when HybHPs are not available, LSPC take control but do not originate new hepatocytes[114].

Further experiments in mice have shown that hepatocytes can change into a biliary ductular phenotype [104, 116] and then later re-differentiate into hepatocytes [117]. Acute activation of Hippo pathway signaling *in vivo* is sufficient to dedifferentiate adult hepatocytes into cells bearing progenitor characteristics, such as ductal markers panCK and CK19, and validated progenitor markers, such as SOX9, MIC1C3, and A6, at very high efficiencies. These hepatocyte-derived progenitor cells demonstrate self-renewal and engraftment capacity at the single-cell level. Hepatocyte-derived progenitors can redifferentiate into the hepatocyte lineage when normal Hippo pathway signaling is re-established *in vivo*[116]. Human and mouse hepatocytes can undergo reversible ductal metaplasia in response to DDC injury, expand as ducts, and subsequently contribute to restoration of the hepatocyte mass[117]. Likewise, after 6 weeks of cholestatic injury by DDC, most HybHPs changed by reducing cell and nuclear sizes and gaining strong expression of SOX9 and the ductal marker OPN. Ten percent of HybHPs lost HNF4α expression, and 2.5% of these cells were incorporated into bile ducts and showed strong CK19 expression. Suggesting that pre-existing ductal cells become oval cells during cholestatic injury but also imply that a small proportion of oval cells originate from trans-differentiating hepatocytes, probably identical to HybHPs[114].

Recently, Pu *et al*[118] described a new marker for a hepatocyte subpopulation named Mfsd2a. This marker is also expressed in periportal hepatocytes. During liver homeostasis, the periportal hepatocyte population decreases. Nevertheless, liver regeneration induced by partial hepatectomy and chronic liver injury significantly stimulates expansion of the Mfsd2aþ periportal hepatocytes, replacing the pericentral hepatocyte population. Mfsd2aþ hepatocytes regressed during cholestatic injury (bile duct ligation and DDC). However, Mfsd2a-CreER might not precisely target the same specific layer of hepatocytes that was targeted by the Sox9-CreER marker used by Font-Burgada[114]. Therefore, the combinatory use of both Sox9- and Mfsd2a-based recombinases could be valuable in understanding the functions of various periportal hepatocyte sub-populations in the future[118].

Evidence of HSC as LSPCs

Studies of fate tracing using a human glial fibrillary acidic protein (GFAP) promoter reported that HSC, a mesenchymal liver cell type that plays a significant role in liver fibrosis, are precursors of liver epithelial cells. Studies suggested that up to 24% of hepatocytes were derived from HSCs in mice that were fed the MCDE diet and had BDL injured livers[98,99,101,119]. However, the fact that GFAP marks CK19-expressing cholangiocytes makes it an unspecific marker[98,99,120]. When this tracing was performed with lecithin-retinol acyltransferase as a more specific HSC/myofibroblast marker[120] with BDL, MCDE diet, DDC diet, CCl_4, or 70% partial hepatectomy conditions, rare HNF4α+ hepatocytes were found that express the Cre reporter (at a frequency of 0.2 per 1000 cells). In 2014, Schuab[101] assessed the contribution of HSC/myofibroblasts to hepatocyte regeneration by performing fate tracing based on platelet-derived growth factor receptor beta (Pdgfrb) expression in mice, a marker that was recently shown to be specifically and efficiently expressed in HSC/myofibroblasts[121]. After mice were fed a CDE diet for 3 wk, the number of fate-traced hepatocytes was negligible in both injured and non-injured livers. These results suggest that HSC/myofibroblasts are not a source of new hepatocytes in CDE-diet-induced chronic liver injury and exclude that HSCs function as epithelial progenitors.

Kordes *et al*[122] transplanted GFP+ HSCs into wild-type rats of two liver injury models with LSPC expansion: partial hepatectomy in the presence of 2-acetylaminofluorene or retrorsine. Transplanted HSCs contributed to liver regeneration in recipient animals by forming approximately 10% to 14% of mesenchymal tissue, progenitor cells, hepatocytes, and cholangiocytes and elevated direct bilirubin levels in blood sera of GUNN rats, indicating recovery from the hepatic bilirubin-handling defect in these animals[122]. These authors did not specify the percentage of HSC-derived hepatocytes but suggest that this is evidence for the characterization of HSCs as LSPCs and for their contribution to tissue repair. However, it should be considered that enzymatic isolation of HSCs from their niche and transplantation into the regenerating liver generates a condition that can promote HSC reprograming in a way that does not resemble *in vivo* behavior (see below)[122].

Evidence of endothelial cells as LSPCs

There is only one report in which lineage tracing of liver sinusoidal endothelial cells (LSECs) was performed. This report used mice carrying Tie2-Cre or VE-cadherin-Cre constructs to trace LSECs in liver regeneration. Tie2-Cre is expressed in the vascular endothelium and VE-Cadherin is expressed in the endothelium of developing and quiescent vessels. Some YFP-positive liver sinusoidal endothelial cells were observed to convert into hepatocytes following a two-thirds partial hepatectomy, accounting for 1% of

all hepatocytes[123].

The dominant role of hepatocytes in hepatic regeneration

According to the majority of previously described LSPC results, when LSPC response is activated, LSPCs do not differentiate into hepatocytes, and the purpose of their expansion is unknown. One hypothesis is that oval cells could be in charge of rebuilding of the bile canaliculi network and liver polarity[114,124]. Inking the ductal tree in models of liver injury has showed that expanding LSPCs are extensions of the pre-existing ductal tree, which further challenges the role of LSPCs in hepatocyte regeneration[114].

No evidence has been provided to either prove or disprove the existence of resident LSPCs. In most of the reports, it seems that only a very low percentage of hepatocytes were traced from LSPCs and would not have a significant contribution to the liver regeneration. It is important to note that the cumulative effects of LSPC-to-hepatocyte differentiation at a constant low rate could hypothetically reach a functionally significant level over time in long-term chronic injury[92]. Therefore, the studies presented here do not rule out a functionally significant contribution of LSPCs in long-term liver injuries, such as chronic hepatitis, in humans.

We can conclude that most studies point towards a dominant role of hepatocytes in regeneration at all levels of liver damage. However, these studies also point to the fact that during severe or chronic damage, hepatocytes are the main source of facultative LSPCs since they must go through a dedifferentiation process to be able to fully reach their regenerative potential.

HCC CELL OF ORIGIN

In many experimental models of hepatic carcinogenesis, LSPC responses precede cancer development, something similar occurs when ductular reactions precede HCC in human cirrhotic livers[125]. Biliary/progenitor markers[126-128] are often linked with poor prognosis and stemness features[128,129]. These observations have led to the idea that progenitors or cholangiocytes might be the cell of origin of HCC[130,131].

LSPCs as the cell of origin of HCC

Very few studies have carried out lineage tracing LSPCs during hepatocarcinogenesis. HNF1β+ LSPCs were traced in mice that were induced to express the reporter 7 d before treatment with carcinogenic DEN at postnatal day 17. HNF1β+ cells were also traced in Mdr2KO animals, which are commonly used as genetic models for spontaneous, inflammation-induced HCC development[132-134], with extensive activation of LSPCs that could undergo malignant transformation. However, the identified tumors never expressed the reporter in either of the HCC models[132]. Another study traced Foxl1-positive cells simultaneously with exposure to diethylnitrosamine (DEN), followed by multiple injections of CCl_4. None of the tumor nodules expressed YFP, indicating that Foxl1-expressing cells are not the origin of hepatotoxin-induced liver tumors[131].

LSPCs were marked by tamoxifen-inducible OPN-iCreERT2 in several HCC models: chronic administration of DEN, DEN injection into 15-day-old animals, DEN and multiple doses of CCl_4. The last model listed mimics hepatocarcinogenesis in the setting of liver fibrosis[135]. The same study also marked K19-CreERT LSPCs in the DEN+CCl_4 HCC induction model. Reporter expression was absent in all tumors[7], confirming that in several genotoxic DEN-induced hepatocarcinogenesis models and in mice traced with various LSPCs markers, HCCs did not arise from LSPCs or cholangiocytes.

As we discussed in the previous section, most of the markers used to trace LPSCs present some grade of unspecificity because they can also be expressed by injured hepatocytes. However, because none of these markers produced HCC tumors that expressed the reporter gene, it is possible to conclude that LSPCs expressing such markers are not the cell of origin of HCC in these hepatocarcinogenic models (Table 2). These studies question the hypothesis that progenitors are the cell of origin of HCC. However, we should note that these studies did not exclude the potential role of LSPCs to tumorigenesis in other models of HCC. Hepatocytes are the ones that metabolically activate DEN, therefore LSPCs may not have been exposed to genotoxic stress in most of these models[131].

Hepatocytes as the cell of origin of HCC

HybHPs are highly proliferative during chronic liver injury, therefore they could be an alternative origin for HCC. This hypothesis was tested tracing HybHPs and ductal cells in three independent mouse models of HCC: DEN-induced HCC[136], MUP-uPA mice fed a high-fat diet (HFD)[137], and the STAM model of diabetes-promoted HCC[138], using the Sox9-CreERT;R26RYFP reporter. DEN is metabolically activated in pericentral/zone 3 hepatocytes (unlike HybHPs) and does not induce LSPCs expansion, however HFD, which induces liver damage and compensatory proliferation in both MUP-uPA and STAM mice, gives rise to extensive LSPC proliferation. Tumor nodules and hyperproliferative lesions were negative for YFP+ cells; suggesting that the source for HCC in these models is different form HybHPs or ductal cells. The authors propose that even with the high rate of proliferation that HybHPs

Table 2 Studies tracing possible cells of origin of hepatocellular carcinoma

Cell	Tracing gene promoter	HCC models in mice	Tumor positive to lineage-traced	Ref.
LSPCs	HNF1β	DEN 17 d postnatal Mdr2KO	NO	[132]
	Foxl1	DEN/CCl_4	NO	[131]
	OPN	DEN 15 d postnatal DEN/CCl_4	NO	[7]
	K19	DEN/CCl_4	NO	[7]
HybHPs + LSPCs	Sox9	DEN MUP-uPA/HFD STAM	NO	[114]
Hepatocytes (mature)	TBG	DEN/CCl_4 DEN/CDE DEN/DDC Mdr2KO	YES (approximately 100%)	[7,131]
HSC	Lrat	DEN/CCl_4 Mdr2KO	NO	[7]

HCC: Hepatocellular carcinoma; LSPC: Liver stem progenitor cell; HSC: Hepatic stellar cells.

present, they lack the metabolic properties needed to participate in HCC initiation. DEN is metabolically activated by CYP2E1, which is expressed only in fully differentiated pericentral zone 3 hepatocytes[139] but absent in HybHPs. The same applies for class 4 genes, which only are expressed by fully differentiated hepatocytes, are needed for de novo lipogenesis and ROS generation through fatty-acid oxidation[137] in HCC development that is dependent on HFD consumption[114].

The evaluation to determine if mature hepatocytes give rise to HCC was made by two groups using an AAV-TBG-Cre promoter to label both periportal and pericentral hepatocytes in a model of HCC induced by DEN, followed by multiple injections of CCl_4. The treatment led to the activation of ductular reactions and to the development of multiple nodules that had the morphology of HCC. Both groups found that 99.7% of tumors[7] and all tumors[131] were lineage-traced as hepatocyte-derived HCC, concluding that hepatocytes are the cell of origin of HCC in this model (Table 2). The HCC nodules in the first study were positive to progenitor and hepatoblast markers AFP and H19, respectively, and most of them expressed high levels of CD133[7]. Therefore, mature hepatocytes can give rise to tumor cells that are positive for biliary/progenitor markers, therefore the idea that progenitors are the cells of origin of HCC, cannot be supported by the expression of biliary/progenitor markers by HCC[131].

Considering the possibility that LSPC origin may only be evident in models of HCC that have higher LSPCs cell presence and turnover. Mu *et al*[7] also used the combination of DEN with CDE or DDC diets. Comparable to the DEN+CCl_4 model, DEN+CDE and DEN+DDC-induced tumors showed high expression of HCC, LSPCs and hepatoblast markers, but they were originated completely from AAV-TBG-Cre+ hepatocytes. Hepatocyte origin could be specific to the HCC models that are dependent on the metabolism of carcinogens in hepatocytes; this possibility was assessed by using the DEN-free model of HCC: Mdr2KO mice, in which all HCCs arose from AAV8-Tbg-Cre-labeled hepatocytes[7]. Confirmation of such results must be performed using other HCC models that do not require the metabolism of carcinogens. All these evidence strongly suggest that HCC is originated from differentiated hepatocytes (Table 2). Further research using alternative labeling strategies will be essential to identify the subpopulation of hepatocytes responsible of HCC origin, such as tracing Axin2+ hepatocytes[131]

HSC as the cell of origin of HCC

HSCs were labeled by Lrat-Cre in mice that underwent injury-driven hepatocarcinogenesis induced by DEN+CCl_4. The authors did not find any tumors derived from HSC Lrat-Cre-labeled cells. Comparable results were obtained in the Mdr2KO model, where no tumor cell was derived from Lrat-Cre-labeled HSCs and all fluorescent cells within tumors were desmin positive and HNF4α and cytokeratin negative[7].

CHARACTERIZING CCO WITH DIFFERENT ASSAYS RESULTS IN DIFFERENT CONCLUSIONS

Depending of which experiments researchers used to delineate the origin of tumor a cell, conclusions were drawn that are controversial. Previously, works have reported that different populations participate in acute and chronic liver damage and that the cellular response is strongly associated with the risk factors and severity of the lesion[140].

With respect to CCOs, it was demonstrated that "any" cell could switch their cellular program to give rise to CSCs after a strong oncogenic stimulus, but it is important to consider that in this work, there was a genetic manipulation of the cells that were transformed with transgenes that encode oncogenic H-Ras and SV40LT[141]. In 2011, Chang and colleagues indicated that oval cell lines transfected with the HBV x gene (HBx) and treated with aflatoxin B1 in vivo, are responsible of HCC. These intrahepatic tumors included HCC cell markers (HepParl, ALB, CK8 and AFP) and mesenchymal cell markers (Vimentin and SMA), which is a stem-like phenotype[142].

In 2016, Kaestner's group used genetic lineage tracing to demonstrate that hepatocellular adenomas (HCA) and HCC are derived from mature hepatocytes in one model that was induced by DEN/CCl4; however,

a disadvantage of these studies was that they yielded too few HCCs for the studies to be considered a good evaluation. Moreover, these HCCs did not correlate well with c-Myc, but other authors have reported that in the liver, transgenic overexpression of c-Myc in murine hepatocytes is sufficient to induce liver tumors[131]. Another study by Mu and colleagues concluded the same result, although they selected a different stem-cell marker promoter[7]. In this study, the research group obtained higher numbers of HCCs that showed good correlation with HCC markers that had been reported by other authors, such as CK19[11,143]. The fact that there was a difference in the number of HCCs obtained when both groups used the same system makes the assay questionable.

In 2016, another group also used the line tracing assay with CD133+ to describe the ability of SCs to display different tumors[74]. Some tumors, especially HCCs, are considered to have a tendency to evolve and grow for more than 30 years, which means they are not common in young populations[144]. Finally, the authors did not have a different evaluation of different the liver cancers that were obtained, such as HCC, HCA and ICC (intrahepatic cholangiocarcinoma).

The lineage tracing assay is used to assess the probable CCO during cancer initiation. During lineage tracing, a specific subpopulation is labeled using different cell-specific promoters *in vivo*. Like other techniques, there are perks and disadvantages accompanying lineage tracing. The first point with lineage tracing is that it only uses mice. Anatomically, there are important differences between human and rodent. The liver is our organ of interest, and in a mouse liver, there are four lobes, denoted as right, left, medial and caudal. A human liver is formed by only two lobes that are separated by the falciform ligament; therefore, the anatomical distribution differs between species, but until now, there was no report of how this fact could affect the development or growth of HCCs. In addition, there is a difference in isoform composition, expression and catalytic activities of drug metabolizing enzymes between animal species. For example, CYP2E1 is the only isoform that has no appreciable differences in expression and catalytic activity among several species. However, CYP2A expression in humans corresponds to the 2A6, 2A7 and 2A13 isoforms, but its expression in mice corresponds to 2A4, 2A5, 2A12, 2A22. Additionally, CYP2B, C and CYP3A have distinct patterns of expression. Another example is the marked differences in the metabolic patterns of testosterone and 7-hydroxycoumarin; the main testosterone metabolites that are observed in both species are 2β-OHT, 6β-OHT and androstenedione. Nevertheless, the last two are reported to have different metabolic rates, 7α-OHT is only detected in mice and rats. Moreover, in the human liver, it had been reported that the amount of 7-hydroxycoumarin sulphate was minimal and that it differs between other species, such as rat, monkey and mice. With that being said, mice tumors that are caused by the induction of specific CYPs may not be of human significance because of the well-known species differences between human and mouse CYP gene regulation[145]. In addition, somatic mouse cells have high telomerase activity and significantly longer telomeres than human cells, suggesting that, in most linages, mouse cells may never experience actual terminal differentiation. Although some expression patterns of HCC in rodents and humans are not closely related, there is a comparison of global gene expression in one study indicative of the reproduction of some molecular pathways and expression of molecular patterns. Therefore, these authors indicate the importance of the identification and use of animal models that allow for comparison and extrapolation of the results to their specific human cancers or their subclasses.

The second problem is using the combination of mouse tumor models and lineage tracing to elucidate the cell of origin. In general, the majority of human epithelial cancers, especially liver cancer, develop very slowly with decades of clonal evolution and accumulation of epigenetic and genetic alterations. It is well known that the diagnosis of HCC occurs in the late stage, generally when the disease is in the symptomatic phase. Thus, the initial point when mutations trigger carcinogenesis is not easy to elucidate. Nonetheless, in mouse models of human cancer, the promoter of the gene of interest is instantly turned on, leading to genetic defects that occur all at once in an entire population of cells. This phenomenon is fundamentally different from what happens in most human cancers, where there is a sequential acquisition of mutations. Ideally, a cancer model should recapitulate the natural course of the disease by introducing sporadic mutations at a low frequency. For example, studies involving line tracing of CD133(+) and (TBG)-Cre do not study or describe the mutations that are gained or appear during the development of HCC[56].

The third problem is that the labeling efficiency in lineage tracing studies is highly variable and is dependent on Cre- or reporter-driving promoters. The efficiency is usually low and the results are often subject to alternative interpretations. It also depends on the promoter, for endogenous promoters, activity in poor differentiated cells could be not enough to drive reporter expression, causing low efficiency and difficult interpretations. For example, when CreER is driven by the endogenous X promoter, this study will conclude that the cells that express X will regenerate into more X cells. However, the SCs can differentiate

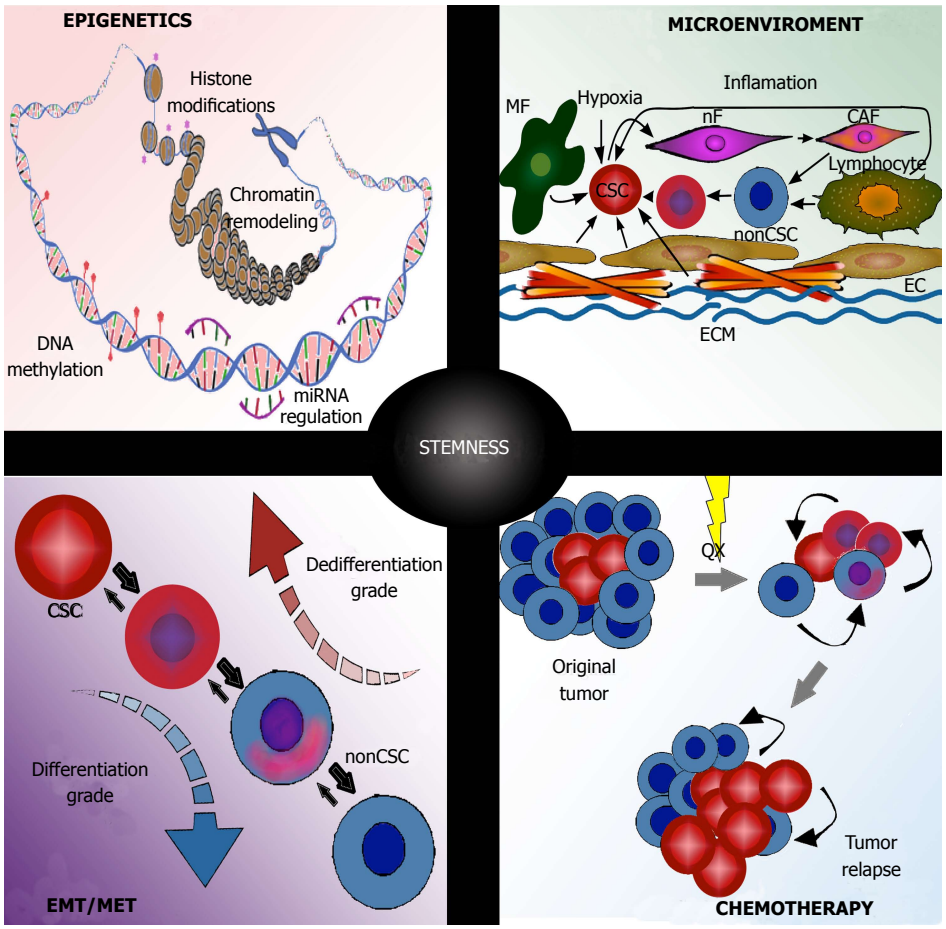


Figure 2 Stimuli that trigger the gain of stemness of non-cancer stem cells in hepatocellular carcinoma. The diagram includes the conditions that are reported to induce stemness: epigenetics (histone modifications, DNA methylation, Chromatin remodeling and miRNA regulation); microenvironment that includes hypoxia, inflammation and cellular populations participation (MF, nF, CAF, lymphocytes, CSC and non-CSC, ECM, EC); epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) and chemotherapy (QX: chemotherapy insult, *i.e.* carboplatin, curcumina and cisplatin). MF: Miofibroblast; nF: Normal fibroblast; CAF: Cancer associated fibroblast; CSC: Cancer stem cell; ECM: Extracellular matrix; EC: Endothelial cells.

into cells other than X cells, and then the no-X cells are not labeled because Cre activation is weak. This problem is conducive to inaccurate or erroneous conclusions, because only some differentiated cells will be tagged by the reporter. Similarly, exogenous or heterologous promoters randomly integrate into the genome and are regulated by different mechanisms than endogenous promoters, leading to expression patterns different from those of the endogenous gene. Due to read-through transcripts or regulatory elements at the insertion site, promoters may drive expression in tissues other than predicted[56].

Finally, all systems need to be optimized because the remaining construct has insertions that could cause effects; in the absence of an inducer could finish in spontaneous back ground recombination. In order to eliminate background recombination, AhcreERT can be used, since Cre transcription is controlled by tamoxifen binding and the Ah promoter. A transgene insertion of Cre recombinase under the control of a specific promoter may alter the function of the endogenous locus *via* activation or silencing. Sensitivity to Cre-mediated recombination of different LoxP-flanked target genes can be quite variable. Unexpected excision or incomplete incorporation of regulatory elements into the driver construct could occur. Lastly, the genetic background and variable maternal/paternal germline expression can also modify Cre activity. Therefore, it is important to optimize the animal models used in this assay[56].

The cell transplant study is another assay that allow know the possible CCO. However the main disadvantage present in this study is the limitation of only one population being selected. The study could not demonstrate the participation of other CSC subpopulations that could potentially participate in tumor development[146]. This study also did not evaluate the CSCs plasticity, which is the ability of CSCs to transition to non-CSCs or vice versa. Another crucial point is the immunophenotype of the animals used for this assay. In general, the assays are performed in immunodeficient mice, which is a different context

than real patient situations. In addition, there are big differences between the original tumor environment (niche), the proteins of the extracellular matrix, non-CSCs, and cells that cooperate within the tumor (immune cells, endothelial cells, *etc.*). One study demonstrated that when HCC cell lines are cultured under standard conditions *in vitro* and then injected subcutaneously into NMRI mice, they dedifferentiated into embryonic-like type cells. Thus, the behavior of CSCs from a distinct origin could probably condition the results[147].

In vitro, clonogenicity and multilineage differentiation have been extensively regarded as assays that demonstrate stem cell potential[148]. Some of the cellular markers that identify liver cells with clonogenic and multilineage potential are EpCAM, Lgr5, CD133, MIC1-1C3, Foxl1 OPN, Sox9 and CD24[76,94,107,149-153]. However, it has recently been suggested that removing liver cells from their *in vivo* environment could trigger the activation of a stem cell state because of damage to the tissue, similarly to what occurs during a regenerative response, and these plasticity properties may not be representative of *in vivo* biology[101,154,155]. Therefore, we mainly focused on discussing *in vivo* experiments, such as repopulation following transplantation and lineage tracing.

ARE CSCs A CELL STATE? OR IS THERE A DYNAMIC INTERCONVERSION EQUILIBRIUM BETWEEN CSCs AND NON-CSCs?

Currently, in respect to the CSC hypothesis, new data proposed that the added concept of plasticity ability explains how CSCs are maintained in the tumor. The proposal is that there is a dynamic interconversion between CSCs and non-CSCs in response to environmental injury, intercellular communication and cues from the tumor niche. These new concepts complicate the perspective of cancer, and it is still controversial because there is no agreement of how it could be validated *in vivo*. Altered conditions could trigger the gain of stemness, including EMT-MET, epigenetics, microenvironment and selective stimuli, such as chemotherapy (Figure 2).

The epithelial-mesenchymal transition (EMT) is defined as the process by which epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties to become mesenchymal SCs. This condition is important, and it is present in numerous developmental processes; it has been described to be implicated in wound healing, fibrosis, and cancer in which non-CSCs gain SC properties and become CSCs. This is a phenomenon that demonstrates high cellular plasticity[156] and has also been seen in hepatocytes. When cells are in the transition to the SCs-like phenotype, some factors could be induced, such as Octamer 3/4 (Oct3/4), sex determining region Y-box 2, c-Myc, and Kruppel-like factor 4 (Klf4)[157]. Moreover, the signaling pathways related to stemness are activated, including the Wnt/β-catenin, Hedgehog, Notch, and TGF-β signaling systems. Targets, such as β-catenin, human telomerase reverse transcriptase, cyclin-dependent kinases, and Myb (from the myeloblastosis gene family), are also activated[46].

The link between EMT and the CSC phenotype has been studied broadly. It had been reported that cells undergoing EMT may resist toxic injuries and chemoradiation therapy, and a series of studies demonstrated that CSCs are more resistant to conventional therapies than differentiated cells. Fillmore and coworkers selected the cells with surface phenotype CD44+/CD24-/low/ESA+ to isolate CSCs from human breast cancer cell lines and demonstrated preferential resistance of CSCs to chemotherapy[158]. In another tumor model (pancreatic cancer), the cells that are rich for the "stem-cell-like" phenotype when they undergo EMT have chemoradiation-resistant characteristics. They also have increased migratory and invasive capacities *in vitro* and *in vivo*[159]. Many stimuli trigger the EMT, such as IL-6, by an inflammatory loop, which accelerates the transformation of normal LSPCs to metastatic CSCs. In addition, Malfettone A and coworkers demonstrated the induction of EMT in HCC cell lines *via* TGF-β stimulus, and that these lines could gain molecular markers and characteristics of CSCs such as invasiveness. The authors also concluded that the partial EMT phenotype confers a higher stemness potential than that of the full EMT[160]. In addition, experimental EMT and derived inflammatory cytokines, such as IL-6, IL-8, TGF-β, and TNF-α, can all promote the manifestation of CSC phenotypes and properties in non-CSCs[161].

Epigenetic is new to cell research that is defined as stable alterations or changes in gene expression that are not caused by changes in DNA sequence and are heritable across every round of cell replication. Changes in expression affect the cellular phenotype and/or differentiation state. One of the main control mechanisms of cellular developmental hierarchies is through epigenetic regulation of the genome. Until now, there were four important epigenetic mechanisms: chromatin remodeling, DNA methylation, histone modifications, and noncoding RNAs, including miRNAs[162].

For the first epigenetic mechanism, histone methylation occurs mainly on lysine and arginine, histone readers use such methylated residues as docking sites. In general, histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36), and histone H3 lysine

79 (H3K79) are associated with gene activation, whereas histone H3 lysine 9 (H3K9), histone H3 lysine 27 (H3K27) and histone H4 lysine 20 (H4K20) are associated with gene repression and heterochromatin formation. It was reported that in the absence of the UTX, one histone, H3K27 demethylase, induces epithelial-mesenchymal transition (EMT)-mediated breast CSC properties by increasing the expression of SNAIL, ZEB1 and ZEB2[163]. In head and neck squamous cell carcinoma (HNSCC), it was reported that the participation of G9, a histone methyltransferase that makes H3K9, triggers the transcriptional repression of E-cadherin, thus influencing the EMT, cell migration, tumorsphere formation and increased expression of CSC markers[164].

DNA methylation results in long-term gene silencing, which is performed by DNA methyltransferases (DNMTs), transfer a methyl group from S-adenosyl methionine to CpG dinucleotides at cytosine bases on gene promoters and regulatory regions. CpG are concentrated in short regions called "CpG islands". Rountree and colleagues showed that TGF-β regulated CD133 expression through inhibition of DNMT1 and DNMT3b and the subsequent demethylation of promoter-1. Another relation between DNA methylation and CSCs was found in breast and colon cancers. It was described that aberrant Wnt/β-catenin enhanced the methylation of promoters of various Wnt inhibitors, such as Wnt inhibitor factor-1 (WIF-1), AXIN2, Dickkopf-related protein (DKK1) and secreted frizzled-related protein 1 (SFRP-1)[165,166]. In HCC, it was described that methylation affects the expression of E-cadherin and some other markers related to the EMT-phenomenon that are highly related to the stemness hallmark. Genes that are commonly epigenetically altered in cancer cells through promoter CpG methylation often show a specific epigenetic regulation pattern in embryonic SCs, termed "bivalent chromatin". This bivalent state could explain the dynamic response to environmental changes, such as differentiation stimulus. Thus, these genes can easily switch between an active or repressed condition. One clear example is during the EMT, where TGF-β stimulus changes the E-cadherin expression state[167].

miRNAs are endogenous non-coding RNAs that have been identified as post-transcriptional regulators of gene expression. These RNAs function by targeting mRNAs for degradation or repressing mRNA translation by binding to the 3′- untranslated region of mRNAs. They have been reported to be involved in the control of self-renewal and the differentiation of embryonic SCs, and they are also implicated in the progression and tumorigenesis of various cancers. Some miRNAs, such as miR-181s, miR-130b, miR-150 and miR-121, have been suggested to be important in epigenetic regulation in liver CCO. In HCC patient samples, a correlation was found in the miR181 family to features of hepatic SCs and progenitor cells. In addition, functional studies showed that forced expression of miR-181s induced stemness in HCC cells with a significant enrichment of the EpCAM+ marker. miR-130b overexpression was found in HCC cells that had a CD133+ marker. Moreover, its overexpression induces high proliferation, self-renewability, tumor initiation and chemotherapy resistance abilities in HCC cells. Moreover, it seems that miR-150 negatively affects the proliferation of CSCs and spheroid formation in HCC cells, probably through modulation of the downstream target c-Myc [168]. Jung and colleagues have identified miR-122 as the regulator of a common network of genes that promote SC self-renewal and HCC proliferation. In human primary hepatocytes, miR-122 is expressed, but it was attenuated in HCC. Finally, miR-122 expression may be associated with the methylation status and RNAPII binding activity of the promoter region of this gene[169]. Currently, the importance of epigenetic turn on or off in cancer is not only for the gain of stemness in non-CSCs, but it is also considered to be important for the regulation of many functions, such as cell plasticity and drug resistance acquisition[170].

Development of resistance represents a major drawback in cancer treatment, indeed, there are a lot of reports of conversion of non-CSCs to CSCs by this way and the treatments include: irradiation (IR), radiochemotherapy and chemoteraphy. IR may cause changes in a tumor microenvironment that can affect invasion and metastasis. IR changes cellular metabolism, leading to EMT and CSC phenotypes. Additionally, in liver cancer, it was reported that EMT is responsible for enhancing the motility and invasiveness of cancer cells; a possible mechanisms for this is that it is mediated by the induction of ROS production, directly through extracellular water radiolysis, or indirectly through intracellular metabolic alterations and mitochondrial dysfunction[171]. Also it had been described that IR could activate stemness pathways in liver heterogeneous no-cancer stem cells by enhancing upregulation of the pluripotency genes Sox2 and Oct3/4, resulting in a high enrichment subpopulation with new abilities such as better spherogenesis and resistance to radiotheraphy[172]

It was also suggested that radiochemotherapy has a side effect of directly transforming non-CSCs into induced CSCs, which possibly contributes to tumor recurrence and metastasis[173]. For example, Vares and coworkers indicate that the combination of progesterone and irradiation could induce a higher cancerous phenotype in MCF-10A cells and increase the proportion of CSCs that give the cells a resistance phenotype[174]. Another study showed that combining

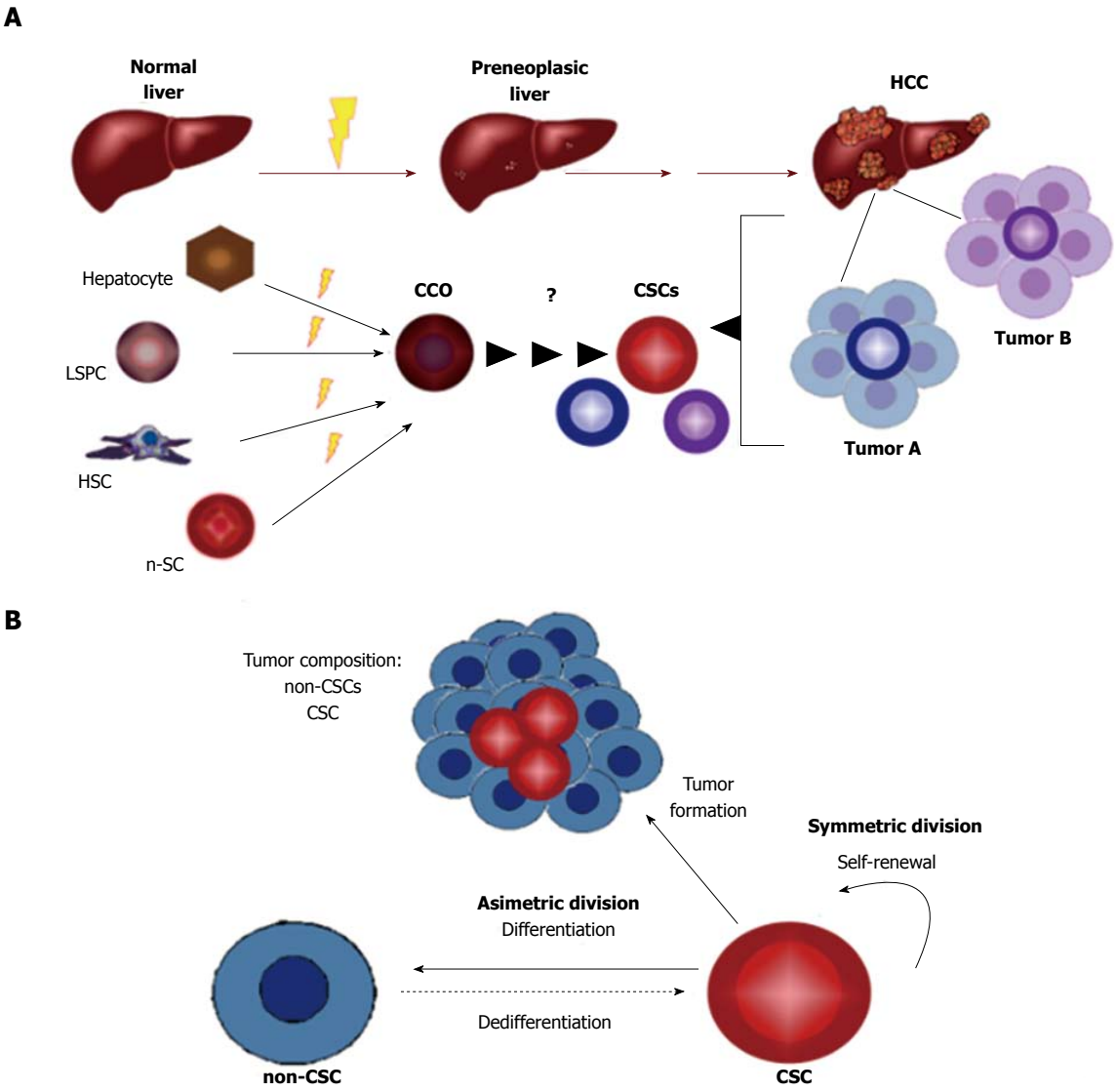


Figure 3 An integrated model of cancer cell of origin and cancer stem cell hypotheses in hepatocellular carcinoma. A: Different cells involved in adult liver regeneration are potential targets of malignant transformation when they received hard insults (Yellow ray, *i.e.* carcinogenic agent, partial hepatectomy). These cells could be considered CCO (Hepatocytes, LSPC, n-SC, HSC), however many studies point to the fact that during severe or chronic damage and consistently in the beginning of HCC, hepatocytes have the main role. The relationship between CCO and CSC is not clear until now. CSCs composition is heterogeneous in HCC tumors. B: The hierarchy hypothesis shows the tumor composition: CSCs and non-CSCs. This includes the plasticity model, which indicates the interconversion by differentiation/dedifferentiation balance between non-CSCs and CSCs. LSPC: Liver stem progenitor cell; n-SC: Normal stem cell; HSC: Hepatic stellar cells.

valproic acid (VA), a histone deacetylase inhibitor, and ionizing radiation can promote the dedifferentiation of ALDH- cells into ALDH+ cells. In ovarian cancer, it had been described that short-term, single treatments of chemotherapy cause an enrichment of CSC-like cells[175], and a similar result was seen in lung adenocarcinoma A549 cells that were exposed to a combination of paclitaxel and being cultivated in serum-free medium [176]. These data propose that non-CSCs can give rise to CSCs through dedifferentiation or induction. These new cells are termed induced-CSCs (iCSCs).

In the case of chemotherapy there are some studies that describe the gain of new iCSCs population after the treatment with anticancer-drugs. For example, Hu and coworkers describe in liver cancer cells treated with carboplatin, significantly increases the ability to form spheres, it seems that the treatment with carboplatin induce some key genes of stemness maintenance such as Oct4 and Sox2[177]. Another example is one study with curcumina, which has been demonstrated in some cancers that suppress cancer cell proliferation, invasion metastasis and angiogenesis, however it was demonstrated that although it induces arrest on glioma cells also induces changes in cell morphology from spindle to round with an overexpression of stem markers CD133 and Nestin. In addition, the round glioma cells show induction of the Sox2/4-Oct4 axis, hallmarks factors of stem cells[178]. Moreover Nör and colleagues discover that cisplatin enhances the fraction of CSCs in head and neck squamous cell carcinoma (HNSCC). The induced-

CSCs overexpress ALDH and CD44, also they report activation of the signaling pathways involved in the stemness *via* IL-6/STAT3[179].

Neighborhood impact on CSCs

It has been reported that pathophysiological changes in the liver during inflammation/regeneration could induce the initiation or promotion of liver cancers[180,181]. Some alterations of the hepatic tumor microenvironment are: increased lymphocyte infiltration, HSC activation and the expansion of LSPC, cell migration, release of free radicals, cytokines, chemokines, and high proliferation rate. These stromal activations induce signaling pathways and cause the accumulation of genetic and/or epigenetic changes. These conditions may cooperatively support and maintain liver CSCs. Thus, the cellular neighborhood is important. There is much evidence indicating that co-cultivation of normal fibroblast and tumor cells facilitates/increases the tumorigenicity of tumor cells. Nevertheless, more recent evidence suggests that CSCs could modify the microenvironment by transforming neighboring fibroblasts into cancer-associated fibroblasts (CAFs), which have increased proliferation rates and unique secretory factors compared with their normal counterparts[182].

In addition, hypoxia favors an increase in the CSC pool *via* HIF-1α and HIF-2α activation, and it also induces EMT by activating EMT-associated signaling pathways, transcription factors or repressors (SNAIL, ZEB1, TWIST and TCF3). Moreover, both hypoxia and reactive oxygen species (ROS) upregulate CSC stress *via* the ROS, TGF-β and TNF-α signaling pathways with the goal of enhancing cancer cell survival and maintaining cancer cell stemness[183]. Another study showed that myofibroblast-secreted factors restore CSC phenotypes in differentiated colon cancer cells *in vitro* and *in vivo*[184].

Tumor angiogenesis is important in providing the tumor mass with nutrients and oxygen and maintaining the CSC population. In one study, it has been shown that vessel-forming endothelial cells within the niche closely interact with Nestin+/CD133+ brain CSCs to keep them in a SC-like state. Another study was interested in the CXCR4/ CXCL12 axis, which forms a chemo attractant gradient that allows normal and CSCs to find their niche. Antagonists against CXCR4, such as Plerixafor (AMD3100) and small peptide CXCR4 inhibitors (T140, TC14012, TN14003), can block the stroma-CSC communication axis thereby inducing the cells to become vulnerable to cytotoxic drugs[185]. For all the evidence that was previously mentioned, some of the investigators were focused on affecting the CSCs niche to dissolve the tumor. However, next studies must consider the heterogeneity in subpopulations and that there might be several distinct CSC niches within one tumor, which may change over time depending on the tumor stage and therapy.

CONCLUSION

There is no doubt that there is controversy about cell interplay during the initiation, maintenance and dissemination of cancer cells and about how to explain the phenomenon of radio/chemotherapy resistance of tumors. The hypothesis of cancer stem cells (CSCs) give us the opportunity to look forward, but now, several new questions have been introduced, such as determining the relationship between OCC and CSC, determining if markers correctly characterize CSCs and determining whether there is a main or single CSC population. This suggests using operational definitions rather than phenotypically defined cells to distinguish the definitions of cancer cell of origin, cancer stem cell, tumor propagating cell and stem/progenitor cell. The controversy is increased if the tissue in which the cancer originates is considered. Hepatocellular cancer, the most prevalent liver cancer, is unique in respect to this contention due to the great regeneration capacity of liver and the controversial role of liver progenitor cells in liver regeneration and cancer initiation.

The studies discussed here question the hypothesis that progenitor cells are the cell of origin of HCC. However, we should note that current studies did not exclude the potential role of LSPCs to tumorigenesis in other HCC models, or human HCC, considering the discussed distance between murine models and the human disease. Mature hepatocytes are emerging as the cell of origin of HCC. Importantly, they can produce tumor cells that are positive for biliary/ progenitor markers. This indicates that, on its own, the expression of such markers by HCC does not support the hypothesis that progenitors are responsible for the origin of HCC (Table 2, Figure 3). Further research using specific labeling strategies will be necessary to identify which subpopulation of hepatocytes contributes to tumorigenesis, such as tracing Axin2+, or Mfsd2aþ hepatocytes.

It is worth noting that, even though some facultative populations (of unknown origin) were induced by liver damage schemes and had a variable degree of participation during liver parenchymal regeneration, including the Sox9, Foxl1, OPN, and HNF1β populations, they are apparently not the targets of malignant transformation during carcinogenesis. Perhaps this shows that an expanding population of cells is not enough to ensure malignant transformation, and other considerations should be made. As we discussed earlier, this could mean that certain metabolic firms are required to be susceptible to malignant transformation. Another probable reason is that such populations are only transiently amplified, thus they only express the lineage marker temporarily, preventing it from being

traced for the extended periods that are needed during HCC development.

As for the results presented by several research groups about the progression of cancer, it seems that two new considerations stand out. The first is that many subpopulations of CSC (and their progeny) have different phenotypes and functions and coexist within the tumor. The second is that the hypothetical interchange of cell populations that have certain CSC phenotypes and possible interchanges with non-CSCs leave us with the fact that the main characteristic of tumor cells is plasticity. Such great plasticity is an advantage when cancer cells are compromised in the face of certain challenges, such as hypoxia or chemotherapy. This leads to the proposition that a CSC-state is a description of a highly adaptable, dynamic intrinsic property of tumor cells instead of a static subpopulation of a tumor. This characteristic that there are many different phenotypic populations within the tumor makes tumors out smart therapeutic protocols that are directed at a single target. Thus, for future research and clinical intervention, it is important to consider more than one marker to identify/target different subpopulation of CSCs. A further avenue of research will be to uncover mechanisms that trigger the interconversion of cell populations that have different phenotypes within the same tumor. In this way, we could find new protocols that first tackle the ability of cancer cells to exhibit plasticity and then lock them into a single, or at least a few, defined subpopulations. Then, we could find therapeutic targets for the locked cell populations.

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REVIEW

Intrahepatic vascular changes in non-alcoholic fatty liver disease: Potential role of insulin-resistance and endothelial dysfunction

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Abstract

Metabolic syndrome is a cluster of several clinical conditions characterized by insulin-resistance and high cardiovascular risk. Non-alcoholic fatty liver disease is the liver expression of the metabolic syndrome, and insulin resistance can be a frequent comorbidity in several chronic liver diseases, in particular hepatitis C virus infection and/or cirrhosis. Several studies have demonstrated that insulin action is not only relevant for glucose control, but also for vascular homeostasis. Insulin regulates nitric oxide production, which mediates to a large degree the vasodilating, anti-inflammatory and antithrombotic properties of a healthy endothelium, guaranteeing organ perfusion. The effects of insulin on the liver microvasculature and the effects of IR on sinusoidal endothelial cells have been studied in animal models of non-alcoholic fatty liver disease. The hypotheses derived from these studies and the potential translation of these results into humans are critically discussed in this review.

Key words: Non-alcoholic fatty liver disease; Endothelial dysfunction; Insulin resistance; Metabolic syndrome

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Core tip: Insulin-resistance participates in the development of endothelial dysfunction and interferes with vascular homeostasis in patients with metabolic syndrome. This has been demonstrated in large conductance vessels, promoting atherosclerosis, but also occurs at a microcirculation level, suggesting an important role for Insulin in controlling vascular resistance and, finally, organ perfusion. We offer an overview of those pre-clinical and clinical studies exploring the liver microcirculation, and discuss the importance of early vascular changes induced by insulin-resistance in non-alcoholic fatty liver disease and in the most common chronic hepatitis in which Insulin-Resistance is a comorbidity.

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INTRODUCTION

Metabolic syndrome (MS) is a cluster of cardiovascular risk factors including glucose intolerance, hypertension, dyslipidemia, and visceral obesity, and has a prevalence of up to 25% in adults over 40 years old[1]. Non-alcoholic fatty liver disease (NAFLD) is the liver expression of MS, and constitutes a chronic disease associated with high cardio-vascular risk, with potential for progression to cirrhosis and hepatocellular carcinoma[2]. In other chronic liver conditions such as hepatitis C virus-related chronic hepatitis[3-5] and cirrhosis[6], MS can be an important comorbidity that potentially worsens liver histology[7] and increases the risk of decompensation[8]. Insulin-resistance (IR) is a common early finding in patients with MS and is the main pathogenic substrate for the development of NAFLD. IR is associated with endothelial dysfunction (ED)[9,10], a major pathogenic factor in arterial hypertension, coronary artery disease and atherosclerosis[11].

Several studies in animal models and humans have demonstrated that insulin action couples vascular and glucose homeostasis[12]. Indeed, in physiologic conditions, Insulin can influence the production of nitric oxide (NO) that mediates to a large part the vasodilating, anti-inflammatory and antithrombotic properties of a healthy endothelium. In patients with MS, the degree of IR parallels the severity of ED with important repercussion on structural and functional changes of the macro- and micro-circulation that may lead to impaired organ perfusion[11]. In the last few years, intrahepatic ED has been demonstrated in several models of liver disease including cirrhosis[13], ischemia-reperfusion[14], endotoxemia[15] and fatty liver[16,17]. As a consequence, vascular changes induced by IR, acting through the development of ED, are of potential therapeutic interest in the context of the most prevalent liver diseases. The present review offers an overview of the molecular mechanisms linking IR with ED in the control of vascular homeostasis, and reports the main biological and clinical findings on this topic in the context of the most common liver diseases.

INSULIN AND ENDOTHELIUM: FROM PHYSIOLOGY TO PATHOPHYSIOLOGY

Biological activity of NO is highly regulated in healthy conditions

NO is the main biochemical mediator of endothelium-dependent vasodilation in blood vessels. In physiologic conditions, it is constitutively synthesized by endothelial nitric oxide synthase (eNOS), whose activation results in a cascade of molecular events leading to smooth muscle relaxation[12]. The vasodilatory actions of NO play a key role in the renal control of extracellular fluid and is essential for the regulation of blood flow and blood pressure[18].

The endothelial NO production by eNOS is tightly regulated at the transcriptional and post-transcriptional level[19]. The expression of eNOS mRNA is largely restricted to the vascular endothelium[20]. Methylation is biologically associated with a marked impairment of promoter activity in mammalian cells and appears to play an important role in endothelial cell-specific expression of the human eNOS gene[21]. The eNOS promoter is heavily methylated in non-endothelial cells in comparison with endothelial cells. Kruppel-like factor 2 (KLF2) is a transcription factor that modulates the expression of multiple endothelial genes, including eNOS and thrombomodulin[22,23]. KLF2 is endothelial-specific and its expression, which is modulated by different flow patterns, confers endothelial protection against inflammation, thrombosis and excessive vasoconstriction[24,25]. Gracia-Sancho *et al*[26] have demonstrated that KLF2 can be activated by SIRT1 and that KLF2 overexpression activates vasoprotective genes in the vascular endothelium[27]. The same group demonstrated that simvastatin upregulates KLF2 expression in whole livers from cirrhotic rats[14] and in sinusoidal endothelial cells in culture[28]. This demonstrates that KLF2-mediated transcriptional regulation at the liver sinusoidal endothelial cells reproduces what occurs at peripheral endothelial cells.

The activity of eNOS is also thoroughly regulated

at the posttranslational level. These include protein-protein interactions, cofactors availability and protein phosphorylation[13]. The impact of these posttranslational modifications in liver endothelial eNOS has been thoroughly studied in animal models of liver disease[13]. Finally, the bioavailability of NO can be affected by the oxidative stress generated by several clinical conditions, and antioxidants have demonstrated the importance of redox environment to maintain microcirculation in conditions of compromised liver perfusion[29].

eNOS and iNOS: From physiology to pathophysiology

In addition to the constitutive form of eNOS, at least two other isoforms have been described: the inducible NOS (iNOS), and the neuronal NOS (nNOS). The potential pathogenic roles of eNOS and iNOS dysregulation have been assessed in several models of liver disease. Both isoforms produce NO, but their intracellular localization, activation, and concentration of NO produced are not the same, resulting in different biologic actions.

Physiological hepatic production of NO is derived from eNOS in response to stimuli such as shear stress and the presence of vasoconstrictors[30,31]. The classical activation of eNOS implies an increase of intracellular calcium (Ca^{2+}) and binding of Ca^{2+}/calmodulin to the enzyme. In addition, a Ca^{2+}-independent pathway regulating eNOS has been recently described. This pathway can be stimulated by several factors, among them shear stress and insulin[32,33]. Both shear stress and insulin increase endothelial NO production *via* activation of PI-3-kinase and protein kinase B (PKB/Akt), which activate eNOS by Ser1179-phosphorylation[34]. In addition, insulin upregulates the transcription of eNOS in endothelial cells[35].

In the liver, eNOS-derived NO targets hepatic stellate cells (HSC), promoting the synthesis of cyclic GMP (cGMP)[36]. The most important target of cGMP is protein kinase cGMP-dependent (PKG), which phosphorylates numerous proteins involved in the regulation of Ca^{2+} homeostasis, among them inositol 1,4,5 triphosphate-receptor. This leads to a decrease in the concentration of intracellular Ca^{2+} in HSC and produces relaxation with enduing decreased intrahepatic vascular resistance[37]. Thus, a physiological production of NO in the healthy liver offsets vasoconstrictor stimuli[38]. Since increased intrahepatic vascular tone is a major factor leading to portal hypertension in cirrhosis, different pharmacological strategies have been explored to increase liver NO availability[39-42].

iNOS was initially identified for its vital role in the immune system. When activated, it produces continuously large amounts of NO since, in contrast to eNOS, the substrate and cofactors are not limiting. iNOS is upregulated in metabolic tissues under different conditions of stress[43]. Although it is important for the immune system, iNOS activity can be harmful for other cell types, including pancreatic β cells[44] and vascular cells[45]. Recent studies have shown that iNOS-derived NO may play a role in the pathophysiology of obesity-induced metabolic dysfunction[43]. Among other mechanisms, it has been shown that iNOS is a critical modulator of PPAR-γ activation (a target of insulin sensitizing drugs)[46] and can decrease insulin sensitivity through S-nytrosilation of the insulin receptor[47]. Indeed, the inhibition of iNOS reduces hyperglycemia, hyperinsulinemia and improves liver insulin sensitivity[48]. Moreover, several studies with animal models have demonstrated that the induction of iNOS can cause ED through increased nitro-oxidative stress[49-51] and downregulation of eNOS[52]. Finally, the inhibition of iNOS in animal models that overexpress this enzyme restores a normal endothelial function[53-55].

Under physiologic conditions, the only NOS expressed in the endothelium of the vessels is eNOS. During inflammation, blood vessels express iNOS and eNOS[56]. Overexpression of iNOS thus contributes to vascular dysfunction.

Insulin-resistance and eNOS activity

The binding of insulin to its receptors at the level of peripheral endothelial cells[57] activates the phosphorylation of the insulin-receptor substrate which initiates a phosphorylation of a series of down-stream substrates, among them the PI3K/Akt pathway[58,59], that finally activate eNOS[60,61]. The result of this set of reactions ultimately produces an increase in eNOS activity and increased production of nitric oxide (NO), leading to vasodilation (Figure 1).

In the presence of IR, the PI3K/Akt pathway (involved in metabolic functions) is impaired, while other pathways of insulin signalling remain unaffected, including the Ras/MAPK pathway (involved in the control of cell proliferation), resulting in an imbalance between insulin functions performed by the PI3K pathways and MAPK[62,63]. This imbalance leads to decreased activation of eNOS and thus lowered production of NO, resulting in ED.

INTRAHEPATIC VASCULAR CHANGES IN NAFLD

Microvascular abnormalities in models of fatty liver: Structural and functional increase of resistance

The isolated liver perfusion technique has been instrumental in the assessment of liver microvascular changes in fatty liver[16] (Figure 2). In several studies, substantial changes in vascular function and liver blood flow have been demonstrated in fatty liver disease, as reviewed elsewhere[64]. Studies in rabbits

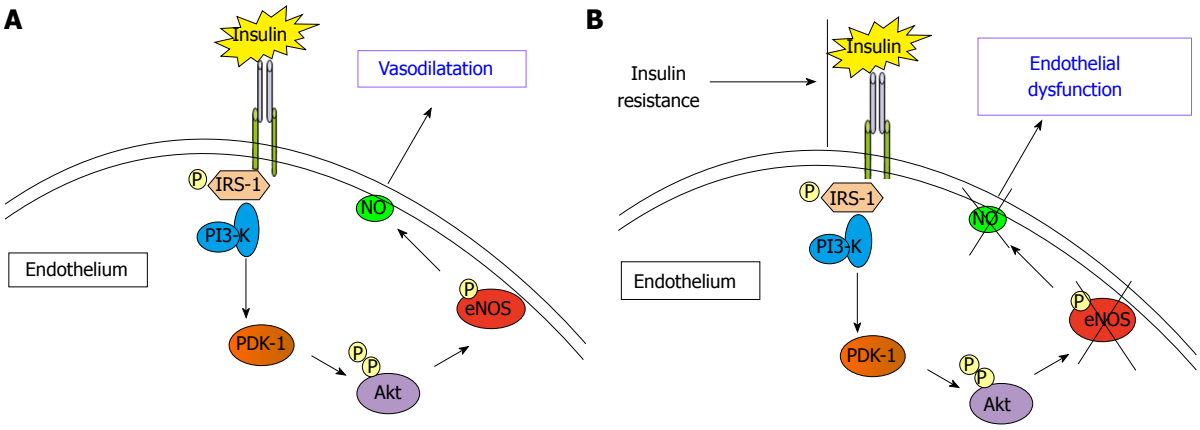


Figure 1 The binding of insulin to its receptor activates a series of phosphorylations of downstream receptors that finally activate nitric oxide-production by endothelial nitric oxide synthase. A: The release of nitric oxide (NO) causes endothelium dependent vasodilation; B: Insulin-resistance causes the reduction of Insulin-induced activation of endothelial nitric oxide synthase (eNOS). This is associated with reduction of NO bioavailability and, finally, endothelial dysfunction.

with diet-induced steatosis of different severity confirmed that reduction in sinusoidal perfusion correlated with the severity of fat accumulation in parenchymal cells[65] and the severity of steatosis had a greater impact on microcirculation. These studies demonstrated that steatosis caused an increase in the mechanical component of intrahepatic vascular resistance to portal blood flow, independent of functional changes potentially induced by IR, a feature that could be observed also in patients with genetic susceptibility to NAFLD[66,67]. The potential impact of these hemodynamic changes on liver perfusion was subsequently explored in rats exposed to a high-cholesterol diet that developed steatosis. Compared to controls, steatotic rats had significantly reduced hepatic microcirculation and tissue oxygenation[68]. Interestingly, in this study, exposure to L-Arginine, the biochemical precursor of NO, improved tissue oxygenation, whereas L-NAME, a NOS inhibitor, further deteriorated hepatic microcirculation and hepatocyte oxygenation. These results allow for two major considerations. First, reduced oxygenation is an important issue if we consider the susceptibility of fatty livers to ischemic injury[69]. Second, all these results, from a biochemical point of view, suggest that NO, the marker of a healthy endothelium, is involved in the modulation of hepatic microcirculatory perfusion and oxygenation in rats with steatotic livers. Along these lines, we had previously demonstrated that intrahepatic ED in isolated and perfused livers from rats with several features of NAFLD and MS predated the development of fibrosis and inflammation[17], suggesting that liver ED contributes to increased intrahepatic resistance very early in the pathogenesis of NAFLD.

Sinusoidal endothelial dysfunction and fibrogenesis

Fibrogenesis is a complex biochemical process which represents the hallmark of any evolving chronic liver disease. Studies with animal models of fibrosis have demonstrated that fibrogenesis parallels neo-angiogenesis, confirming the importance of endothelial cells in this phenomenon[70]. In particular, the paracrine crosstalk of sinusoidal endothelial cells with hepatocytes, hepatic stellate cells and Kupffer cells is determinant in initiating and maintaining the fibrogenic reaction[71]. However, among all these cytotypes, there remains debate as to which cell initiates the process and when exactly these cells start changing their function towards a pathologic phenotype. In addition, the role of IR on this crosstalk and its impact on the pathogenesis and evolution of NAFLD has not been adequately addressed by *in vitro* and *in vivo* studies. Recently, Miyao *et al*[72], by using mice models of NAFLD/non-alcoholic steatohepatitis, confirmed that sinusoidal endothelial injury appeared in the steatotic phase, preceding the activation of Kupffer cells, hepatic stellate cells and, in turn, inflammation and fibrosis[72]. This suggests that sinusoidal endothelial cells may have a gatekeeper role in the progression from simple steatosis to steatohepatitis, but this would require confirmatory explorations. Interestingly, these functional changes in the endothelium parallel a replacement of regular sinusoidal anatomy into appearing disorganized and characterized by abnormal vascular interconnections[73]. Furthermore, the recent observation that fibrosis may be sustained by an abnormal activation of coagulation[74-76] suggests the theoretical scenario in which the loss of the anticoagulant properties of endothelium can play a mechanistic role in fibrogenesis even in NAFLD. Indeed, Kopec *et al*[77] demonstrated that steatosis due to 3 mo of high fat diet is associated with a pro-coagulant imbalance that has a cause-effect relation with the severity of liver damage. The real impact of all these microvascular abnormalities in the progression to

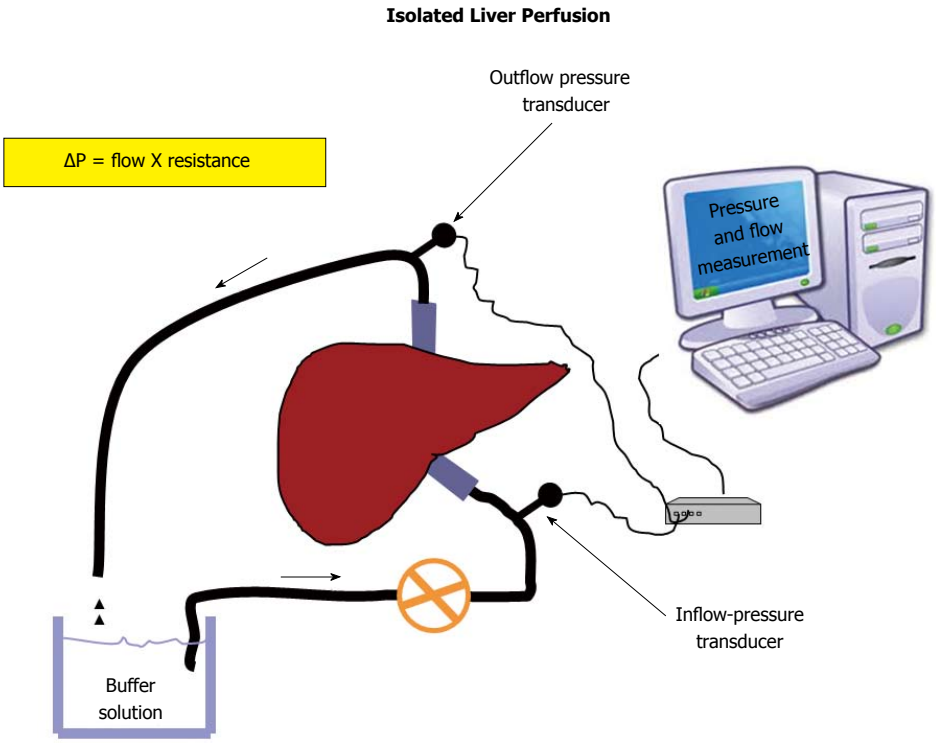


Figure 2 Livers are isolated and perfused at a constant velocity. Any change of pressure will be directly proportional to the resistance offered by sinusoids to the flow of a buffer solution according to Ohm's low applied to fluid-dynamic (ΔP = flow X resistance). This allows measuring the intrahepatic resistance offered by sinusoids.

steatohepatitis and cirrhosis is an intriguing question, and their role in the pathogenesis of NAFLD remains in need of further investigation.

Pathogenic link between intrahepatic microvascular abnormalities and IR

Many mechanisms observed in patients with IR, among them, lipotoxicity[78], oxidative stress[79,80], changes in local renin-angiotensin system[81], increased sensitivity to adrenergic stimuli of vascular smooth muscle cells[82], glucotoxicity (*via* oxidative stress, increased flow, activation of diacylglycerol, among others[83,84]) and inflammation[85,86], could explain the development of ED.

In a rat model of simple steatosis, we demonstrated the presence of insulin resistance in the liver sinusoidal endothelium that was mediated, at least in part, by the upregulation of iNOS[16]. As occurs in the peripheral circulation, in the normal liver, insulin results in liver vasodilation. In rats with fatty liver, insulin-dependent vasodilation in the liver vasculature was significantly impaired as compared to livers from control rats. This was partially restored in rats treated with the iNOS specific inhibitor 1400 W. Moreover, the insulin sensitizer metformin also restored hepatic vascular sensitivity to insulin while diminishing hepatic iNOS expression. Recently, the interaction between sinusoidal ED and iNOS has been explored in a rat model of endotoxemia (characterized by an overexpression of iNOS). These data further demonstrated that iNOS upregulation can induce, *per se*, sinusoidal ED[87]. All these findings support that in the hepatic vasculature, IR can be detected early in the course of the disease and may contribute to disease progression. A recent work by Gonzalez-Paredes *et al*[88]. confirmed the occurrence of intra-hepatic ED in rats with several features of MS and disclosed an important role of oxidative stress and cyclooxygenase end products in determining these functional abnormalities of the vasculature after 6 weeks of exposure to a high fat diet[88].

FROM BENCH TO BEDSIDE: POTENTIAL CLINICAL CONSEQUENCES

The effects of IR on hepatic vasculature could be of relevance in the pathogenesis and progression of NAFLD. The development of ED induced by IR may promote a pro-fibrogenic, pro-inflammatory and a pro-thrombotic environment, and impair regeneration after liver injury; aspects all related with the transition from steatosis to steatohepatitis and cirrhosis[72]. Unfortunately, in humans, the vascular abnormalities described in the liver of animal models with IR have been poorly investigated. Several authors have shown a correlation between IR and the severity of NAFLD[89], and have tested pharmacologic and non-pharmacologic strategies to improve IR in patients with NAFLD[90]. However, the association of these results with intra-

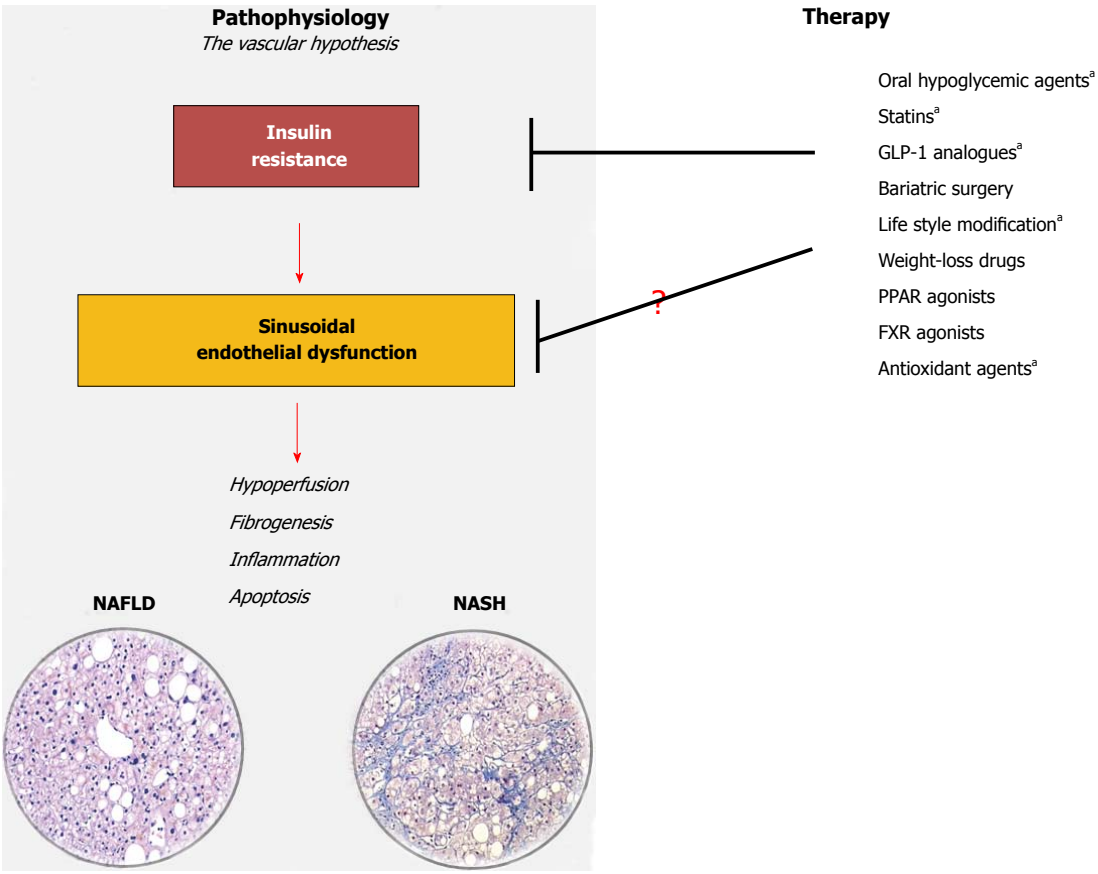


Figure 3 The vascular-hypothesis of liver damage in non-alcoholic fatty liver disease considers sinusoidal endothelial dysfunction due to insulin-resistance a key factor for the initiation and perpetuation of liver damage from simple steatosis to steatohepatitis. Any strategy of treatment ameliorating insulin-resistance may be efficacious in ameliorating sinusoidal endothelial dysfunction. Drugs marked with [a] are those with a proven efficacy on liver microcirculation (Ref. [16,42,95,103,104]). (Histological images are courtesy of Dr. Marco Maggioni, IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy). NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

hepatic vascular abnormalities, although theoretically possible, has not been demonstrated so far.

The study of intrahepatic ED in clinical practice is demanding and probably represents the Achilles' heel to translating experimental observations of vascular abnormalities from animal models to humans. Catheterization of the suprahepatic vein and measurement of the hepatic venous pressure gradient (HVPG) and hepatic blood flow with indicator-dilution techniques is the most reliable tool to measure hepatic resistance to portal blood flow[91]. These measurements can be more informative if performed dynamically, after stimulating the hepatic circulation by increased splanchnic blood flow, for example with a test meal. The rationale for this is that if ED occurs in the liver, the liver circulation is less efficient in accommodating an increase in blood flow, with a consequent abrupt increase in portal pressure[92]. Unfortunately, the use of invasive methods in patients with early disease is challenging. Notwithstanding, Francque *et al*[93] published a series of 50 patients with biopsy-proven NAFLD who underwent liver hemodynamic studies. They found that up to 28% of patients had a HVPG over 5 mmHg (the threshold indicating sinusoidal portal hypertension), even though only one patient had histological documentation of cirrhosis. In that series, HVPG significantly correlated with the degree of steatosis, suggesting that the ballooning of hepatocytes causes narrowing of sinusoidal spaces and consequently increases intrahepatic resistance to portal blood flow, in keeping with the experimental observations in animal models of steatosis. The investigation of intra-hepatic hemodynamic changes induced by IR has been addressed by studies including patients with cirrhosis and comorbidities related to MS. Cirrhosis is frequently associated with IR[6,94]. Berzigotti *et al*[8] have demonstrated that obesity is an independent risk factor for higher portal pressure and disease progression in patients with cirrhosis (independent of the etiology) and might suggest that clinical features of IR could worsen the degree of intrahepatic ED. Interestingly, the same authors demonstrated that a program of weight loss through diet and physical exercise reduced portal pressure in overweight/obese patients with cirrhosis with positive results[95].

Beyond HVPG measurement, some authors have separately explored less invasive methodologies or biomarkers of inflammation, coagulation, platelet

activation that may be linked to ED in NAFLD. In human studies, imaging techniques such as Doppler flowmetry[96], positron emission tomography[97] and magnetic resonance[98] have demonstrated the potential for investigating intravascular changes and hepatic perfusion within the steatotic liver. It has been much harder to identify biomarkers specific for intrahepatic ED. Selectins (such as P-Selectin, E-Selectin), von Willebrand Factor, Isoprostans, asymmetric dimethyl-arginine, endothelins and a series of molecules involved in the inflammatory and hemostatic activity of the endothelium have been used by cardiologists to describe and monitor ED[99]. However, unless these biomarkers are measured at the level of the hepatic veins or, alternatively, be discriminable by their organ-origin, the impairment of all these tests can only be interpreted as consequence of IR/MS but is not specifically liver-related. This underlines once again that NAFLD is the liver expression of a systemic disease. As matter of fact, several authors have demonstrated that the severity of NAFLD correlates with some key features of the systemic cardiovascular risk described in patients with MS/IR, among them, peripheral ED measured by vasodilatory response of the brachial artery to ischemia[10], mild chronic inflammation and low fibrinolytic activity[100,101]. Presently, it would be hard to recommend a pharmacologic strategy specifically targeting intrahepatic ED. However, any strategy of treatment recommended for NAFLD[102] which can modulate MS/IR has the potential to benefit sinusoidal endothelial cells in this clinical setting (Figure 3). Furthermore, the ongoing research on new drugs targeted against apoptosis, inflammation, fibrogenesis could offer in the next future an alternative/adjuvant therapy to contrast the downstream effects of the vascular changes induced by IR.

CONCLUSION

Several studies with animal models have demonstrated that IR is associated with narrow sinusoids and sinusoidal endothelial dysfunction, which cause both a mechanical and functional increase of hepatic vascular resistance to portal blood flow, even in the absence of cirrhosis. Due to the high prevalence of metabolic syndrome in patients with chronic liver diseases, the influence of these vascular changes on the natural history of NAFLD and of cirrhosis of other etiologies is highly plausible and should be explored by specifically designed human studies. This could certainly result in new strategies for the treatment of patients with chronic liver disease.

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REVIEW

Epidemiological and clinical perspectives on irritable bowel syndrome in India, Bangladesh and Malaysia: A review

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Abstract

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder, common in clinic and in the community. It has a significant impact on both society and patients' quality of life. The epidemiology, clinical presentation, and management of IBS may vary in different geographical regions due to differences in diet, gastrointestinal infection, socio-cultural and psychosocial factors, religious and illness beliefs, symptom perception and reporting. Although previous reviews and consensus reports on IBS in Asia have been published, Asia is quite diverse socio-demographically. In this context, India, Bangladesh and Malaysia share some similarities, including: (1) large proportion of the population living in rural areas; (2) rapid development and associated lifestyle changes in urban areas; and (3) dietary, cultural and religious practices. The present review explores the clinical and epidemiological data on IBS from these three major nations in South and South-East Asia. In-depth review of the literature revealed important differences between IBS in the East, as revealed by studies from these three countries, and the West; these include a predominantly rural profile, differences in bowel habit and symptom profile, raising concern with regards to diagnostic criteria and subtyping of IBS, higher dietary fiber consumption, frequent lactose malabsorption, parasitosis, and possible overlap between post-infectious IBS and tropical sprue. Moreover, the current perception on difference in prevalence of the disorder in these countries, as compared to the West, might be related to variation in survey methods.

Key words: Epidemiology; Functional gastrointestinal

disorders; Irritable bowel syndrome; Asia; Symptom; Constipation; Diarrhea

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Core tip: The epidemiology, clinical presentation and management of irritable bowel syndrome (IBS) may vary in different geographical regions due to differences in diet, gastrointestinal infection and infestations, socio-cultural and psycho-social factors, religious and illness beliefs, symptom perception and reporting. Asia is geographically and socio-demographically diverse. In this context, India, Bangladesh and Malaysia share some similarities: (1) large population living in rural areas; (2) rapid development and associated lifestyle changes in urban areas; and (3) dietary, cultural and religious practices. The present review aims to explore clinical and epidemiological data on IBS from these three major nations in South and South-East Asia.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a bowel disorder in which chronic abdominal pain or discomfort (the latter having been removed in the Rome Ⅳ criteria) is associated with irregularity in stool form and passage in the absence of any organic cause[1]. IBS has a significant impact on patients' quality of life due to physical suffering, psychological co-morbidity, social disability and economic non-productivity[2,3]. Though the global pooled prevalence of IBS has been estimated to be 11.2%[4], this figure has been questioned due to significant heterogeneity in published studies[5]. The prevalence, however, may be lower in the East. As the pathophysiology of IBS is poorly understood, the management generally focuses on the amelioration of symptoms and improvement in the quality of life. At present, the drugs available for the treatment of IBS have only a modest effect on symptom improvement and may not alter the natural history of the condition. The management strategy recommends a positive diagnosis, consideration of the patients' perspective, development of a good doctor patient relationship, identification of contributing factors, critical appraisal of the efficacies of various drugs according to the subtype of IBS and continuing care[6-8].

In view of the recent and growing interest in the gut microbiome and gastrointestinal (GI) diseases, including IBS, data from Asian research (where infection and infestations are common) may be able to offer specific epidemiological insight. Although previous reviews and consensus reports on IBS in Asia have been published[8], Asia remains a geographically and socio-demographically diverse region. Furthermore, a significant portion of the population in Asia live in rural areas, but data from rural epidemiological studies are scant. In this context, India, Bangladesh and Malaysia, three major nations in the South and South East region of Asia, share some similarities: (1) significant proportions of their populations residing in rural areas; (2) rapid development and associated lifestyle changes in urban areas; and (3) dietary, cultural and religious practices that are somewhat similar. Another similarity between these three countries is the language used for medical education and scientific publications. Since the medical education in these three countries are in the English language and the scientific studies are also published in English, local languages were not needed for data extraction from published literature. Hence, the present review aims to explore clinical and epidemiological data on IBS from a South and South-East Asian perspective.

EPIDEMIOLOGY

Prevalence

Several population-based studies have been conducted to explore the epidemiology of IBS in India, Bangladesh, and Malaysia using different diagnostic criteria (Table 1 and Figure 1). In contrast to several studies from the West, most of which are either internet and telephone surveys with their inherent bias, the majority of the eastern studies were house-to-house surveys. Based on these studies, the reported prevalence of IBS varies from 4.2%-7.5%, 7.7%-12.9% and 11%-14% in India[9-12], Bangladesh[13-15] and Malaysia[16,17], respectively.

In addition to the studies summarized in Table 1, a few house-to-house surveys, conducted primarily on chronic constipation, provided data on constipation-predominant IBS (IBS-C) as well. In a house-to-house survey among 505 subjects aged 9-64 years from Chandigarh, India the prevalence of constipation by Rome Ⅱ criteria was 16.8%[18]. In another Indian study among 925 constipated patients presenting to general physicians, 75.6% had functional constipation and 24.4% had IBS-C[19]. It has been reported that the prevalence of IBS in Asian countries is rising, with more developed nations, such as Japan and Singapore, documenting a comparable prevalence to that in western countries[20].

These data suggest that IBS might be related to economic development associated with lifestyle changes, fast living, and psychological stress associated with reduced T regulatory cell response. However, the differences in prevalence across the three countries and within the countries may be a result of variation in the

Table 1 Prevalence of irritable bowel syndrome in India, Bangladesh and Malaysia

Ref.	Study site	Sample size	Study type	Criteria	Prevalence	Male/female ratio
Shah *et al*[9]	Mumbai, India Urban	2549	Community/healthy subjects	Manning	7.5%	1/0.87
Ghoshal *et al*[10]	Multiple sites, India	4500	Community	Clinical	4.2%	1/0.93
Makharia *et al*[11]	Haryana, India	4767	Community	Rome Ⅲ	4%	1/1.5
Ghoshal *et al*[12]	Uttar Pradesh, India, rural	2876	Community	Rome Ⅲ	6.8%	1/1.09
Masud *et al*[13]	Natore, Bangladesh Rural	2426	Community	Rome Ⅰ	8.5%	1/1.84
Perveen *et al*[14]	Dhaka, Bangladesh, urban	1503	Community	Rome Ⅱ	7.7%	1/1.28
Perveen *et al*[15]	Sylthet, Bangladesh urban and rural	3000	Community	Rome Ⅲ	12.9%	1/1.09
Rajendra *et al*[16]	West coast, Malaysia, urban	1179	Community	Rome Ⅱ	14%	1/1.4
Lee *et al*[17]	East coast, Malaysia rural		Community	Rome Ⅲ	10.9%	1/1.18

diagnostic criteria used, socio-demographic differences in the populations studied, survey methods and the study instrument. A previous systematic review and meta-analysis of 260960 subjects from 80 studies across the globe suggested that the pooled prevalence of IBS of all studies was 11.2% (95% confidence interval (CI): 9.8%-12.8%)[4]. However, a more recent systematic review and meta-analysis demonstrated that the estimation of global prevalence of IBS may be inaccurate due to significant heterogeneity between the studies[5].

Urban versus rural prevalence

An urban lifestyle is reported to be associated with greater psychological stress, and thus may be associated with a higher prevalence of IBS compared to rural living. Community-based epidemiological studies on IBS have been published both from the urban and rural population in India, Bangladesh and Malaysia. Rural surveys in India have demonstrated an IBS prevalence of 4.2% (Rome Ⅲ criteria) among 2774 subjects in three villages of Uttar Pradesh, the northern part of India[12], and 4% (Rome Ⅲ criteria) in another northern Indian study[11]. In Bangladesh, an IBS prevalence of 8.5% (Rome Ⅰ criteria) was reported from a rural household survey among 2426 subjects[13]. In the East Coast of Peninsular Malaysia, a 10.9% prevalence of IBS (Rome Ⅲ criteria) was documented among rural subjects of Malay ethnicity[17].

Interestingly, urban surveys in these three countries did not show a significant variation in the prevalence of IBS from the rural communities. A 7.7% prevalence of IBS (using the Manning criteria) was observed among 2549 randomly selected subjects in urban Mumbai, India[9], while an IBS prevalence of 7.7% (Rome Ⅱ criteria) was reported in a survey of 1503 adult subjects in Dhaka city, Bangladesh[14]. In the West Coast of Peninsular Malaysia, investigators reported a relatively high prevalence rate of IBS at 14% (Rome Ⅱ criteria) among 1179 multi-ethnic adult subjects in an urban setting, but not much higher than the previous rural study[17]. In this multi-ethnic study, there were no differences in prevalence among ethnic Malays, ethnic Chinese and ethnic southern Indians, who were of comparable socio-demographic status[17].

The observations from these studies in South Asia appear to contrast with reports, which indicate that IBS is more common in urban as compared to rural populations[21-23]. Population-based studies comparing prevalence of IBS in urban and rural communities have shown differences in Chinese (urban 10.5% *vs* rural 6.14%)[21], Israeli Bedouins (9.4% *vs* 5.8%, $P < 0.01$)[22] and Italians (urban 9.9% *vs* rural 4.4%)[23]. It is uncertain if the lack of difference in IBS prevalence between urban and rural communities highlighted in this review of Indian, Bangladeshi and Malaysian studies is related to lack of head-to-head comparison using uniform diagnostic criteria and study design. Furthermore, previous population studies in Malaysia, using similar methodologies and diagnostic criteria (Rome Ⅱ) have shown significant differences in the prevalence of uninvestigated dyspepsia between urban and rural communities[24].

Sex-related issues

Early institution-based studies from India showed that IBS was 2-4 times more common among male adults compared to female adults[25-27] but subsequent population-based studies revealed mixed findings. The male:female ratios in relation to the prevalence of IBS in India, Bangladesh and Malaysia are highlighted in Table 1. It is important to note that among all these studies that reported female predominance from these three Asian countries, female to male ratios were not as wide as reported from the West. The lack of an obvious female predominance amongst IBS subjects appears to be common in several Asian reports[8,20,28-30]. This is in contrast to the studies from the West, which consistently reported 2-3 times greater prevalence of IBS in women than in men[3].

Although a meta-analysis of community-based studies reported that IBS is 67% higher in females

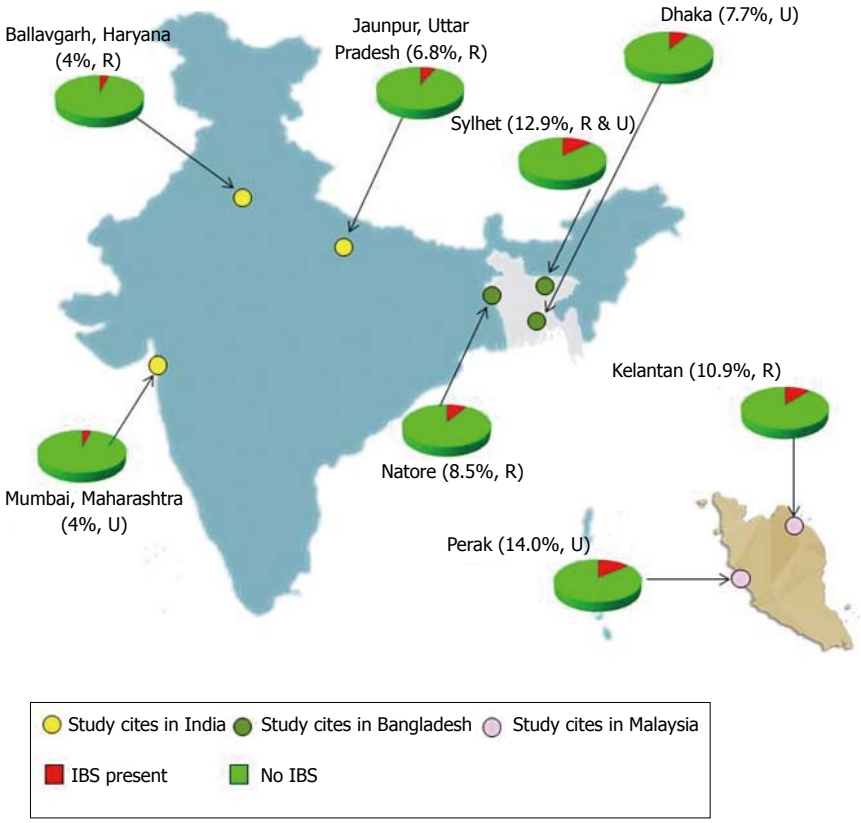


Figure 1 Map of India, Bangladesh and Malaysia showing sites of epidemiological studies on IBS in these countries and its prevalence. IBS: Irritable bowel syndrome; R: Rural; U: Urban.

compared to males (OR = 1.67; 95%CI: 1.53-1.82), no significant sex-related differences were observed in South Asia[4]. A recent meta-analysis on a Global prevalence of IBS found that the pooled prevalence rates were higher among females (10.2%) compared to males (8.8%). The prevalence rates of IBS were higher among females than males in each region of the globe separately[5]. However, as the authors of this meta-analysis acknowledged that due to significant heterogeneity in the number of available studies in different parts of the world and the methods, pooled estimates are expected to be biased.

SYMPTOM PROFILE AND DIAGNOSIS OF IBS

The Rome criteria are currently the most widely accepted for establishing a diagnosis of IBS. However, studies from India and Bangladesh have reported that frequency and/or severity of abdominal pain, a cardinal symptom in the Rome criteria, may not be so common among their population with IBS. A multi-centric study involving 2785 patients visiting physicians at 30 centers across India with chronic lower GI symptoms, no `alarm features' and negative investigations for organic causes has been published; in that study, abdominal pain and discomfort was present in only 70% of subjects[10]. Other institution-based studies on IBS subjects from India have reported abdominal pain in a range from 33%[31] to 70%[32]. As abdominal pain is a pre-requisite for the symptom-based diagnosis of IBS by the Rome criteria, these criteria may have a low sensitivity for diagnosing IBS in India.

In another multi-center, Indian study (MIIBS) using various diagnostic criteria for IBS, the Manning criteria had the highest sensitivity (91%), followed by Asian criteria (74.5%), Rome Ⅰ (68%), Rome Ⅲ (52.5%) and Rome Ⅱ (40%) to diagnose IBS[33]. The higher sensitivity of the Manning and Asian criteria might have been due to the fact that "abdominal pain" was not absolutely necessary for the diagnosis of IBS by both of these criteria. Furthermore, "abdominal bloating" had been included in combination with "abdominal pain or discomfort" by the Asian criteria[8]. When multiple diagnostic criteria were applied in a rural population in Bangladesh, IBS prevalence of 1.8%, 7.3%, 8.8%, and 7.7% was found using Manning ⩾ 3, Manning ⩾ 2, Rome Ⅰ and Rome Ⅱ criteria, respectively. The IBS prevalence varied according to the criteria applied, with rates as high as 27.9% using "Manning ⩾ 2 without pain" and down to 1.8% using "pain with Manning ⩾ 3"[34].

A systematic review previously demonstrated that the Rome Ⅱ criteria had a poor sensitivity for diagnosing IBS in primary care, ranging between 31% to 65%, with the lowest sensitivity rates derived from studies in India[35]. The lack of accuracy of a

symptom-based criterion for diagnosing IBS may be more relevant for patients in India and Bangladesh for at least two reasons. Firstly, IBS patients often do not report abdominal pain as the predominant symptom in this region. Secondly, these criteria were originally developed and validated in Western populations, who are known to differ from Eastern populations with regard to socio-cultural issues, language, symptom perception and epidemiology[36]. Considering the fact that the Rome Ⅳ criteria did not include even abdominal discomfort or bloating as the essential symptom to diagnose IBS (Table 2), the sensitivity of the Rome Ⅳ criteria is expected to be lower in these three countries[37].

A recent study by the Rome Foundation-Asian working team[38] on 1805 consecutive unselected functional gastrointestinal disorder patients presenting to the primary or secondary care centers in 11 Asian centers using a culturally adapted Rome Ⅲ Diagnostic Questionnaire initially translated and validated in 9 Asian languages[39,40] identified 9 symptom clusters by principal component factor analysis with varimax rotation. Authors concluded that though IBS and chronic constipation were the dominant symptom clusters, bowel symptom cluster with meal trigger and a gas cluster were the other prominent groups that were in line with the old Asian concept that identified epigastric localization of pain, and emphasized meal- and gas-related symptoms[41].

Upper abdominal symptoms and overlap with functional dyspepsia in IBS

IBS and functional dyspepsia (FD) have common patho-physiological mechanisms, like visceral hypersensitivity, abnormal central processing of sensory perception, GI dysmotility and psychological factors; these two disorders are, therefore, expected to coexist[42]. Figure 2 shows the prevalence of IBS-FD overlap, IBS and FD in three population-based studies in India and Bangladesh[9,12,15]. These population-based studies from India and Bangladesh demonstrated that 3.5% to 4.5% of the general adult population of India and Bangladesh have dyspepsia-IBS overlap (Figure 2). In a community-based survey conducted among 3000 people in rural and urban populations in a district of Bangladesh, 42% of FD subjects had IBS and 27% IBS subjects had FD. Overall, bowel symptom frequency scores were higher in IBS-FD than IBS alone (10.01 ± 4.17 *vs* 8.16 ± 3.84, $P < 0.000$)[15].

In an urban survey of adults from Malaysia, subjects with IBS were more likely to report symptoms of gastroesophageal reflux disease (GERD) compared to healthy controls [56% *vs* 29%, odds ratio (OR) = 3.06][16]. Environmental factors such as diet may be a contributory factor for upper GI symptoms in IBS sufferers among South Asians. In a study in Mumbai, India, > 99% of the population consumed chili in their diet and 75% of subjects consumed it in a moderate to high quantity[9]. Chili is known to aggravate not only IBS symptoms[43] but has been shown to independently predict dyspepsia in a multi-ethnic population in Malaysia[44]. Furthermore, GERD and *H. pylori* infection are recognized to be more prevalent among ethnic Indians/South Asians compared to other ethnic groups in Asia[45,46]. It is likely that these factors may lead to a greater overlap of IBS-FD and IBS-GERD among South Asians compared to other ethnic groups in Asia.

SUBTYPING OF IBS

IBS sub-classification into diarrhea- and constipation-predominant (IBS-D and IBS-C, respectively) are important for determining appropriate therapeutic strategies. However, studies in India, Bangladesh and Malaysia have reported that the majority of IBS patients could not be easily classified into distinct sub-types. In a multi-centric Indian study, 53% and 47% of 2656 subjects with lower GI symptoms had subjective perceptions of constipation and diarrhea, respectively[10]. However, when stool frequency-based criteria of constipation (stool frequency < 3/wk) and diarrhea (stool frequency > 3/d) were applied to the same subjects, 19% had constipation, 4% had diarrhea and 57% had indeterminate symptoms. In that study, the median stool frequency was similar (2 times/d) in patients who had perceived themselves to have either constipation or diarrhea[10].

In a rural community-based study at Uttar Pradesh, northern India, 83% of the IBS subjects remained unclassified using the Rome Ⅲ criteria. Interestingly, the application of Asian criteria, instead of the Rome Ⅲ criteria, reduced the indeterminate sub-type of IBS subjects in rural India from 83% to 12%[12]. Similarly, the MIIBS study of 1618 subjects with lower GI symptoms found that by stool frequency criteria, 78% subjects remain unclassified, whereas by patient perception and stool form (Bristol stool scale) criteria only 28% and 16% of patients remain unclassified, respectively[33]. In a rural study in Bangladesh, 64% of IBS subjects diagnosed by the Rome Ⅱ criteria could not be sub-typed[13]. In Malaysia, a rural survey of ethnic Malays similarly demonstrated that the majority of IBS subjects fell into a "mixed" category with the Rome Ⅲ criteria as follows: 20.8% IBS-C, 16.7% IBS-D and 58.3% mixed pattern[17].

These studies suggest that several popularly used criteria may be poor at classifying IBS into constipation- and diarrhea-predominant types in Asia. Community-based studies in India and Bangladesh have shown that > 90% of the general population have a bowel motion of at least once per day[10,47,48]. These differences in bowel habit between South Asians and Westerners may be due to differences in dietary pattern, fiber intake, intestinal transit time and frequency of lactose malabsorption as compared to the Western population[49]. In a study from India,

Table 2 Rome III and Rome IV criteria for diagnosis of irritable bowel syndrome[37]

Rome III criteria	Rome IV criteria
At least 3 mo, with onset at least 6 mo previous to recurrent (at least 3 d/mo) abdominal pain or discomfort associated with 2 or more of the following	Recurrent abdominal pain, on average, at least 1 d per week in the last 3 mo, associated with 2 or more of the following
Improvement with defecation	Related to defecation
Onset associated with a change of frequency of stool	Associated with a change in frequency of stool
Onset associated with a change in form of stool	Associated with a change in form (appearance) of stool

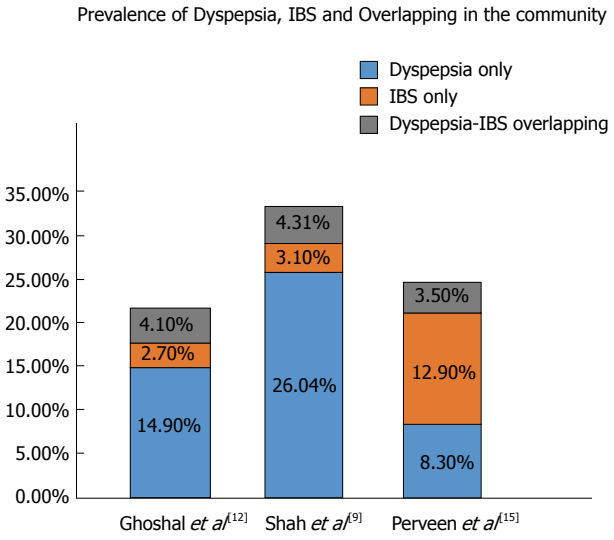


Figure 2 Prevalence of IBS, dyspepsia and overlap syndromes in community studies in India and Bangladesh. IBS: Irritable bowel syndrome.

the mean fiber intake was found to be similar at 52 g in both IBS and healthy controls[50]. This is higher than the recommended daily fiber intake (20-30 g in adults) and actual daily dietary fiber intake (13.3 g) in the West[51-53]. Furthermore, the mean intestinal transit time of 39.85 h among northern Indians contrasts significantly to that of 83.4 h in Caucasian adults[54].

PATHOPHYSIOLOGICAL INSIGHTS

Inflammation and IBS

Current evidence suggests that the pathogenesis of IBS is multi-dimensional consisting of gut microbial dysbiosis, including small intestinal bacterial overgrowth (SIBO), visceral hypersensitivity, intestinal mucosal immune activation, dietary intolerance, increased intestinal permeability, abnormal gut-brain interaction including cognitive dysfunction, psychosocial distress and altered GI motility[55-57]. Low-grade inflammation plays a major role in the pathophysiology in a subset of patients with IBS[57]. This has been explored in several studies in India, Bangladesh and Malaysia. IBS patients in a tertiary care hospital in Bangladesh were found to have a significant increase in chronic inflammatory cells, mast cells and lymphoid follicles compared to control group of healthy subjects ($P < 0.05$). Mast cells but not the number of chronic inflammatory cells, mucosal and submucosal lymphoid follicles, were significantly increased in post-infectious (PI)-IBS compared to non-PI IBS in that study[58]. These findings are in accordance with similar reports of Western IBS patients[56,59]. Mast cell activation is associated with epithelial and neuromuscular dysfunction, visceral hypersensitivity, increased epithelial permeability and altered motility[56,59].

In an Indian case-control study including 221 patients with IBS and 273 age- and sex-matched controls, an interleukin (IL)-1 receptor antagonist under-producer genotype was associated with IBS and its over-producer protective. Moreover, higher levels of IL-1α and IL-1β were found in patients with SIBO, and IL-1α and IL-1β were associated with bloating and loose stools. The findings of that study clearly show the role of inflammation in the pathogenesis of IBS in general and diarrhea-predominant IBS in particular[60]. Several case-control studies, though under-powered, on genetic polymorphisms in candidate pro-/anti-inflammatory and xenobiotic metabolizing genes support the role of inflammation in the pathogenesis of IBS[61-64].

Imbalance in body serotonin (5HT), 95% of which is in the gut, plays a major role in the pathogenesis of IBS, with its deficiency contributing to slow gut transit and constipation, and excess causing diarrhea[65]. In an Indian case-control study, SLC6A4 was found as a potential candidate gene involved in the pathogenesis of IBS[66]. The frequency of SLC6A4 polymorphism and higher levels of 5HT were significantly associated with IBS[66].

The association between microscopic colitis (MC) and IBS has similarly been described. In a recent systematic review and meta-analysis, the pooled prevalence of MC in Western IBS patients was 7.2% (95%CI: 1.5%-17.2%) and the prevalence varied between 2.4% to 11.5% in individual studies[67]. In the only report from Bangladesh, lymphocytic colitis (LC) was found in 37% of 60 patients with diarrhea-predominant IBS, with a complete absence of collagenous colitis. The distribution of LC was restricted in proximal colon in 68.2% patients, in the left colon in 9.1%, and diffuse in 22.7%[68]. A study of 120 subjects in a Malaysian tertiary institution found a very low (1.3% only) prevalence of collagenous colitis among 120 IBS subjects. However, IBS-D patients had a higher prevalence of moderate non-specific, microscopic inflammation compared to controls (14.9% *vs* 2.2%, $P = 0.005$), suggesting that either occult

infection or other causes of inflammation may be contributory towards IBS-D sub-types[69].

In an Indian case series on 29 patients with MC presenting with chronic large bowel diarrhea, of 7, 4, 7 and 11 patients with collagenous colitis, LC, minimal change colitis and microscopic colitis not otherwise specified, 28.6%, 25%, 14.3% and 33.3% had abdominal discomfort, suggesting that they would fulfill the Rome Ⅲ criteria for IBS[70]. In another Indian study among 400 patients with chronic watery diarrhea, 3.7% had microscopic colitis (33% had collagenous colitis and 67% had lymphocytic colitis) and 10/15 of these patients were clinically diagnosed with IBS[71]. Data from the institutional-based studies suggest that MC is rare amongst South and South-East Asian IBS patients, perhaps in keeping with the lower prevalence of inflammatory bowel disease (IBD) in this region[72].

Protozoa and IBS

The presence of amoebic cysts in the stool of a patient with chronic lower GI symptoms creates confusion among physicians, and they often label such patients as suffering from chronic non-dysenteric amebic colitis. A multi-centric study on IBS from India showed that of 1237 patients reporting treatment with metronidazole in the past, 672 (24%) experienced temporary improvement[10]. Two studies in India explored this issue among IBS patients[73,74]. No significant differences were found in the proportion of amoeba in the stool (18% *vs* 18%), serologic evidence of infection (42% *vs* 41%), colonoscopic abnormalities (7% *vs* 3%) or histologic abnormalities among 144 IBS and 100 asymptomatic subjects. Additionally, there was no significant difference in response to therapeutic trial with metronidazole among the symptomatic patients who were cyst-positive or -negative at 6 mo[73]. In a second study of 154 inmates of a leprosy rehabilitation center, detection of amoeba among IBS and controls was found to be 50% and 19%, respectively. All strains of amoeba isolated were found to be non-pathogenic and there was no relationship between detection of amoeba and symptoms of IBS at 12 mo follow-up[74]. These studies suggest that non-pathogenic amoeba is an innocent by-stander in IBS subjects, which are inappropriately termed as "chronic amoebiasis" and often treated with anti-amoebic drugs unnecessarily.

Another common gut parasite found in the intestinal tract of humans and animals, *Blastocystis* sp, has been associated controversially with IBS, possibly as a result of irregular shedding of parasites in stool and variation in fecal detection. A recent study examining parasitic infestations using PCR techniques reported a significantly higher prevalence of *Blastocystis* sp (17% *vs* 5.5%), corroborated by increased cytokines for inflammation (IL-3, IL-5 and IL-10) in IBS patients compared to healthy controls in Malaysia[75]. Furthermore, IBS patients with increased stool frequency were found to have a greater quantity of *Blastocystis* cysts, indicating that the severity of IBS symptoms correlated with the quantity of parasitic infestation[76]. These studies from Malaysia indicate that persistent chronic parasitic infection, which is often undetectable using standard culture or laboratory techniques, may be an additional contributory factor to microscopic gut inflammation in IBS in Asians.

In the light of these observations of protozoal diseases among South and South East Asian patients with IBS, it is not surprising that a multi-centric Indian study showed that 44% of the subjects with chronic lower GI symptoms took metronidazole with temporary symptom improvement among 54%[10]. Additionally, a randomized placebo controlled trial among 45 IBS subjects who were allocated to ispaghula husk (60 d), metronidazole (metronidazole 10 d and placebo 50 d) and placebo (60 d) found that metronidazole provided symptom relief in IBS compared to placebo without affecting recto-sigmoid motility[77].

Helminthiasis and IBS

Helminthic infestation is common in Bangladesh and India. In a report from rural Bangladesh, 70% of 252 children had been found to have soil-transmitted helminthiasis by the age of 2 years[78] . In southern India, a report suggested that 62% of all age groups had hookworm infestations[79]. Intestinal helminthiasis causes a shift in the immune system towards a T-helper 2 cell response[80,81]. This may be associated with a low-grade, protracted inflammation, which is recognized in a subset of IBS patients. Previous reviews have speculated that a high intestinal helminthic/parasitic infestation may be the cause of a low prevalence of IBS in spite of a high incidence of acute infective diarrhea in Asia[82]. Furthermore, the "hygiene hypothesis" speculates that a high childhood infection rate is responsible for the low prevalence of IBS in the developing countries.

PI-IBS and PI-MAS

PI-IBS has been defined as an acute-onset IBS (by Rome criteria) that develops after the individual, who has not previously met the Rome criteria, experiences a GI infection with two or more of the following characteristics: fever, vomiting, diarrhea, and a stool culture positive for an infectious agent[83,84]. The pooled incidence of PI-IBS was found to be 10% in a systematic review and meta-analysis of predominantly Western studies[85]. Data on PI-IBS from Asian countries are scant, whereas acute gastroenteritis is common[86]. Studies from China and Korea have revealed that the incidence of PI-IBS is 8%-15%[87,88]. The incidence rate for acute diarrhea among those aged > 14 years in parts of India and Bangladesh have been reported as 60 per 100 person-years and between 15.2 to 135.7 per 100 person-years, respectively[89-91].

Despite a high incidence rate of acute diarrhea

in the Indian subcontinent, there is no published study on PI-IBS. Furthermore, the relatively low prevalence of IBS in the Indian subcontinent, despite a high burden of acute diarrheal illness, appears enigmatic[83,86]. However, a condition similar to PI-IBS, post-infectious malabsorption (PI-MAS) or tropical sprue has been recognized in the Indian sub-continent for some time. A prevalence rate of 8%-20% of PI-MAS was previously reported in rural southern India following an episode of acute infectious diarrhea[92]. Both PI-IBS and PI-MAS have similar clinical features, etiopathogenesis, and potential response to treatment with antibiotics[93]. Furthermore, both PI-IBS and PI-MAS have been associated with SIBO[94,95] and abnormal small intestinal permeability[96].

It is likely then, as IBS is a symptom-based diagnosis or that patients with mild malabsorption syndrome can be misdiagnosed as IBS, particularly of the diarrhea-predominant type, unless malabsorption is carefully excluded by appropriate investigations. In most studies on PI-IBS, PI-MAS has not been carefully excluded using tests for malabsorption. Considering the fact that PI-MAS or tropical sprue has been described recently following an attack of acute infectious diarrhea in developed countries like the United States, this issue needs consideration in developed and temperate countries as well[97]. Hence, there may be some overlap between PI-IBS and PI-MAS in the Indian sub-continent[83,86,96] and some cases of PI-MAS may be erroneously diagnosed as cases of PI-IBS. In a recent study from Dhaka, Bangladesh, 10% of 23 patients with acute gastroenteritis fulfilling Rome Ⅲ criteria for IBS in the absence of previous IBS were found to have PI-MAS on investigation[98].

SIBO and IBS

SIBO and IBS have similar clinical features. Routine investigations conducted to exclude organic diseases in IBS, such as colonoscopy and blood tests, do not rule out SIBO. Several studies have reported the presence of SIBO in IBS patients in India, as shown in Table 3[95,99-101]. Ghoshal *et al*[99] reported that 19% of 80 IBS patients had SIBO, diagnosed by upper gut aspirate culture with a colony count > 10^5 CFU/mL. In a case-control study among 225 Indian IBS patients, 11% had SIBO by glucose hydrogen breath test (GHBT), whereas 1% of 100 healthy subjects had SIBO[100]. In the third study, 9 (13%) of 69 IBS patients had SIBO by GHBT. There was no significant difference in the frequency of SIBO among subjects with and without lactose intolerance and IBS[101]. In the fourth study, 11 (8.5%) of 129 IBS subjects had SIBO compared to 1 (2%) of 51 healthy subjects[95].

An evidence-based review documented that the prevalence of SIBO in patients with IBS varies widely due to different criteria for defining a positive breath test and the methodology employed. It is 28%-84% with lactose hydrogen breath test (LHBT), 2%-31% with GHBT and 2%-6% based on culture[102]. Most of the studies used GHBT to diagnose SIBO in India. GHBT had a sensitivity and specificity of 44% and 82%, respectively, when the culture of the upper gut aspirate was taken as the gold standard in a study on patients with malabsorption syndrome in India[103]. However, in a recent study on 80 patients with IBS (Rome Ⅲ criteria), GHBT had a sensitivity of 27% and specificity of 100% considering quantitative jejunal aspirate culture as the gold standard[99]. The lower sensitivity of GHBT among patients with IBS as compared to malabsorption syndrome might be related to lower bacterial colony counts in proximal small bowel among the former rather than the latter group of patients.

Further evidence for the association between SIBO and IBS was provided by a randomized, placebo controlled trial from India, of norfloxacin in IBS cases with SIBO compared to those without SIBO. In this study, the response rate (negativity of Rome Ⅲ criteria at 1 mo) of norfloxacin and placebo among IBS patients with and without SIBO was 87.5% (7/8) and 0% (0/7), 25% (8/32) and 0/33 (0%), respectively. Overall, 37.5% of 40 IBS patients responded to norfloxacin[99].

Food intolerance including lactose malabsorption and IBS

In recent years, dietary fermentable oligo, di, monosaccharide, and polyols (FODMAP) have been incriminated in contributing to the symptoms of IBS[104]. However, studies on dietary FODMAP in the pathogenesis of IBS symptoms and their exclusion in its treatment have been scant from India, Bangladesh and Malaysia, except for lactose as an isolated FODMAP. In a multi-centric Indian study, self-reported milk intolerance was present among 32% of 2785 subjects with lower GI symptoms. Patients with IBS-D reported milk intolerance more often than those with IBS-C[10]. In a rural community-based study in Bangladesh, a regular dairy intake was present in 61.7% of 2542 subjects, but 13.8% reported milk intolerance. Among 593 subjects with IBS, 12.6% reported milk intolerance and they showed a higher frequency of frequent bowel motion and loose or watery stool ($P < 0.025$)[13].

However, the diagnostic accuracy of self-reported milk intolerance to diagnose lactose malabsorption was reported as having a sensitivity, specificity, positive predictive value and negative predictive value of 53%, 61%, 26% and 55%, respectively, in a case-control study in India among 112 IBS patients and 53 healthy controls[101]. In the same study, the frequency of lactose malabsorption diagnosed either by LHBT or lactose tolerance test was similar among IBS (82%) and healthy controls (77%). Nevertheless, symptoms of lactose intolerance were more frequent among IBS (55%) compared to healthy subjects (34%).

Table 3 Studies on small intestinal bacterial overgrowth in India

Ref.	Number of IBS patients	Diagnostic criteria of IBS	Number of controls	Method of diagnosis of SIBO	% of SIBO in IBS	% of SIBO in controls
Ghoshal *et al*[99]	80	Rome Ⅲ	-	Culture of upper jejunal aspirate	19	-
Rana *et al*[100]	225	Rome Ⅱ	100	GHBT	11.1	1
Gupta *et al*[101]	69	Rome Ⅱ	-	GHBT	13	-
Ghoshal *et al*[95]	129	Manning	51	GHBT	8.5	2

GHBT: Glucose hydrogen breath test; IBS: Irritable bowel syndrome; SIBO: Small intestinal bacterial overgrowth.

In an Indian study, the researchers demonstrated that lactose malabsorption diagnosed using 25-g lactose dose is clinically more meaningful than that diagnosed using the conventional 50-g lactose dose, which is quite a non-physiological dose; in fact, lactose malabsorption diagnosed using 25-g lactose dose was associated with 10 times higher rate of clinical improvement following milk withdrawal than that diagnosed using 50-g dose[105].

More recently, investigators were able to demonstrate a similar frequency of C/T -13910 and G/A-22018 lactase gene polymorphism between Indian IBS patients and healthy controls[106]. These studies indicate that the prevalence of lactose malabsorption is high in South Asians, with no difference between IBS and non-IBS adults. However, the clinical consequences of lactose malabsorption are likely to be exaggerated in patients with IBS who often have underlying abnormalities in motility and visceral sensation and a reduced pain threshold.

Of the other FODMAP agents, a northern Indian study showed that 14.4% patients with IBS, particularly IBS-D, as compared to 2.4% of healthy controls had fructose malabsorption as demonstrated by fructose hydrogen breath test[107]. Importance of dietary factors in occurrence of IBS in the third world countries has been shown in a Brazilian study as well[108]. More studies on FODMAP diet and IBS, and their elimination in its treatment are needed from South Asia.

Psychological comorbidity and IBS

With rapid development and urbanization in many of the South Asian countries, including India, Malaysia and Bangladesh, it is anticipated that an increase in urban-related psychopathology may contribute to IBS symptoms, severity and clinic visit in these nations. In a cross-sectional study of 248 adult patients in a referral center in Malaysia, a higher prevalence of anxiety and depression was observed in FD, non-erosive reflux disease and IBS patients compared to a healthy control group (43.5%, 45.2% and 67.7% *vs* 14.5%, $P < 0.001$; and 22.6%, 33.9% and 38.7% *vs* 6.5%, $P < 0.0001$). IBS patients had a higher rate of anxiety than FD patients ($P = 0.01$) and non-erosive reflux disease patients ($P = 0.02$), while no significant differences in depression rates were observed among all three groups of functional GI disorders[109].

In a similar study from India, a greater prevalence of major depressive (47.3% *vs* 5.1%, $P < 0.001$), somatoform (50% *vs* 14.6%, $P < 0.001$) and panic disorder (44% *vs* 11.6%, $P < 0.001$) were identified in IBS patients compared to healthy controls[110]. Whilst these studies in Malaysia and India have indicated an association between psychological disorders and IBS, a causal relationship could not be considered from these studies as patients with any chronic disorder are expected to have more psychological issues than healthy subjects. Furthermore, data on the efficacy of psychopharmacological therapy among Indian subjects with IBS have not been convincing. A prospective double-blind randomized controlled study among 90 IBS patients showed that oxyphenonium bromide and imipramine was not superior to placebo in improving symptoms nor the health-related quality of life ($P > 0.05$)[111]. Nevertheless, about one-third of patients with lower GI symptoms in India were reported to be on tricyclic antidepressants in a previous survey[10].

Healthcare seeking/consultation behavior

Several community-based studies in India and Bangladesh have investigated healthcare seeking behavior among IBS subjects in both rural and urban areas. Table 4 shows the consultation rates of IBS patients in India and Bangladesh. Physician consultation rates were 34.9%[13] and 65.5%[14] of IBS subjects in rural and urban Bangladesh, respectively, with no significant sex differences (68.6% *vs* 61.2%, $P > 0.05$). The main predictor of consultation was the presence of multiple dyspeptic symptoms. The studies from India have alluded to a similarly high consultation rate (Table 4), with higher consultation rates for male IBS subjects (70% *vs* 44%, $P = 0.00001$)[9]. The types of healthcare practitioners consulted were explored in one study and found to include physician of modern medicine (MBBS and above) 3.1%, homeopathic physicians 5%, ayurvedic physicians 8.3% and Unani practitioners 0.8%[12]. These studies from India and Bangladesh suggest that availability, accessibility, and cost of the consulting physician may be important determinants of the consultation behavior of the IBS patients.

CONCLUSION

This review has highlighted several unique aspects

Table 4 Consultation rate of IBS patients in different population-based studies in India and Bangladesh

Ref.	Study areas/ country	Number of IBS patients	Diagnostic criteria of IBS	Consultation Rate	Reason of consultation	Nature of consulting doctors
Shah *et al*[9]	Urban/India	190	Manning	28.94%	-	-
Ghoshal *et al*[12]	Rural/India	FGIDs	Rome Ⅲ	17.20%	-	MBBS and above (3.1%) Homeopathic (5%) Ayuervadic (8.3%) Unani (0.8%)
Masud *et al*[13]	Rural/Bangladesh	593	Manning	39.0%	Altered stool passage Sense of incomplete evacuation Mucus in stool	
Perveen *et al*[14]	Urban/Bangladesh	116	Rome Ⅱ	65.50%	Multiple dyspeptic symptoms	

IBS: Irritable bowel syndrome; FGIDs: Functional gastrointestinal disorders.

of IBS in the populations of India, Bangladesh and Malaysia. The prevalence of IBS is comparable in urban and rural areas of these three countries. A male predominance has been reported in institution-based studies in India, but a sex-based disparity has not been observed in community studies. Upper abdominal symptoms and FD are common among IBS subjects in the community. The sensitivity of the Rome criteria to diagnose IBS is lower in India. Many patients with IBS cannot be subtyped into constipation- and diarrhea-predominant sub-types based on popularly used criteria developed in the West, and a stool subtype and the Asian criteria appear to be more sensitive.

From a pathophysiological perspective, chronic gut infections, gut microbial dysbiosis, and SIBO appear to have important roles in IBS in South Asia. In spite of the high incidence of acute infective gastroenteritis, the low prevalence of IBS in India and Bangladesh remains somewhat enigmatic. PI-IBS, PI-MAS and SIBO may have a great degree of overlap in adults and may need specific investigations to differentiate them. Therefore, a role of the gut-specific antibiotics, such as rifaximin, cannot be over-estimated. Healthcare consultation by IBS subjects in India and Bangladesh are high in both rural and urban areas.

Future direction

Further epidemiological studies using the same instruments and survey method are needed for valid comparison of the prevalence of IBS in the East and the West, as well as a head-to-head comparison in the rural and urban areas in Asian countries. Further studies are also needed on the symptom profile and subtyping of IBS in Asian countries using clinical, Rome and Asian-based criteria. Considering the high burden of acute infectious diarrhea in the Indian Subcontinent and frequently reported cases of tropical sprue, more studies are needed on the pathophysiology of PI-IBS and PI-MAS.

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ORIGINAL ARTICLE

Basic Study

Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis

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Abstract

AIM

To investigate gender-specific liver estrogen receptor (ER) expression in normal subjects and patients with hepatitis C virus (HCV)-related cirrhosis and hepatocellular carcinoma (HCC).

METHODS

Liver tissues from normal donors and patients diagnosed with HCV-related cirrhosis and HCV-related HCC were obtained from the NIH Liver Tissue and Cell Distribution System. The expression of ER subtypes, ERα and ERβ, were evaluated by Western blotting and real-time RT-PCR. The subcellular distribution of ERα and ERβ was further determined in nuclear and cytoplasmic tissue lysates along with the expression of

inflammatory [activated NF-κB and IκB-kinase (IKK)] and oncogenic (cyclin D1) markers by Western blotting and immunohistochemistry. The expression of ERα and ERβ was correlated with the expression of activated NF-κB, activated IKK and cyclin D1 by Spearman's correlation.

RESULTS

Both ER subtypes were expressed in normal livers but male livers showed significantly higher expression of ERα than females ($P < 0.05$). We observed significantly higher mRNA expression of ERα in HCV-related HCC liver tissues as compared to normals ($P < 0.05$) and ERβ in livers of HCV-related cirrhosis and HCV-related HCC subjects ($P < 0.05$). At the protein level, there was a significantly higher expression of nuclear ERα in livers of HCV-related HCC patients and nuclear ERβ in HCV-related cirrhosis patients as compared to normals ($P < 0.05$). Furthermore, we observed a significantly higher expression of phosphorylated NF-κB and cyclin D1 in diseased livers ($P < 0.05$). There was a positive correlation between the expression of nuclear ER subtypes and nuclear cyclin D1 and a negative correlation between cytoplasmic ER subtypes and cytoplasmic phosphorylated IKK in HCV-related HCC livers. These findings suggest that dysregulated expression of ER subtypes following chronic HCV-infection may contribute to the progression of HCV-related cirrhosis to HCV-related HCC.

CONCLUSION

Gender differences were observed in ERα expression in normal livers. Alterations in ER subtype expression observed in diseased livers may influence gender-related disparity in HCV-related pathogenesis.

Key words: Estrogen receptor α; Estrogen receptor β; Hepatitis C virus-related cirrhosis; Hepatitis C virus-related hepatocellular carcinoma; Sex and gender; Normal liver

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Core tip: Our study, for the first time, demonstrates gender-based differences in basal expression of estrogen receptor α (ERα) in the liver of normal males and females. Altered nuclear and cytoplasm liver ER subtype protein expression was also observed in hepatitis C virus (HCV)-cirrhosis and HCV-related hepatocellular carcinoma (HCC) that correlate with the inflammatory and oncogenic markers, activated NF-κB and cyclin D1, implicating their role in chronic cirrhosis and malignant transformation. These findings may have an impact on future planning strategies for the development of novel treatments or prognostic markers by targeting ERs or their signaling pathways during chronic HCV-related cirrhosis and HCC development.

Iyer JK, Kalra M, Kaul A, Payton ME, Kaul R. Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis. *World J Gastroenterol* 2017; 23(37): 6802-6816 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6802.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6802

INTRODUCTION

Hepatitis C virus (HCV) infection is a major health problem in the United States[1]. HCV-infection tends to be asymptomatic for long periods of time during which fibrosis and cirrhosis can develop, resulting in end-stage liver disease[2,3]. No viable vaccine is currently available for HCV prevention and, while newer and expensive direct acting antivirals are available for treating early HCV-infection, transplantation is the only effective treatment for patients with advanced or end stage liver disease[4,5]. HCV-infection related fibrosis and cirrhosis are associated with the development of chronic liver disease that eventually leads to hepatocellular carcinoma (HCC), the most common type of liver cancer[6-8]. The latest epidemiological data shows that HCC has the fastest growing death rate in the United States[9]. Thus, there is an urgent need to develop novel, effective and affordable therapeutics to treat chronic HCV-infections.

Understanding the contribution of host factors that directly or indirectly interact with HCV is central to finding new treatment modalities[10]. Striking gender disparity in HCV and HCC pathogenesis has led to the investigation of estrogen as one of the key host players in modulating the course of HCV-pathogenesis. Epidemiological and clinical studies have shown that chronic HCV-infections are more prevalent in males and progress more rapidly to cancer development as compared to females[11,12]. Similar to males, postmenopausal females lacking circulating estrogen show accelerated progression of HCV-infection to fibrosis and HCC development[12,13]. In contrast, physiological estrogen levels seen in premenopausal females, have been associated with less severity and slow progression through all stages of HCV infection[11,14]. Direct evidence of the therapeutic potential of estrogen has come from experimental studies demonstrating that administration of 17β-estradiol inhibits HCC development in *in vitro* and *in vivo* animal models[15,16]. However, clinical trials evaluating hormonal therapy using selective estrogen receptor modulators (SERMs) have shown inconsistent outcomes in HCC patients[17-19]. These inconsistencies may be attributed to the differences in the expression of estrogen receptors (ERs) in the liver[20].

Liver is a sexually dimorphic organ and expresses the ERs, ERα and ERβ, making it responsive to the

actions of estrogen[21,22]. These receptors classically function as transcription factors, shuttling between the cytoplasm and nucleus for regulating the expression of various genes involved in cell cycle, proliferation, apoptosis and inflammation[23]. More recently, the non-classical role of ERs in cell signaling has become increasingly evident[24,25]. The two subtypes, ERα and ERβ share significant structural homology and ligand binding properties and yet function very differently, often antagonizing each other's actions[26]. Thus, the relative expression of the two subtypes can have a significant impact on net cellular responses to estrogen.

In breast cancer, ERα:ERβ expression ratio is thought to play a key role in estrogen-dependent tumor development[27,28]. Like breast cancer, aberrant increase in ER gene expression has been reported in liver tumors when compared to normal or non-tumor parts of the liver in HCC patients[29,30]. However, there is very limited information available on the relative expression of ERα and ERβ subtypes and ERα:ERβ expression ratio in livers of normals, HCV-related cirrhosis and HCV-related HCC.

We hypothesized that basal liver ERα and ERβ expression differs in males *vs* females and this differential expression dictates host susceptibility to chronic HCV or its progression to hepatocellular carcinoma. In the present study, we evaluated the expression of ERα and ERβ in the livers of normals, HCV-cirrhosis and HCV-related HCC at the mRNA and protein levels using different techniques. We further determined the correlation of ER subtype expression with the levels of inflammatory and oncogenic markers like NF-κB and cyclin D1. Our findings show that the basal expression of ER subtypes is different between normal males and females. Furthermore, sub-cellular expression of both ER subtypes is altered in HCV cirrhosis and HCV-related HCC livers as compared to normals and correlates with the expression of inflammatory and oncogenic markers. This altered expression of ER subtypes in the liver may contribute to the progression of cirrhosis and cancer development during HCV-pathogenesis.

MATERIALS AND METHODS

Patients

Explant liver tissues from normal donors and HCV-related cirrhosis and HCV-related HCC patients (Tables 1-3) were obtained from the NIH Liver Tissue and Cell Distribution System (LTCDS) at the University of Minnesota. Liver explants were aseptically collected under the institutional review board (IRB) guidelines of University of Minnesota. The study was conducted at Oklahoma State University-Center for Health Sciences under IRB guidelines. In order to avoid interference of other co-factors, patients co-infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV) or with a history of alcohol consumption or drug use were excluded from the study. Liver explants were snap frozen in liquid nitrogen and stored at -80 ℃ until further use.

Western blot analysis

Whole tissue lysates were prepared by homogenizing approximately 200 to 300 mg of liver tissues in liquid nitrogen followed by lysis in buffer comprising of 50 mmol/L Tris-HCl (pH 8.0), 400 mmol/L KCl, 10 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin and 1 μg/mL leupeptin. The homogenates were centrifuged at 30000 × *g* for 1 h at 4 ℃. The supernatants were collected and stored at -80 ℃ until further use. Cytoplasmic and nuclear fractions were prepared from liver tissues using the NE-PER kit (Pierce Chemical Co., Rockland, IL, United States) as per the manufacturer's instructions. Protein concentrations were determined by Bicinchoninic acid assay (Pierce Chemical Co., Rockland, IL, United States). Thirty micrograms of protein from each lysate (whole tissue lysate, cytoplasmic or nuclear tissue lysates) was resolved on 4%-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, United States). The separated proteins were electrophoretically transblotted on to a 0.2 μm nitrocellulose membrane. Purified recombinant ERα and ERβ proteins were included as positive controls. The membrane was blocked in 5% non-fat dry milk made in TBS (20 mmol/L Tris-HCl, pH 7.6 and 136 mmol/L NaCl) containing 0.1% Tween-20 for 2 h and probed with antibodies specific for human ERα (rat monoclonal against human ERα, H222, Fitzgerald Industries International Inc., Concord, MA, United States), human ERβ (mouse monoclonal against amino acids 1-153 of human ERβ, 14C8, Affinity Bioreagents, Golden, CO, United States), phosphorylated IκB-kinase (IKK) αβ (rabbit polyclonal against a phosphopeptide corresponding to amino acids surrounding Ser176/180 of human IKKα, Cell signaling Technology, Danvers, MA, United States), IKKα (rabbit polyclonal against a peptide corresponding to 20 amino-terminal amino acids of human IKKα, Cell signaling Technology, Danvers, MA, United States), Cyclin D1 (rabbit polyclonal against amino acids 1-295 of human cyclin D1, H-295, Santa Cruz Biotechnology, Dallas, TX, United States), phosphorylated NF-κB (mouse monoclonal against a phosphopeptide corresponding to amino acids surrounding Ser536 of human NF-κB p65 subunit, Cell signaling Technology, Danvers, MA, United States), NF-κB (rabbit monoclonal against a peptide corresponding to amino acids near the amino-terminus of human NF-κB p65 subunit, Cell signaling Technology, Danvers, MA, United States) and β-actin (rabbit polyclonal against a peptide corresponding to amino acid 20-33 of actin, Sigma-Aldrich, St. Louis, MO, United States). After incubation with alkaline phosphatase conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, United States), the protein

bands were detected by enhanced chemiluminescence (Pierce Chemical Co., Rockland, IL, United States). To ensure the purity of cytoplasmic and nuclear fractions, the blots were also probed with anti-GAPDH (rabbit monoclonal against a peptide corresponding to a part of the carboxyl terminus of human GAPDH, Cell signaling Technology, Danvers, MA, United States) and anti-histone H1 (mouse monoclonal against nuclei from biopsies of myeloid leukemia cells, EMD Millipore, Billerica, MA, United States) antibodies respectively. Images of the protein bands were digitally captured with Alpha Innotech instrumentation (Alpha Innotech Corp, San Leandro, CA, United States) and quantified with Image analysis program, ImageJ (NIH, Bethesda, MD, United States). The phosphorylated forms were expressed as a ratio of the phosphorylated protein to total protein.

Immunohistochemistry

Immunohistochemistry was performed on normal and diseased explant liver tissue sections to confirm the protein expression of ERα, ERβ and cyclin D1. Formalin-fixed liver tissues were embedded in paraffin and sectioned into 4-μm thick sections. Non-enzymatic antigen retrieval was performed in citrate buffer (pH 6.0) using a microwave procedure. Slides were allowed to cool down for 20 min at room temperature and endogenous peroxidase was quenched with Dako EnVision system kit according to manufacturer's instructions (Dako, Carpintaria, CA, United States). The sections were washed and incubated for 30 min with 0.5% Triton X-100 in PBS followed by 5% rabbit serum. The tissue sections were incubated overnight with primary antibodies against ERα (rabbit polyclonal against a peptide corresponding to the carboxyl terminus of mouse ERα, MC-20, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, United States), or ERβ (mouse monoclonal against peptide corresponding to the carboxyl terminus of ERβ, PPG5/10, 1:20 dilution, Dako, Carpinteria, CA, United States) at 4℃. Slides were washed with PBS containing 0.05% Tween and treated for one hour at room temperature with corresponding HRP labeled secondary antibodies (Dako, Carpinteria, CA, United States). The tissue sections were stained with 3,3'-diaminobenzidine and the nuclei were counterstained with Mayer's hematoxylin. A similar immunostaining procedure was used for cyclin D1 (rabbit polyclonal antibody against amino acids 1-295 of human cyclin D1, H-295, 1:150 dilution, Santa Cruz Biotechnology, Dallas, TX, United States). For negative controls, sections were incubated with PBS instead of the primary antibody. The stained sections were visualized using Nikon microscope (Nikon H600 L) and sections with staining of ⩾ 10% of the cells were defined as positive.

Real-time RT-PCR

Total RNA was isolated from liver tissues with Trizol (Thermo Fisher Scientific, Waltham, MA, United States) followed by purification on RNeasy columns (Qiagen, Valencia, CA, United States) as per the manufacturer's instructions. Total RNA from each sample was treated with DNase (Thermo Fisher Scientific, Waltham, MA, United States) and then reverse-transcribed to synthesize cDNA using the High capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, United States). The expression of target genes was evaluated by quantitative real-time PCR using commercially available ERα and ERβ specific Taqman primers and probe assay mixes (Applied Biosystems, Foster City, CA, United States) on an ABI StepOne Real-Time PCR System (Applied Biosystems Foster City, CA, United States). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was included as the endogenous control. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method using the normal group for normalization[31].

Statistical analysis

The statistical methods of this study were performed and reviewed by Dr. Mark E. Payton from Department of Statistics, Oklahoma State University, Stillwater, United States. Data obtained from Western blotting and real time RT-PCR experiments were analyzed statistically using SAS software v9.4 (SAS Institute Inc., Cary, NC, United States). Statistical significance between appropriate groups was determined by non-parametric analyses that included the Kruskall-Wallis test and Mann Whitney *U* test. Post-hoc analyses were performed using Fisher's Protected Least Significant Difference comparisons using rank data test. Correlations of ER subtypes with other proteins were analyzed by using the Spearman's rank correlation test. $P \leqslant 0.05$ was considered statistically significant. All graphs were made and depicted using the GraphPad Prism v6 software (GraphPad Software Inc, La Jolla, CA, United States).

RESULTS

Basal expression of ERα is significantly higher in liver of normal males than females

Due to the well-established gender disparity in HCC prevalence, we initiated this study by investigating the expression of ERα and ERβ in normal males and females. Whole tissue lysates of liver explants obtained from normal females and normal males (Table 1) were evaluated by Western blotting using validated ERα and ERβ directed antibodies (Figure 1A). Densitometric analysis of protein bands showed comparable ER subtype expression in normal male and female tissues when the gender data was pooled (Figure 1B). However, when ER subtype expression data was segregated based on gender, we found that there was a significantly higher expression of ERα in males as

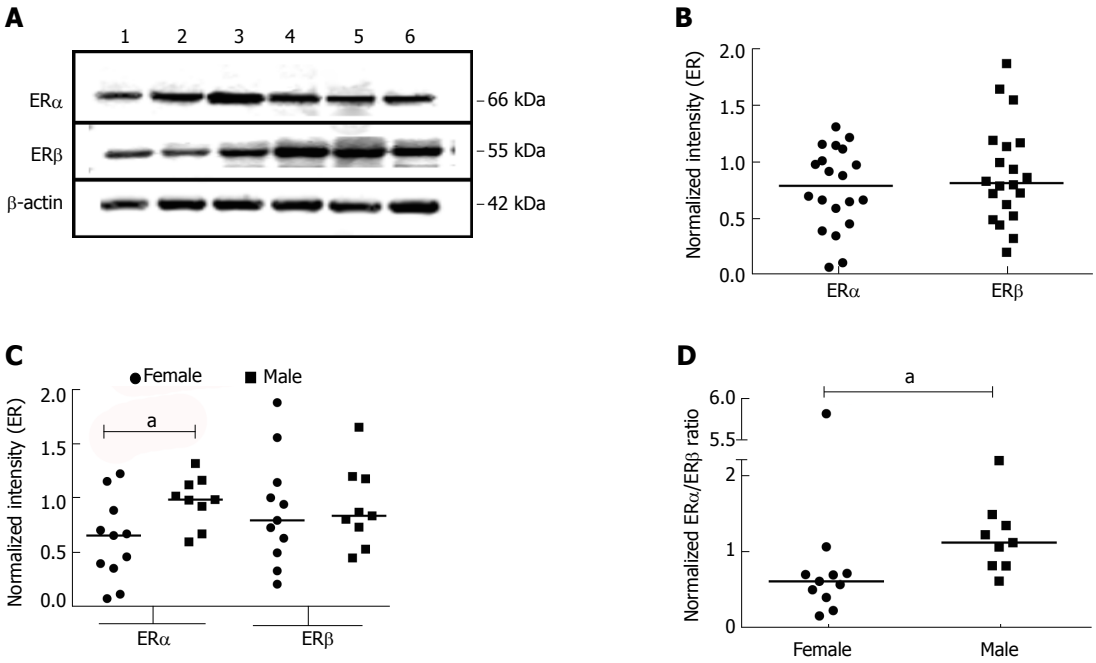


Figure 1 Expression of estrogen receptor subtypes in whole tissue lysates from normal male and female subjects. A: Whole tissue lysates from liver tissues of normal donors were subjected to Western blotting and probed with antibodies against ERα, ERβ and β-actin. A representative blot is shown. B-D: The bands corresponding to ERα, ERβ and β-actin were quantified by densitometric analyses using ImageJ. Each symbol represents one individual. Expression of the ER subtypes was normalized to the expression of β-actin and plotted (B). Gender-based ER subtype expression was evaluated by segregating the gender and plotted (C). The ERα:ERβ expression ratio was also plotted for each gender group (D). $^{a}P < 0.05$ was considered significant. ER: Estrogen receptor.

Table 1 Age ranges of normal and diseased subjects in whole tissue lysates

Group	No. of subjects	No. of females	Age range (yr)	No. of males	Age range (yr)
Normal	20	11	42-67	9	34-64
HCV	14	4	53-60	10	43-55
HCC	14	1	56	13	44-66

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 2 Age ranges of normal and diseased subjects in mRNA analysis

Group	No. of subjects	No. of females	Age range (yrs)	No. of males	Age range (yrs)
Normal	9	8	42-67	1	64
HCV	10	3	43-60	7	43-53
HCC	10	0	N/A	10	44-59

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 3 Age ranges of normal and diseased subjects in nuclear and cytoplasmic tissue lysates

Group	No. of subjects	No. of females	Age range (yr)	No. of males	Age range (yr)
Normal	10	5	43-67	5	50-64
HCV	12	3	51-60	9	46-53
HCC	16	0	NA	16	44-66

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

compared to females (Figure 1C). No difference was found in the expression of ERβ between males and females. Since physiological outcomes are determined by the expression of both, ERα and ERβ, we compared the ERα:ERβ expression ratio between male and females and found that males showed a significantly higher ERα:ERβ expression ratio compared to females (Figure 1D).

Altered expression of ERα is observed in males with HCV-related cirrhosis and HCV-related HCC

The expression of ER subtypes was compared in liver explants of normals, HCV-related cirrhosis and HCV-related HCC (Tables 1 and 2) by real time RT-PCR and Western blotting (Figure 2). At the transcriptional level, there was an increase in the liver mRNA expression of ERα (ESR1) and ERβ (ESR2) subtypes in chronic HCV and HCV-related HCC as compared to normals (Figure 2A and B). This change in expression significantly decreased the ER subtype ratio in the HCV-related cirrhosis group compared to normal (Figure 2C), suggesting that HCV infection may result in altering the expression of ER subtypes.

When we measured ER subtype protein levels by Western blotting, we could not detect any significant differences among the three groups when the data was evaluated without gender segregation (Figure 2 D-G). However, upon separating males and females,

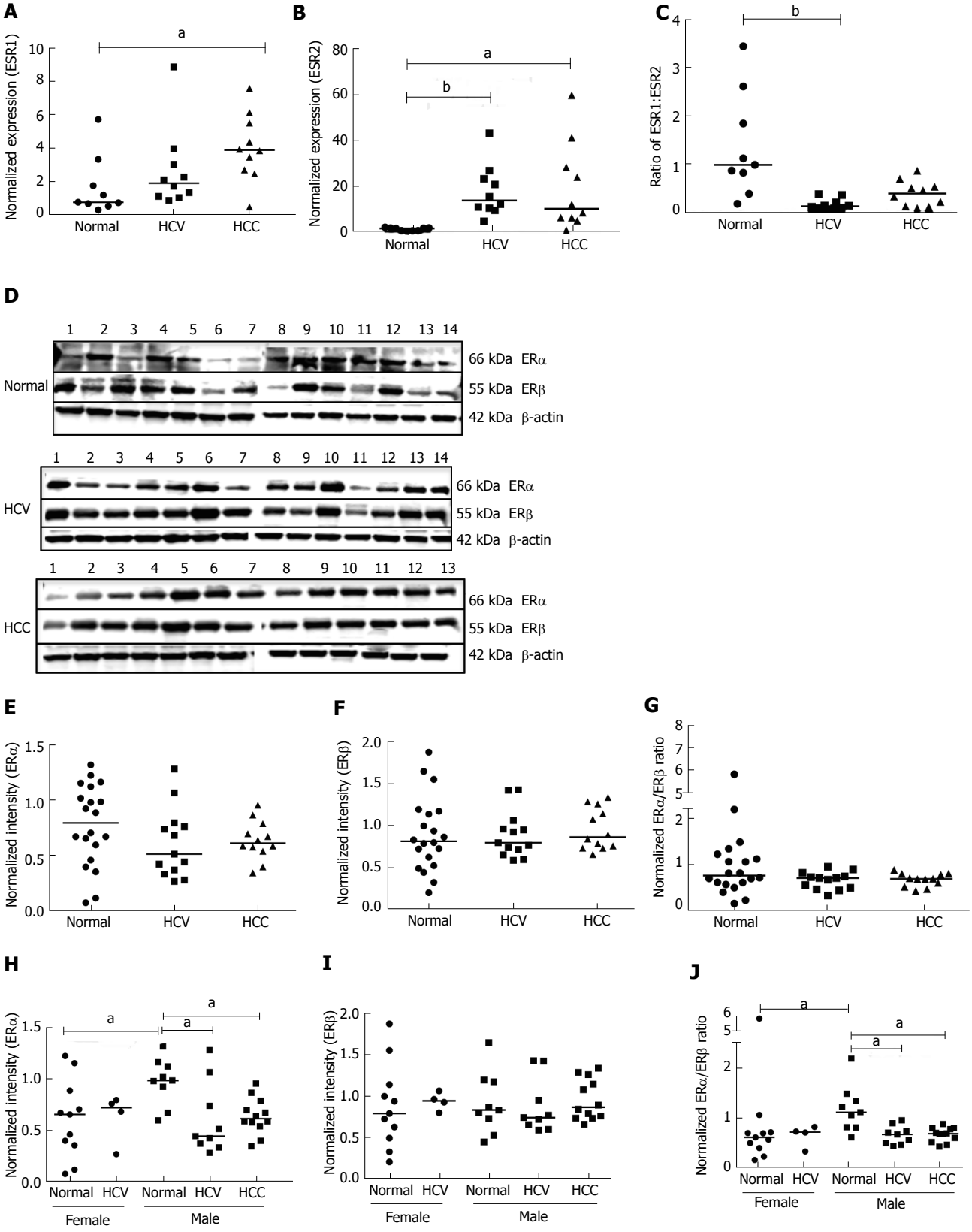


Figure 2 Expression of estrogen receptor subtypes from normal and diseased subjects. A-C: Total RNA extracted from normal, HCV-related cirrhosis and HCV-related HCC livers was subjected to RT-PCR. The abundance of ESR1 (A) and ESR2 (B) transcripts was determined by real time PCR and normalized to the expression of HPRT. ESR1:ESR2 expression ratio was also plotted for the three groups (C). [a]$P < 0.05$ or [b]$P < 0.001$ was considered significant. D: Whole tissue lysates from liver tissues of normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against ERα, ERβ and β-actin. Representative blots for each group are depicted. E-J: The bands corresponding to ERα, ERβ and β-actin were quantified by densitometric analyses using ImageJ. Expression of the ERα and ERβ was normalized to the expression of β-actin and plotted (E and F). The ERα:ERβ expression ratio was also plotted for normal, HCV and HCC group (G). Expression of ER subtypes and ERα:ERβ expression ratio in the male population of normal, HCV and HCC groups was plotted separately (H-J). [a]$P < 0.05$ was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

Table 4 Spearman correlations of ERα and ERβ expression in whole tissue lysates with nuclear cyclin D1, nuclear phosphorylated NF-κB and cytoplasmic phosphorylated IKK expression

	R-coefficient (*P* value)		
	Normal	HCV	HCC
Correlations with WTL ERα			
ERβ	0.4154 (0.0692)	0.81944 (0.0006)[b]	0.70339 (0.0107)[a]
Nuclear cyclin D1	0.62189 (0.0549)	0.88842 (0.0075)[a]	-0.29785 (0.3737)
Nuclear pNF-κB	0.02757 (0.9397)	0.84435 (0.0169)[a]	0.19351 (0.5686)
Cytoplasmic pIKK	-0.21577 (0.5494)	-0.09787 (0.8346)	-0.37679 (0.2534)
Correlations with WTL ERβ			
Nuclear cyclin D1	-0.03251 (0.9290)	0.92648 (0.0027)[a]	-0.70135 (0.0162)[a]
Nuclear pNF-κB	-0.18056 (0.6176)	0.71732 (0.0696)	0.41196 (0.2080)
Cytoplasmic pIKK	0.24608 (0.4931)	-0.21801 (0.6386)	-0.03438 (0.9201)

[a]$P < 0.05$, [b]$P < 0.001$. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor; WTL: Whole tissue lysates.

we observed a significant decrease in the expression of ERα in diseased livers (HCV as well as HCC) compared to normal livers (Figure 2H) but no difference in the expression of ERβ (Figure 2I) in males. This resulted in a significant decrease in ERα:ERβ expression ratio in male diseased livers (HCV as well as HCC) compared to normal male livers (Figure 2J). Among the females, we could compare the expression of ER subtypes between normals and HCV-related cirrhosis only as there were no females in the HCV-related HCC category. We found that there were no significant differences in protein levels of ERα and ERβ between normal livers and HCV-related cirrhotic livers in females (Figure 2H and I). We further observed a positive-correlation between the expression of ERα and ERβ in HCV-related cirrhosis and HCV-related HCC group based on gender-pooled data (Table 4).

Thus the results from the mRNA and protein analyses performed in whole tissue extracts showed different trends. Since classical ERs shuttle between the nuclear and cytoplasmic compartments of the cells and mediate their regulatory functions by binding to estrogen response elements on DNA, we evaluated the expression of ER subtypes in nuclear and cytoplasmic extracts of liver tissues.

Increased nuclear translocation of ER subtypes is observed in HCV-related cirrhosis and HCV-related HCC

The distribution and expression of ERα and ERβ in nuclear and cytoplasmic extracts of normal, HCV-related cirrhosis and HCV-related HCC group is depicted in Figure 3. We observed a higher expression

Table 5 Spearman correlations of ERα and ERβ expression in nuclear tissue lysates with nuclear cyclin D1 and nuclear phosphorylated NF-κB expression

	r-coefficient (*P* value)		
	Normal	HCV	HCC
Correlations with nuclear ERα			
Nuclear ERβ	0.63880 (0.0468)[a]	0.63539 (0.0264)[a]	0.73192 (0.0008)[b]
Nuclear cyclin D1	0.12049 (0.7402)	0.75728 (0.0043)[a]	0.55714 (0.0202)[a]
Nuclear pNF-κB	-0.62968(0.0511)[c]	0.207 (0.5186)	0.4793 (0.0516)[c]
Correlations with nuclear ERβ			
Nuclear cyclin D1	0.44012 (0.2031)	0.64445 (0.0237)[a]	0.51223 (0.0355)[a]
Nuclear pNF-κB	-0.52091 (0.1226)	0.10243 (0.7514)	0.25861 (0.3162)

[a]$P < 0.05$, [b]$P < 0.001$, [c]$P < 0.055$. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

Table 6 Spearman correlations of ERα and ERβ expression in cytoplasmic tissue lysates with cytoplasmic pIKK

	r-coefficient (*P* value)		
	Normal	HCV	HCC
Correlations with cytoplasmic ERα			
Cytoplasmic ERβ	0.61233 (0.0599)	0.74376 (< 0.0001)[a]	0.79765 (0.0158)[b]
Cytoplasmic pIKK	-0.66837 (0.0346)[a]	-0.00698 (0.9828)	-0.56014 (0.0194)[a]
Correlations with cytoplasmic ERβ			
Cytoplasmic ERα	0.61233 (0.0599)	0.91363 (< 0.0001)[b]	0.79765 (0.0001)[b]
Cytoplasmic pIKK	-0.60909 (0.0616)	-0.29717 (0.3482)	-0.60848 (0.0095)[a]

[a]$P < 0.05$, [b]$P < 0.001$. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

of ERα in the nuclear extracts of normal males as compared to normal females but the increase was not statistically significant, probably due to the small sample size (Supplementary Figure 1). There was no change in the expression of nuclear ERβ. These observations in normals for the expression of nuclear ERα and ERβ were similar to the results observed in whole tissue extracts.

When we compared the expression of ER subtypes in nuclear and cytoplasmic extracts of normal and diseased livers, we could not perform statistically relevant gender analyses due to the small sample size and scarcity of female subjects in the diseased groups. Hence we have reported our results without gender segregation. We observed a significant increase in the expression of nuclear ERα and ERβ in HCV-related HCC and HCV-related cirrhosis (Figure 3A, B and D) respectively when compared to normals. However, there was no significant difference in the expression of cytoplasmic ERα, while HCV-related HCC group showed a significantly higher expression of cytoplasmic ERβ compared to HCV-related cirrhosis group (Figure 3C and E). An increase in the nuclear:

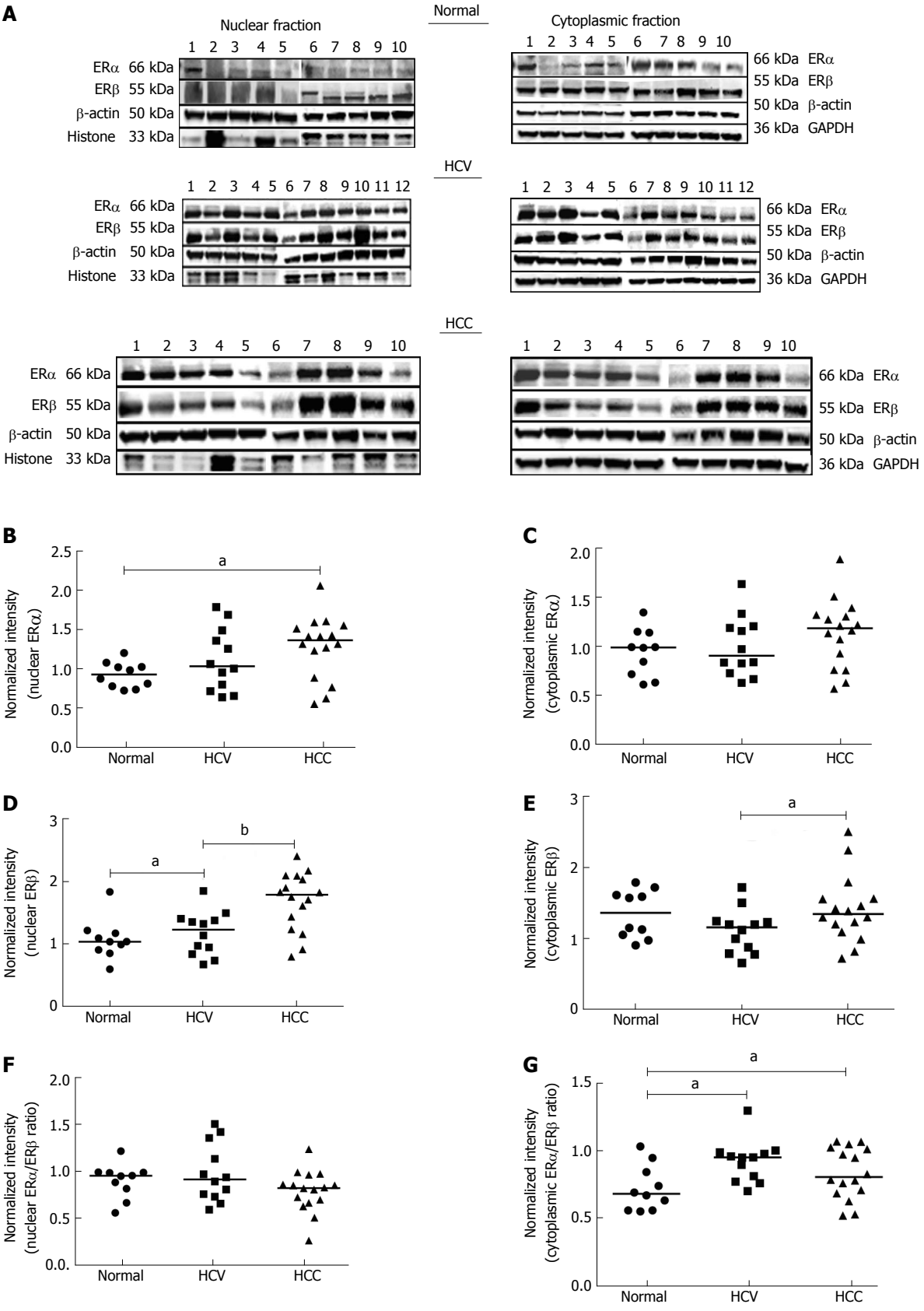


Figure 3 Expression of estrogen receptor subtypes in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against ERα, ERβ, β-actin, histone (nuclear lysates only) and GAPDH (cytoplasmic lysates only). Representative blots for each group are depicted. B-G: The bands corresponding to ERα, ERβ and β-actin were quantified by densitometric analyses using ImageJ. Expression of the ERα and ERβ was normalized to the expression of β-actin and plotted with (B and C). The ERα:ERβ expression ratio was also plotted for normal, HCV and HCC group (D). Expression of ER subtypes and ERα:ERβ expression ratio in the male population of normal, HCV and HCC groups was plotted separately (E-G). $^{a}P < 0.05$ or $^{b}P < 0.001$ was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

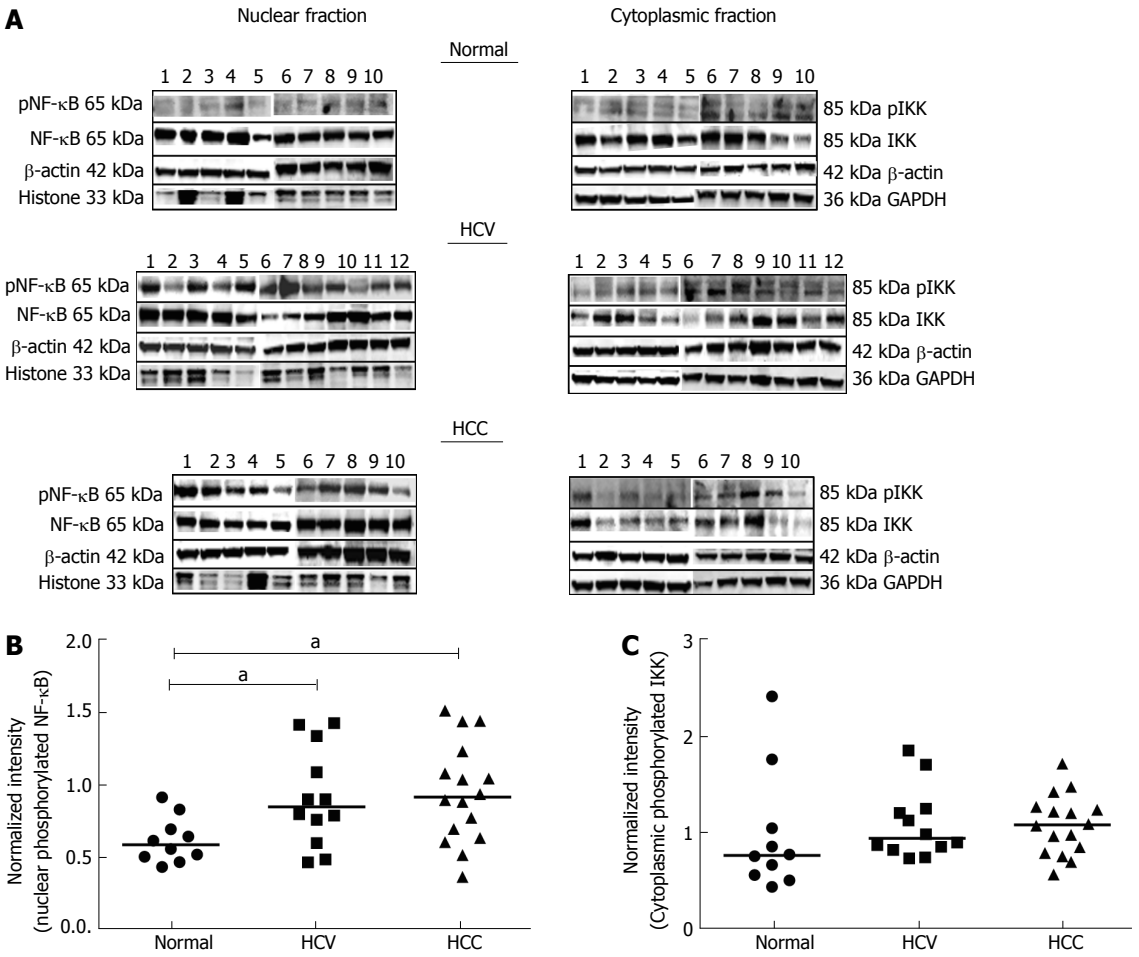


Figure 4 Expression of pNF-κB and pIKK in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting. Nuclear tissue lysates were probed with antibodies against pNF-κB, NF-κB, β-actin, and histones while cytoplasmic tissue lysates were probed with antibodies against pIKK, IKK, β-actin and GAPDH. Representative blots for each group are depicted. B and C: The bands corresponding to pNF-κB, NF-κB, pIKK, IKK and β-actin were quantified by densitometric analyses using ImageJ. The ratio of pNF-κB:NF-κB was normalized to the expression of β-actin and plotted (B). The ratio of pIKK:IKK was normalized to the expression of β-actin and plotted with gender pooled (C) subjects. $^{a}P < 0.05$ was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

cytoplasmic ratio of both, ERα and ERβ, in the diseased groups were observed compared to the normals (Supplementary Figure 1) suggesting that there is increased transcriptional activation of ER subtypes in the progression to HCV-related cirrhosis to HCV-related HCC. Furthermore, there was a significant increase in the ratio ERα:ERβ in the cytoplasmic compartment of the HCV-related cirrhosis and HCV-related HCC groups compared to normals (Figure 3G) but there was no significant difference in the ratio of the nuclear ER subtypes (Figure 3F).

HCV-related cirrhosis and HCV-related HCC group show higher activation of NF-κB

HCV infection is known to trigger inflammatory pathways and livers displaying chronic inflammation are more at risk of developing hepatocellular carcinoma[32,33]. Most inflammatory pathways involve NF-κB, hence we evaluated the activation of the NF-κB signaling pathway by detecting the phosphorylation of the p65 subunit and IKK; the kinase that phosphorylates IκB, resulting in its dissociation from NF-κB and degradation by the proteasome. Livers chronically infected with HCV and HCV-related HCC tissues showed significantly higher expression of phosphorylated NF-κB (Figure 4A and B) compared to normals. There was an increase in the expression of pIKK from normal livers to HCV cirrhosis and HCV-related HCC, but the increase was not statistically significant (Figure 4C). We also observed a weak negative correlation between nuclear ERα and pNF-κB in normal liver tissues (r = -0.62968, P = 0.0511; Table 5). On the contrary, HCV-related HCC group demonstrated a weak positive correlation between nuclear ERα and pNF-κB (r = 0.4793, P = 0.0516; Table 5). In addition, expression levels of pIKK and the cytoplasmic ER subtypes showed a strong negative correlation in the HCC group (ERα: r = -0.56014, P = 0.0194; ERβ: r = -0.60848, P = 0.0095; Table 6).

ERα levels correlate with expression of cyclin D1 in both HCV cirrhosis and HCV-related HCC

In order to study the correlation of ERs with oncogenic markers in diseased livers, we determined the

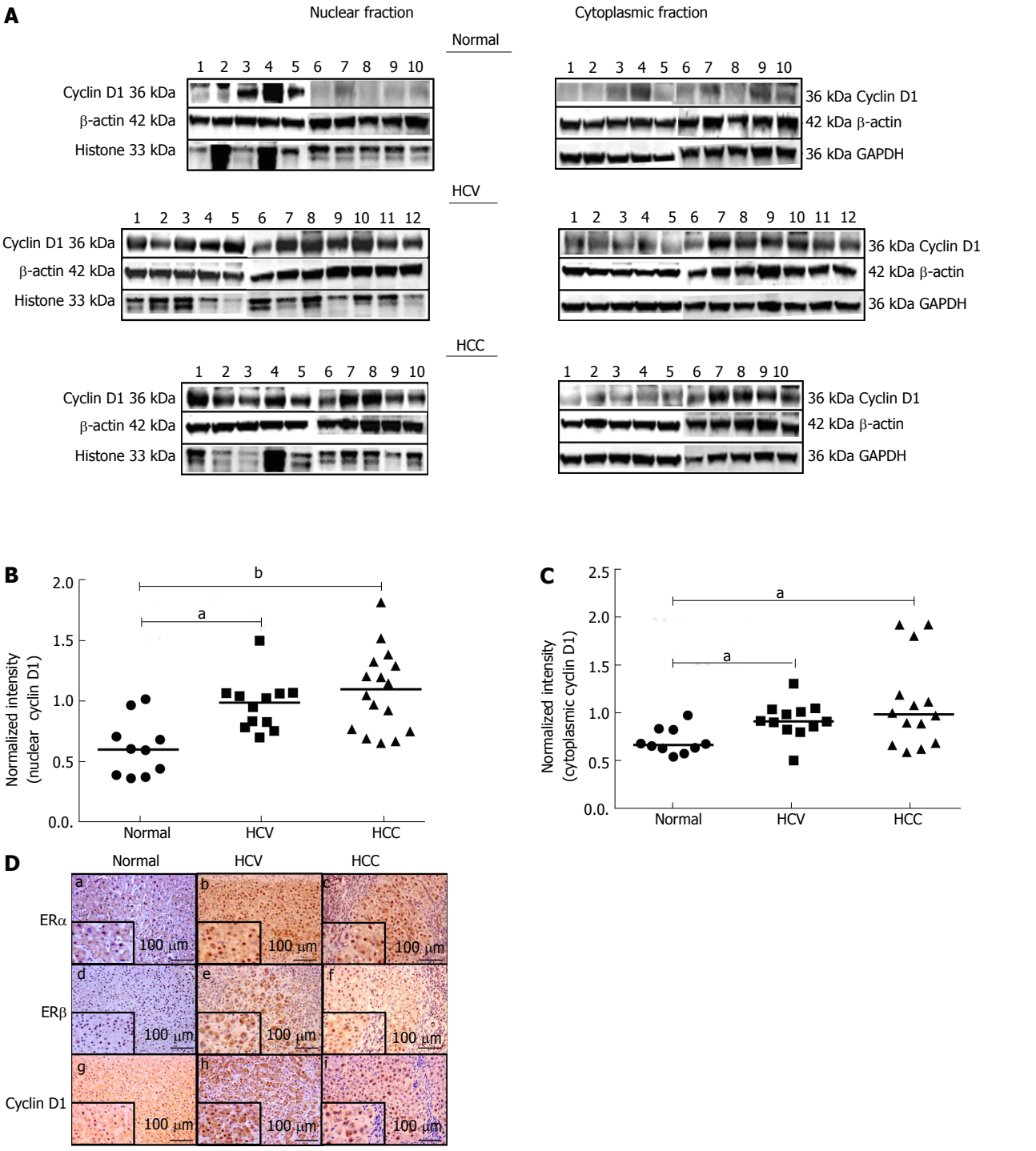


Figure 5 Expression of cyclin D1 in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against cyclin D1, β-actin, histones (nuclear lysates only) and GAPDH (cytoplasmic lysates only). Representative blots for each group are depicted. B and C: The bands corresponding to cyclin D1 and β-actin were quantified by densitometric analyses using ImageJ. Expression of cyclin D1 was normalized to the expression of β-actin in nuclear (B) and cytoplasmic (C) lysates and plotted. $^{a}P < 0.05$ or $^{b}P < 0.001$ was considered significant. D: Immunohistochemical staining of ERα, ERβ and cyclin D1 in normal (a, d, g), HCV-related cirrhosis (b, e, h) and HCV-related HCC (c, f, i) is depicted. A magnified region of the stained tissue section is depicted as an inset in each corresponding image. Scale bar = 100 μm. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

expression of cyclin D1 in the nuclear and cytoplasmic liver tissue extracts of normals, HCV-related cirrhosis and HCV-related HCC group by Western blotting (Table 3). As shown in Figure 5, there was a significant increase in the expression of cyclin D1 in both, the nuclear and cytoplasmic fractions of diseased livers, suggesting that chronic HCV infection primes hepatocytes to proliferate. A positive-correlation between the expression of nuclear ERα and ERβ with nuclear cyclin D1 was observed in both HCV-related cirrhosis (ERα: $r = 0.75728$, $P = 0.0043$; ERβ: $r = 0.64445$, $P = 0.0237$; Table 5) and HCV-related HCC

(ERα: $r = 0.55714$, $P = 0.0202$; ERβ: $r = 0.51223$, $P = 0.0355$; Table 5) groups but not in normals, signifying the importance of ER subtypes in cyclin D1 activation (Table 5). We further determined the expression of ER subtypes and cyclin D1 by immunohistochemistry (IHC). As observed with Western blotting, IHC showed over-expression of ERα and cyclin D1 in HCV-related cirrhosis and HCV-related HCC groups (Figure 5D, Supplementary Tables 1-3).

DISCUSSION

Various epidemiological studies around the globe have recognized the role of gender bias in the progression of HCV infection to chronic liver disease and cirrhosis due to poor therapeutic responses or further development of HCV-related HCC in these patients[34]. Remarkable differences have been observed in the incidence, progression of HCV-related chronic liver disease and outcome of interferon therapy in males, postmenopausal females and premenopausal females[13,35]. This has generated a significant interest in the scientific community to investigate the role of estrogen as the key host factor in HCV-related pathogenesis. Recent studies in postmenopausal women with HCV-related fibrosis clearly show clinical benefits from estrogen replacement therapy in conjunction with standard HCV treatment[12]. One of the most compelling evidence is the inhibition of HCV life cycle by 17β-estradiol in an ERα dependent manner in human liver cell lines[36,37]. Raloxifene, a SERM, treatment has been shown to improve the efficacy of antiviral treatment in HCV-infected postmenopausal women[38]. These findings suggest a protective role of estrogen affecting not only viral replication but also HCV pathogenesis in the host.

Despite the recognition of estrogen as a potentially significant host factor in HCV pathogenesis, little progress has been made to understand the underlying mechanisms on how ER subtypes may be involved in progression to HCV chronic disease in some patients and rapid clearance of HCV in others. Perhaps this gap in understanding is one of the major factors contributing to the current debatable potential of estrogen therapy[17,19]. In addition, most of the clinical studies did not monitor ERα and ERβ expression in the liver of the enrolled patients or the hormonal status of females at the time of recruitment or during treatment[20]. Some studies evaluated ER subtype expression by measuring mRNA levels in the liver or performing less specific ligand binding assays[39, 40].

One of the most important premises of the current study was to evaluate the gender-based (males *vs* females) ER subtype protein expression in the liver of normals (basal expression) and further evaluate the changes in expression of these subtypes in HCV-related cirrhosis or HCV-related HCC patients. Further, we employed subcellular fractionation (nuclear verses cytoplasmic) protein studies to examine the relative expression of ER subtypes and correlate their presence with activation of NF-κB or cyclin D1 in these fractions. Our findings in whole tissue extracts of normal livers indicated that males express significantly higher levels of ERα protein compared to females but comparable levels of ERβ. We also found a significant increase in the ERα:ERβ expression ratio in males as compared to females. To our knowledge, this is the first report demonstrating gender-based differences in ERα at the basal level thereby affecting the ratio of ER subtypes in the liver. Whether these differences in ER subtype expression at basal levels predispose males to severe and fast disease progression of HCV infection needs further investigation.

We were able to detect ERα and ERβ transcripts in normal, HCV-related cirrhosis and HCV-related HCC groups. Similar observations were made in a Korean population study where they observed ERα and ERβ mRNA transcripts in more than 95% of HCV-related HCC liver tissues[40]. There was an increase of ERα and ERβ transcripts in diseased livers compared to normals but there were no changes in the protein levels of ERs among the groups in whole tissue lysates. However, gender analyses of ER expression in whole tissue lysates revealed that there was a significant decrease in ERα but not ERβ expression only in diseased male patients suggesting its disease specific role in these patients. These findings are in agreement with Villa *et al* who showed that there was reduction or even loss of wild-type ERα in male HCV-related HCC livers but an increase in the expression of variant ERα, which we have not evaluated[41,42].

Further, ER expression in the nuclear and cytoplasmic fractions from gender-pooled diseased livers differed when compared to normals. In the nuclear fraction, we found a higher expression of ERα and ERβ subtypes in HCV-mediated HCC and HCV-mediated cirrhosis tissues respectively compared to normal liver tissues, which is in contrast to the findings in the whole tissue extracts. HCV-mediated HCC tissues also showed significantly higher expression of cytoplasmic ERβ only when compared to HCV-cirrhosis tissues. We found a significant increase in the ERα to ERβ ratio in the cytoplasmic fraction than in the nuclear fraction of both HCV-related cirrhosis and HCV-related HCC livers as compared to normal livers. The clinical relevance of these changes in ER subtype expression in the subcellular compartments needs to be further investigated. ER protein levels have been shown to vary under physiological states during breast tumor progression and hormonal therapy[43]. A recent study in the MCF7 cell-line has shown that estrogen stimulation involves dynamic changes in protein subcellular spatial distribution rather than changes in total protein abundance and propose that cells may be transformed by perturbations in nucleocytoplasmic shuttling of nuclear hormone receptors and other proteins[44,45]. In addition to breast cancer, ERs have also been implicated to play important roles

in gastrointestinal cancers like gastric, colon and colorectal cancers[46-48]. Since ERs primarily function as nuclear receptors and act as transcription factors for gene regulation, we propose that evaluating the expression of these proteins in cellular fractions as opposed to the whole tissue lysates would be a more accurate representation of their biological activity. Furthermore, our immunohistochemistry analysis also supported the increase in nuclear and cytoplasmic expression of ERα in both groups of diseased livers compared to normal livers (Supplementary Table 1). The expression of cytoplasmic ERβ was comparable among all the three groups by immunostaining. However, we observed a loss of nuclear ERβ in some HCC tissues (Supplementary Table 2). Loss of ERβ was also reported by Iavarone *et al*[39], who evaluated the expression of ER subtype mRNA in liver cancer tissues. Additionally, in breast, colon and prostate cancers, there are reports that indicate that presence of ERβ leads to better prognosis[49-52]. These findings about differential expression of nuclear or cytoplasmic ERβ expression in HCV cirrhosis or HCC can be substantiated only by increasing the sample size in the study.

Inflammation has been associated with the initiation and progression of hepatocarcinogenesis[53]. Since HCV infection triggers inflammation, and chronic inflammation promotes carcinogenesis, NF-κB has been viewed as an important player in inflammation-induced cancer[54,55]. In the current study, we found a significant increase in activated NF-κB in HCV-related cirrhosis and in HCV-related HCC liver tissues. Significant increase in ERs in the cytoplasmic fraction of diseased livers may modulate the activity of pIKK thereby influencing the activation of NF-κB and inflammation outcome. ERα and NF-κB have been shown to directly interact in the nuclei of U2-OS cells treated with 17β-estradiol and aberrant ERα and NF-κB expression correlated with invasion and metastasis in HCC[56,57]. 17β-estradiol, *via* ERα, can also inhibit the transport of p65 to the nucleus and downregulate inflammation[58]. Detailed mechanistic studies are warranted to explore the cross talk between ERα and NF-κB to unfold the nature of their interaction in a HCV infection setting.

Unlike mammary carcinoma, the target genes and mechanism for ER regulation in liver are not well studied. There is a correlation between dysregulation in ER subtype with the overexpression of cyclin D1[59]. In our study, we observed increased expression of cyclin D1 in HCV-related cirrhosis and HCV-related HCC compared to normal livers and the expression of nuclear cyclin D1 correlated with the expression of nuclear ER subtypes in diseased livers. These findings along with other studies suggest that ERs may play a role in dysregulation of cellular proliferation by participating in increased transcription of cyclin D1 that aids in the progression of HCV infection to cirrhosis and eventually to hepatocellular carcinoma[60]. Cyclin D1 expression can also be regulated by ERα *via* non-genomic signaling. Treatment of HepG2 cells with 17β-estradiol activates the MAPK/ERK pathway by membrane-localized ERs resulting in increased expression of the cyclin D1 gene[61]. Apart from its role in cell cycle progression, cyclin D1 has recently been recognized to play a role in influencing sex steroid metabolism by increasing estrogen levels and decreasing androgen levels[62]. This is the first study that correlates the expression of ER subtypes with cyclin D1 in HCV-related cirrhosis and HCV-related HCC.

Increased expression of different isoforms or variants of ERα in diseased livers has been reported by mRNA studies and their involvement to activate oncogenic signaling pathways in cell lines has been reported[63,64]. The variants, ERα36 and ERα46 have been shown to modulate signaling pathways linked to proliferation and apoptosis[65, 66]. Differential expression of ERα36 has been observed in various cancers like breast, gastric, and endometrial cancer[67] and its expression correlates with a worse prognosis in breast cancer[68] but its role in HCV-mediated HCC has not been thoroughly investigated. Various ERβ variants have also been detected in different tissues but their role in carcinogenesis has not been evaluated as extensively as the ERα variants[69]. The present study focused to fill the gap in knowledge for the expression of wild type ERα and ERβ in normal and diseased livers. However, the expression of variant ER receptors also needs to be investigated to understand their role, if any, in HCV-related carcinogenesis. In addition to ER subtypes, the expression of androgen receptors should also be evaluated as HCV infection enhances androgen receptor-mediated signaling in an *in vitro* model[70] but it is not clear if androgen receptors play a role in carcinogenesis due to existing conflicting reports[71,72].

Finally, in the current study, we have demonstrated for the first time that the basal expression of wild-type ERα in the liver is higher in males than females. Furthermore, the increased sub-cellular expression of ER subtypes observed in diseased livers correlates with the expression of inflammatory and oncogenic markers. This altered expression of ER subtypes in the liver may contribute to the progression of cirrhosis and cancer development during HCV-pathogenesis. These observations have been made in end stage HCV-related cirrhosis and HCC. In future, early disease or stage specific prospective longitudinal studies that include a larger sample size (in both males and females category) can be conducted to further clarify the role of nuclear or cytoplasmic ER expression at early stages of HCV-related cirrhosis or HCV-related HCC. Impact of gender associated ER subtype ratio distribution in the liver can also be studied relative to oncogenic markers and that may help us to monitor host therapeutic responses, viral clearance or improved prognosis during the carcinogenesis process. In conclusion, we expect that by evaluating the expression of sex

hormone receptors and understanding the signaling pathways they affect, gender and stage-related personalized therapies can be designed to treat and manage chronic HCV infections and HCV-related HCC.

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COMMENTS

Background

Hepatitis C virus (HCV) infection-related fibrosis and cirrhosis lead to the development of chronic liver disease that can ultimately result in hepatocellular carcinoma (HCC). According to epidemiological and clinical studies, chronic HCV infection mediated cirrhosis is more prevalent in males and progresses to cancer development faster as compared to premenopausal females who show better therapeutic responses and clear the infection. However, females with menopausal estrogen deficiency may have reactivation of the HCV infection resulting in accelerated progression to fibrosis and HCC development, confirming estrogen related etiology. Previous hormonal therapies in HCC have been inconclusive perhaps due to them poor understanding of estrogen receptor (ER) subtype expression in the normal livers from both males and females and also how changes in the expression of ERs may contribute to HCV-related disease progression. In the current study, they evaluated the basal liver ERα and ERβ expression in males versus females and have studied the changes in liver ER subtype expression and their correlation with inflammatory and proliferative markers in HCV-related cirrhosis and HCV-related HCC

Research frontiers

Gender disparity in immunity to chronic infectious disease, autoimmunity and cancer development is increasingly being recognized in various clinical and experimental studies. Systemic estrogens in females play an important role in host innate immunity, however, the contributions of estrogen receptors are poorly recognized. In addition to hormonal cancers like breast, prostate and uterus, ER subtypes are being increasingly implicated in non-hormonal tissue cancers as well that include liver, colon, pancreas and stomach.

Innovations and breakthroughs

This is the first report demonstrating gender-based differences in the basal expression of ERα in the liver that also results in change in ER subtype ratio. In addition to ER subtype expression in the whole liver tissue extracts, subcellular fractionation of liver extracts for expression of ER subtypes, inflammatory and proliferative markers in nucleus and cytoplasm of both, normal and diseased livers, were also evaluated. To our knowledge, this is the first study that correlates the expression of ER subtypes with cyclin D1 in livers of HCV-related cirrhosis and HCV-related HCC.

Applications

Investigating gender associated changes in ER subtype ratio in the liver during the course of HCV-related disease progression along with oncogenic marker expression may help us to monitor gender specific host therapeutic responses, viral clearance and cancer development more effectively. Furthermore, these ER subtype expression studies will further complement existing treatments and pave the way to develop gender and stage-related personalized therapies that are highly needed to treat and manage chronic HCV infections and HCV-related HCC.

Terminology

Cirrhosis of the liver is damage caused by chronic Hepatitis C viral infection. Cirrhosis involves replacement of normal liver tissue with scar tissue that affects liver function and leads to end-stage disease.

Peer-review

Authors investigated ER expression in liver hepatocytes from normal subjects and patients with hepatitis C virus-related cirrhosis and Hepatocellular Carcinoma. The expression of ER subtypes, ERα and ERβ, were evaluated for the first time in relation to gender.

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ORIGINAL ARTICLE

Basic Study

Glycosylation-related gene expression in HT29-MTX-E12 cells upon infection by *Helicobacter pylori*

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Abstract

AIM

To identify glycosylation-related genes in the HT29 derivative cell line, HT29-MTX-E12, showing differential expression on infection with *Helicobacter pylori* (*H. pylori*).

METHODS

Polarised HT29-MTX-E12 cells were infected for 24 h with *H. pylori* strain 26695. After infection RNA was isolated from both infected and non-infected host cells. Sufficient infections were carried out to provide triplicate samples for microarray analysis and for qRT-PCR analysis. RNA was isolated and hybridised to Affymetrix arrays. Analysis of microarray data identified genes significantly differentially expressed upon infection. Genes were grouped into gene ontology functional categories. Selected genes associated with host glycan structure (glycosyltransferases, hydrolases, lectins, mucins) were validated by real-time qRT-PCR analysis.

RESULTS

Infection of host cells was confirmed by the isolation of live bacteria after 24 h incubation and by PCR amplification of bacteria-specific genes from the host cell RNA. *H. pylori* do not survive incubation under the adopted culture conditions unless they associate with the adherent mucus layer of the host cell. Microarray analysis identified a total of 276 genes that were significantly differentially expressed ($P < 0.05$) upon *H. pylori* infection and where the fold change in expression was greater than 2. Six of these genes are involved in glycosylation-related processes. Real-time qRT-PCR demonstrated significant downregulation (1.8-fold, $P < 0.05$) of the mucin MUC20. REG4 was heavily expressed and significantly downregulated (3.1-fold, $P < 0.05$) upon infection. Gene ontology analysis was consistent with previous studies on *H. pylori* infection.

CONCLUSION

Gene expression data suggest that infection with *H. pylori* causes a decrease in glycan synthesis, resulting in shorter and simpler glycan structures.

Key words: Glycosylation; Adherent mucus; Gastric; HT29-MTX-E12; *H. pylori* strain 26695; Transcriptomics

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Core tip: Few studies on *Helicobacter pylori* (*H. pylori*) infection focus on glycosylation-related genes in the host cells yet key cell-cell interactions are likely mediated through surface glycoconjugates. We use HT29-MTX-E12 cells, a promising and novel model of the stomach epithelium, to investigate the transcriptomic effects of *H. pylori* infection. HT29-MTX-E12 cells produce a thick adherent mucus layer and show a level of pluripotency that gastric cells naturally present and which some other model cell lines do not. Furthermore both *H. pylori* strain 26695 (lacks BabA adhesin) and HT29-MTX-E12 host cells (TLR2-negative) have some features atypical of more common models of *H. pylori* infection.

Cairns MT, Gupta A, Naughton JA, Kane M, Clyne M, Joshi L. Glycosylation-related gene expression in HT29-MTX-E12 cells upon infection by *Helicobacter pylori*. *World J Gastroenterol* 2017; 23(37): 6817-6832 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6817.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6817

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a major cause of peptic ulcer disease and chronic gastritis, and is a causative agent for both gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma[1-3]. Gastric cancer is the second most common cause of cancer-related deaths and is the fifth greatest killer worldwide after tuberculosis. Eradication of *H. pylori* infection with antibiotics may cause as much as a 40 % decrease in the cases of gastric cancer[4]. Despite its occurrence in approximately two-thirds of the world's population, infection with *H. pylori* is asymptomatic in the majority of cases. How *H. pylori* manages to establish chronic infection in the hostile environment of the human stomach and to evade a vigorous immune response is not known.

An acute response to *H. pylori* infection is characteristically marked by the induction of interleukin (IL)-8. This is evident in both human biopsies and cell line studies. An acute response is followed by a chronic response which is marked in humans by the production of IgA autoantibodies to IL-8[5]. A rhesus macaque model of *H. pylori* infection showed a dramatic change in gastric epithelial morphology over the first few hours of infection but a restoration of normal morphology within 24 h despite the continued presence of the pathogen[6]. These morphological changes were associated with modified O-glycan structures. It has been shown previously that transcript levels of *B3GNT5* were upregulated upon *H. pylori* infection of gastric cell lines MKN45 and AGS and that overexpression of this gene, associated with glycosylation of glycolipids, led to increased sialyl Lewis x presentation and adhesion of *H. pylori*[7]. Furthermore, host cells in asymptomatic infected human patients appear to modify O-glycans in order to counter the effects of the invading *H. pylori*[8].

H. pylori resides in the stomach where there is a thick adherent layer of mucus. While most invading pathogens are excluded from epithelial surfaces by the mucous layer, *H. pylori* survives in the mucous layer and can penetrate it to attach to the underlying cells. The main mucin components of mucus in the stomach are MUC5AC, MUC6, MUC1 and MUC16[9-11] with *H. pylori* displaying a distinct tropism for MUC5AC[12,13]. Although *H. pylori* may reside in the mucous layer it is through adhesion to the underlying epithelial cells that the bacteria exert their harmful/inflammatory effects. *H. pylori* binds to cells through adhesins, such as BabA that binds Lewis b and H-type 1 antigens on epithelial cell glycoproteins or glycolipids. They can also use the SabA adhesin to bind to sialyl Lewis x and sialyl Lewis a antigens, though this is expected to play a greater part in chronic infection. More recently LabA has been identified which binds to LacdiNAc structures present on MUC5AC[14]. Only a small fraction of *H. pylori* associated with infected cells is found internally[15], instead the bacteria inject the effector protein CagA and subvert host cell signalling[16,17].

Transcriptomic studies, through the use of high density DNA microarrays, have been applied to various aspects of *H. pylori* infection including analysis of human biopsies[18-23], analysis of human tissue after eradication treatments[24,25], the analysis of animal models[6,26,27] and the analysis of various cell

lines[7,28-30]. High variability in the data sets has been attributed to different model systems, different host cells and the diverse nature of the different *H. pylori* strains used.

Human cell lines, isolated from the gastrointestinal tract (GIT), have been commonly used to study *H. pylori* infection. The responses of different gastric cell lines to *H. pylori* infection have been detailed in a recent paper[31]. The HT29 colon cell line and its derivatives have been used frequently as a model of the gastrointestinal tract because the cells are multipotent and can be directed along different paths of selection and differentiation. Some HT29 derivatives have an absorptive (enterocyte-like) phenotype while others have a mucus-secreting (goblet-like) phenotype. This makes HT29 cells particularly suited to the study of glycan structures in the gastrointestinal tract and mucus-associated effects on bacterial infection. The HT29-MTX-E12 (E12) cell line is a HT29 derivative selected on the basis of tight junction formation and adherent mucus production, which has many of the characteristics of a gastric cell[32-36]. In particular, differentiated E12 cells express MUC5AC, MUC1, MUC 6 and only trace amounts of MUC2[35], a composition which more closely resembles the stomach mucin makeup than the intestine.

The aim of this study was to determine the transcriptomic response of the mucus-secreting E12 cells to *H. pylori* infection with special focus on the glycomic response. Because known *H. pylori* adhesins bind glycosylated structures on the cell surface and in the mucous layer and glycosylation is known to play a major role in general host-pathogen and cell-cell interactions, it is of interest to know how glycosylated structures may change in response to infection.

MATERIALS AND METHODS

E12 culture

E12 cells, a kind gift from Per Artursson of the University of Uppsala, were maintained in flasks in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (Life Technologies), 2 mmol/L L-glutamine (Life Technologies), 1% nonessential amino acids and antibiotics (penicillin (50 U/mL) and streptomycin (50 μg/mL). Cells (2×10^6) were seeded on transwell filters (0.4 μm pore polycarbonate membrane insert) in 6-well plates. Cells were incubated at 37 ℃ in a humidified 5 % CO_2 atmosphere and media was added every second day. Transepithelial electrical resistance (TEER) measurements were taken at 20 d. Antibiotics were removed from the cells at least 24 h before infection assays.

H. pylori culture

Bacteria (*H. pylori* strain 26695) were plated on Columbia blood agar (Oxoid) and grown for 48 h in a microaerophilic environment using gas packs (Oxoid, BR0038). Bacteria were harvested from plates into Brucella broth, the OD_{600} adjusted to 0.1 and grown in T25 tissue culture flasks at 37 ℃ under microaerophilic conditions with shaking at 70-80 rpm until the OD_{600} reached 0.6. Gram staining of bacterial cultures was used to check coccoid levels were less than 5%. Washed bacteria were resuspended to an OD_{600} of 1.8 with antibiotic-free RPMI.

Infection protocol

On day 20 E12 cells in transwells were washed with HBSS and 2 mL antibiotic-free complete DMEM media was added to the base of each well and 1 mL of RPMI to the apical surface of each well. *H. pylori* do not survive in DMEM[37] but will in RPMI. On day 21200 μL bacteria (6×10^8) were added to each well at a multiplicity of infection (MOI) of 300. Plates were incubated at 37 ℃ for 24 h. Cells were harvested for RNA isolation. Replicate transwells were washed gently with sterile PBS and harvested using trypsin EDTA as described previously[35,38]. Serial dilutions of the trypsinised cells were plated in triplicate on Columbia blood agar plates and incubated under microaerophillic conditions. Colonies were counted after 4-5 d incubation to determine the number of bacteria (CFU/mL) that were associated with the cells. Though some infections resulted in low IL8 transcript levels this did not appear to correlate with low CFU values.

RNA isolation and microarray hybridisation

RNA for microarray hybridisations and qPCR analysis was isolated by TRIzol extraction followed by standard RNA purification protocols (Qiagen). RNA (100 ng) was labelled with biotin using the Affymetrix 3' IVT Express Protocol. All hybridisations were carried out using the hybridisation and wash solutions as recommended in the Affymetrix 3' IVT Express Protocol and were carried out by DNA Vision, Charleroi, BE.

Microarray analysis

Spot intensities provided (DNA Vision) were normalised and analysed in GeneSpring (Ver 12.3). Data were log transformed and quantile normalised. Subsequently all normalised data was baseline transformed to the median of all samples. Intensity values for all entities (54675) were initially filtered to exclude values which were less than 50 in all three samples of both conditions (uninfected, infected). Significantly differentially expressed genes were identified based on log values using an unpaired *t*-test (variance assumed equal)[39], with a FDR cut-off value of 0.05. Multiple test correction was carried using the Benjamini and Hochberg correction. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[40] and are accessible through GEO Series accession number GSE74492 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74492). Where microarray data is

graphed, the raw signals for uninfected samples were the actual spot intensities and for the infected samples the signals were corrected using the fold change determined from normalised data for each gene. Standard deviations are shown for the three samples in each condition using raw spot intensities (uninfected) and raw spot intensities corrected for fold change (infected). Where the microarray data is shown as a log fold change, standard deviations were generated using propagation of errors rules.

For Gene Ontology analysis, DAVID (Database for Annotation, Visualization and Integrated Discovery, v6.7) was used[41] (http://www.david.ncifcrf.gov/). The number of significant genes (6034) was reduced further using more stringent parameters (FDR < 0.01, fold change > 1.2). Where directional analysis was carried out, only the top 500 upregulated genes (FDR < 0.005, fold change > 1.2) were included in the GO analysis and this was compared with whatever number of downregulated genes (714) had *P*-values below the *P*-value of the 500th upregulated gene (FDR = 0.0046) in order to maintain a balanced comparison. GO analysis also excluded GO terms where the number of genes in the GO term was more than 10% of all the genes on the array; this therefore removed broader gene ontology terms which were not generally informative. These restrictions were not imposed for GO analysis of the glycosylation-related genes because of the lower number of genes involved.

qRT-PCR

qRT-PCR validation was carried out on RNAs from samples collected independently of the microarray samples. The RNA samples for qRT-PCR validation were also from several different infections: this adds some of the variability seen in the data and is an issue that is discussed further in the manuscript. For real time qRT-PCR RNA (1 μg) was reverse transcribed using the VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. PCR was carried out using diluted template (2 μL) and GoTaq® qPCR Master Mix (18 μL) on a Mx3000P qPCR System (Stratagene). Cycling conditions for all primer pairs was 95 ℃ for 5 min followed by 40 cycles of 95 ℃ 10 s and 60 ℃ 10 s. Dissociation curves were determined using a temperature gradient from 95 ℃ to 56 ℃. Primer sequences were either retrieved from primer repositories (PrimerBank, https://pga.mgh.harvard.edu/primerbank/ and qPrimerDepot, https://primerdepot.nci.nih.gov/), designed using Primer3 (http://primer3plus.com/web_3.0.0/primer3web_input.htm) or taken from relevant papers (Supplementary Table 1 and references therein). Several primer pairs were tested for their potential as housekeeping genes and geNorm analysis[42] was used to select an optimal set of three genes for subsequent normalisation (*GAPDH*, *RPLP0*, *UAP1*). All fold change values were calculated within geNorm which also generated standard deviations based on the rules for the propagation of errors. Standard two-tailed *t*-tests were used to determine where fold changes were significant. As an approximate measure of gene expression level, cycle threshold (Ct) values determined by qRT-PCR for each gene (uninfected cells) were compared to a Ctmax, in this case *IL32* the lowest expressed of our selected genes

RESULTS

This study investigated the effects of *H. pylori* (strain 26695) colonisation on the transcriptome of E12 cells, a specialised derivative of the HT29 cell line. The cell line forms a functional layer of polarised cells and produces an adherent mucous layer when grown on transwells for 21 d.

Microarray analysis

E12 cells were grown on transwells for 21 d and infected with *H. pylori* strain 26695 at a MOI of 300. Cells were harvested for RNA isolation after incubation at 37 ℃ for 24 h. *H. pylori* maintains viability during infection despite its normal requirement for a microaerophilic environment, possibly as a consequence of close association with the mucous layer surrounding the E12 cells. Microarray analysis of all samples showed separate clustering of infected E12 (n = 3) and uninfected E12 samples (n = 3) (Supplementary Figure 1). The infected samples, originating from triplicate wells in a single infection experiment, clustered very tightly. Although greater variability was seen in the uninfected E12 samples, this is probably because these samples originate from two separate infection experiments.

Confirmation of a global modulation of E12 genes in response to H. pylori *infection*

Statistical analysis of the microarray data suggested significant differential expression of 6034 genes (FDR < 0.05) upon infection. Of these, 276 unique genes (< 5 %) showed fold changes greater than two (see Table 1 for the top 15 upregulated and downregulated genes; Supplementary Table 2 for all 276 genes). GO analysis of a reduced list (2639 genes) of regulated genes (FDR < 0.01, fold change> 1.2) is presented in Figure 1A. This analysis was sensitive to the selected fold change cut-off therefore we considered it appropriate to choose quite a low value (1.2-fold). To provide some additional perspective, GO analysis was repeated separately on the most significantly upregulated and downregulated genes (data not shown). This analysis gave an idea of the direction in which each significant process (as shown in Figure 1A) was regulated. GO analysis of the top 500 upregulated genes (FDR < 0.005, fold change > 1.2) revealed

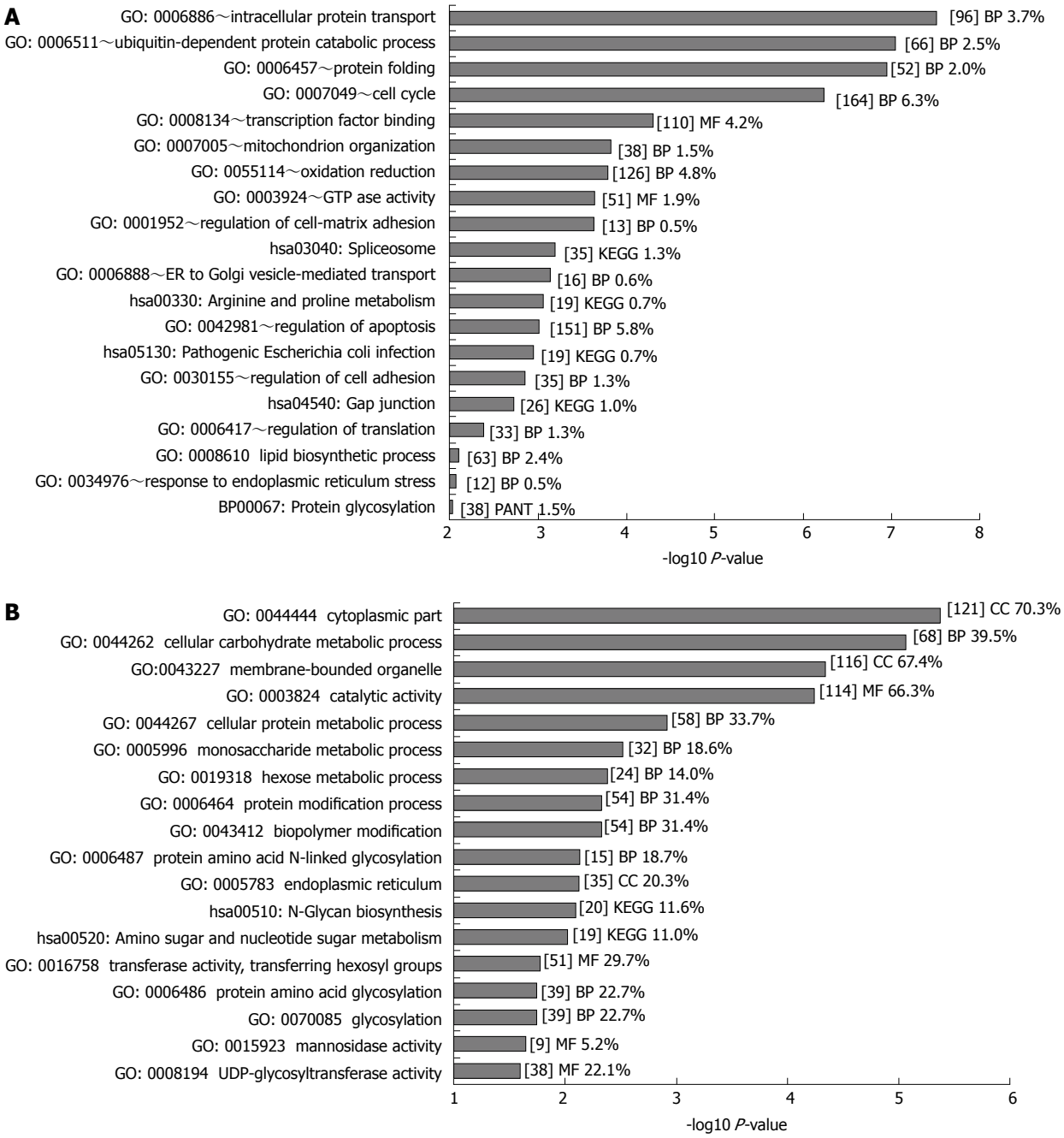


Figure 1 GO analysis of significant differentially expressed genes. A: GO analysis of 2639 genes deemed significantly differentially expressed ($P < 0.01$, fold change > 1.2) was carried out in DAVID. Only GO terms with a *P*-value below 0.01 are shown (-log10 *P*-value > 2). GO terms were excluded where the number of genes belonging to that GO term exceeded 10% of all genes. B: GO analysis of 374 significantly differentially expressed glycosylation-related genes ($P < 0.001$) was carried out in DAVID. Only GO terms with a *P*-value below 0.1 are shown (-log10 *P*-value > 1). Some Panther (PANT) gene ontology and KEGG pathway terms were included from the DAVID analysis. The number of genes included in each term is given within square brackets [ ] together with the percentage of significant genes relative to the total number of genes of that term on the array. BP: Biochemical process; MF: Molecular function; CC: Cellular component.

significant processes related to protein ubiquitination, protein folding, cell redox homeostasis, oxidative phosphorylation and the mitochondrial membrane. GO analysis of the top downregulated genes (FDR = 0.005, fold change > 1.2, 714 genes) revealed significant processes associated with ECM-receptor interaction, focal and cell adhesion, phosphorylation and modulation of the cytoskeleton. Some GO processes included genes with approximately equal numbers of up and downregulated genes: these additional GO terms included Golgi vesicle mediated transport, dolichol-linked oligosaccharide biosynthetic processes, phosphorylation-related signalling, apoptosis, cell junction function, molecular adaptor activity, cell cycle, transcription, antigen presentation and apical junction complex. These terms are consistent with the reported effects of *H. pylori* on cells and therefore support our supposition that the observed effects on glycosylation,

Table 1 Differentially expressed genes associated with *Helicobacter pylori* infection of E12 cells

Gene symbol	*P*-value	Fold change	Gene name
Top 15 upregulated			
[1]*CLIP1*	0.0025	5.42	CAP-GLY domain containing linker protein 1
C10orf118	0.0031	5.21	chromosome 10 open reading frame 118
TOP1	0.0352	5.09	topoisomerase (DNA) I
LARS	0.0054	5.02	leucyl-tRNA synthetase
CDC27	0.0073	4.95	cell division cycle 27 homolog (S. cerevisiae)
STIP1	0.0042	3.92	stress-induced-phosphoprotein 1
MALAT1	0.0077	3.82	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)
IL32	0.0021	3.54	interleukin 32
SLC39A6	0.0204	3.33	solute carrier family 39 (zinc), member 6
AHNAK2	0.0070	3.25	AHNAK nucleoprotein 2
HSP90AB1	0.0014	3.24	heat shock protein 90 kDa alpha (cytosolic), B1
ACTR2	0.0035	3.20	ARP2 actin-related protein 2 homolog (yeast)
EIF4G1	0.0349	3.16	eukaryotic translation initiation factor 4 gamma, 1
ACBD3	0.0253	3.14	acyl-CoA binding domain containing 3
CCND1	0.0222	3.09	cyclin D1
Top 15 downregulated			
PDK4	0.0011	3.25	pyruvate dehydrogenase kinase, isozyme 4
BCAT1	0.0042	3.24	branched chain amino-acid transaminase 1, cytosolic
PELI1	0.0059	3.24	Pellino homolog 1 (Drosophila)
CYP3A5	0.0015	3.12	cytochrome P450, family 3, subfamily A, polypeptide 5
HPGD	0.0032	3.10	hydroxyprostaglandin dehydrogenase 15-(NAD)
LRRC31	0.0030	3.05	leucine rich repeat containing 31
KIAA1984	0.0082	2.97	KIAA1984
HNRNPD	0.0054	2.86	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)
IL28A	0.0026	2.85	interleukin 28A (interferon, lambda 2)
DDX60	0.0026	2.84	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
PHF14	0.0021	2.82	PHD finger protein 14
LOC100288092	0.0025	2.82	Hypothetical protein LOC100288092
ZNF207	0.0027	2.82	zinc finger protein 207
NCOA2	0.0025	2.81	nuclear receptor coactivator 2
AHSA2	0.0064	2.72	AHA1, activator of heat shock 90 kDa protein ATPase homolog 2 (yeast)

[1]qRT-PCR analysis showed *CLIP1* was downregulated.

the focus of this study, relate to infection by *H. pylori*.

Modulation of glycosylation-related genes in response to H. pylori infection

In order to study changes in the glycome of E12 cells upon *H. pylori* infection we examined those gene probes on the Affymetrix U133 Plus 2 Array that were associated with a glycosylation role, selected with reference to the Consortium for Functional Glycomics (CFGv3) array. We identified 170 significantly differentially expressed genes upon *H. pylori* infection that were glycosylation related (see Table 2 for the top 20 upregulated and downregulated genes; Supplementary Table 3 for all 170 genes). Only 6 (< 4 %) of these genes showed fold changes greater than 2-fold. The most upregulated gene from the microarray analysis was *LGALS7/7B*, (lectin, galactoside-binding, soluble, 7/7B, 2.3-fold) and the most downregulated gene was *REG4* (regenerating islet-derived family, member 4, 2.5-fold) followed by *OGT* (O-linked N-acetylglucosamine (GlcNAc) transferase, 2.2-fold) and *AMIGO2* (adhesion molecule with Ig-like domain 2, 2.2-fold). These small changes in glycosylation-related genes are not uncommon in significant processes that involve glycosylation[7,43].

GO analysis of differentially regulated glycosylation-related genes was referenced against this Affymetrix array-derived glycome to determine which GO terms, relevant to glycosylation, were significant in the *H. pylori* infected E12 cells (Figure 1B). The most relevant terms were nucleotide sugar metabolism, N-glycan biosynthesis, cell death and galactose metabolic process, all of which generally related to upregulated genes, and hexose metabolic process, mannosidase activity and UDP-glycosyltransferase activity, which related to downregulated genes (Figure 1B).

Changes in terminal sugars of E12 glycoconjugates upon H. pylori infection

Microarray signal strength is not a robust measure of gene expression because probes bind with different affinities to the array. However, fold change measurements are robust because the same probe is used to compare two conditions. In this and the following sections the fold change differences between infected and uninfected gene levels are therefore quantitative. However, this data alone omits useful information about the expression level of one gene relative to another, always bearing in mind that two probes could have very different binding efficiencies for

Table 2 Glycosylation-related differentially expressed genes associated with *Helicobacter pylori* infection of E12 cells

Gene symbol	Fold change	*P* value	Gene name
Top 20 upregulated			
LGALS7/7B	2.31	0.0115	Lectin, galactoside-binding, soluble, 7 /7B
UGGT1	1.98	0.0059	UDP-glucose glycoprotein glucosyltransferase 1
ST3GAL5	1.90	0.0035	ST3 beta-galactoside alpha-2,3-sialyltransferase 5
PARM1	1.87	0.0026	Prostate androgen-regulated mucin-like protein 1
NAGK	1.79	0.0016	N-acetylglucosamine kinase
VSIG1	1.71	0.0240	V-set and immunoglobulin domain containing 1
EDEM2	1.69	0.0032	ER degradation enhancer, mannosidase alpha-like 2
MPDU1	1.69	0.0029	Mannose-P-dolichol utilization defect 1
DPM2	1.61	0.0015	Dolichyl-phosphate mannosyltransferase polypeptide 2, reg. s/u
LMAN2	1.60	0.0021	Lectin, mannose-binding 2
GMPPB	1.59	0.0019	GDP-mannose pyrophosphorylase B
ALG3	1.59	0.0214	Asn-linked glycosylation 3, alpha-1,3- mannosyltransferase hom.
GALK2	1.57	0.0027	Galactokinase 2
DDOST	1.53	0.0094	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase
MCAM	1.53	0.0111	Melanoma cell adhesion molecule
B3GALT6	1.53	0.0097	UDP-Gal:betaGal beta 1,3-galactosyltransferase polypeptide 6
SLC35B1	1.51	0.0025	Solute carrier family 35, member B1
ST6GALNAC4	1.50	0.0024	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 4
GMPPA	1.49	0.0031	GDP-mannose pyrophosphorylase A
ALDOC	1.48	0.0020	Aldolase C, fructose-bisphosphate
Top 20 downregulated			
REG4	2.46	0.0018	Regenerating islet-derived family, member 4
OGT	2.25	0.0037	O-linked N-acetylglucosamine (GlcNAc) transferase (OGT)
AMIGO2	2.22	0.0039	Adhesion molecule with Ig-like domain 2
GALNT12	2.09	0.0033	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12
EXT1	1.99	0.0256	Exostosin 1
LY75	1.86	0.0075	Lymphocyte antigen 75 (CLEC13B)
PIGZ	1.82	0.0072	Phosphatidylinositol glycan anchor biosynthesis, class Z
UGP2	1.79	0.0320	UDP-glucose pyrophosphorylase 2
MANEA	1.79	0.0225	Mannosidase, endo-alpha
ALG13	1.78	0.0138	Asparagine-linked glycosylation 13 homolog (S. cerevisiae)
GBAP1	1.75	0.0035	Glucosidase, beta, acid pseudogene 1
SLC35A3	1.73	0.0071	Solute carrier family 35 (UDP-N-acetylglucosamine transporter), member A3
ST3GAL4	1.71	0.0032	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
B4GALNT3	1.71	0.0129	Beta-1,4-N-acetyl-galactosaminyl transferase 3
HMMR	1.70	0.0039	Hyaluronan-mediated motility receptor (RHAMM)
B4GALT6	1.69	0.0134	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 6
MAN2A1	1.60	0.0034	Mannosidase, alpha, class 2A, member 1
LGALS2	1.58	0.0127	Lectin, galactoside-binding, soluble, 2
MAN1A1	1.53	0.0021	Mannosidase, alpha, class 1A, member 1
MUC13	1.53	0.0038	Mucin 13, cell surface associated
CD164	1.52	0.0015	CD164 molecule, sialomucin
B4GALT5	1.51	0.0071	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5

the array. In Figure 2, where three selected groups of glycosylation related genes are presented, a gene has only been included if microarray analysis showed that it was either significantly differentially expressed or that it was represented on the array by more than one probe. In addition a signal strength cut-off was set to 20 to exclude weak genes which were less likely to be relevant (though technically significant in the original analysis). For sialylation (Figure 2A) *ST6GALNAC1*, *NEU1*, *SLC35A1*, *ST3GAL5* and *NANP* were significant but because they were each represented by only one probe their absolute expression levels relative to the other genes shown may be less representative. Where a gene was not significant but represented by multiple probes (*e.g.*, *ST3GAL2*), the data was considered sufficiently reliable to warrant presenting expression levels relative to the other genes.

Terminal glycosylation of cell surface glycoconjugates directly determines the interaction of bacteria with the host cell. Therefore, sialylation and fucosylation were expected to be key processes affected by *H. pylori* infection. Terminal sialylation of glycoconjugates is a consequence of sialyltransferase expression and therefore can be predicted based on the transcript expression levels of the sialyltransferase enzymes from the microarray data. *ST3GAL4*, which can sialylate most glycoconjugate types and is believed to be involved in sialyl Lewis x and sialyl T antigen formation, was significantly downregulated (1.7-fold) whereas *ST3GAL5*, which is involved in ganglioside GM3 synthesis in glycolipids, was significantly upregulated (1.9-fold) on *H. pylori* infection (Figure 2A). *ST6GALNAC1* (down, 1.3-fold) and *ST6GALNAC4* (up, 1.8-fold) were also significantly differentially

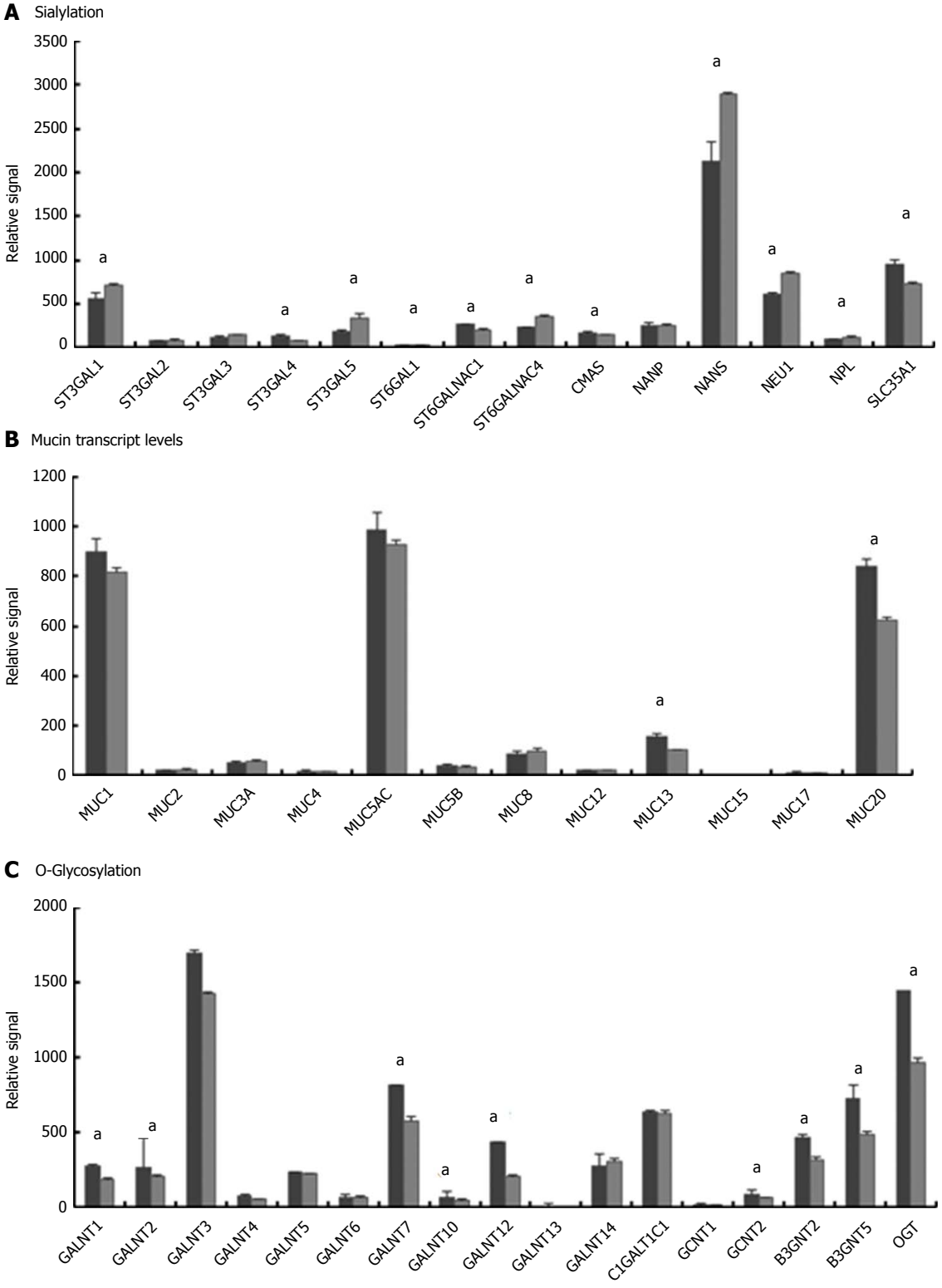


Figure 2 Transcript levels determined by microarray analysis of selected glycosylation related processes. A: Sialylation-related genes; B: Mucin genes; C: O-glycosylation genes. Raw signals reported for each gene were the strongest signals where multiple probes were available (except for MUC2 where the negligible signal from the single probe, indicating very weak expression, was confirmed by qRT-PCR). All signals for uninfected samples were raw values whereas all infected samples were extracted using fold change values of normalised data as detailed in Materials and Methods. Uninfected (dark grey) or Infected (light grey). Error bars show standard deviations (n = 3). ([a]FDR < 0.05).

expressed upon *H. pylori* infection. *ST6GALNAC4* is involved in the biosynthesis of ganglioside (GD1A from GM1B) and the transfer of NeuAc to GalNAc residues of O-glycans to form the stunted sialyl Tn antigen. Based on signal intensities, ST3GALs 2-5 were all moderately expressed (raw control intensity < 500), *ST3GAL1* was highly expressed (> 500) and *ST3GAL6* was negligibly expressed (< 10). ST6GALs were lowly

Table 3 qRT-PCR significance of selected genes

Gene	Fold Change	Regulation	Significance
GNE	1.84	Down	[1]
MUC20	1.77	Down	[1]
REG4	3.06	Down	[1]
MAN2A2	1.76	Down	[1]
MAN2B2	1.91	Down	*NS P* < 0.1
SLC35A2	1.53	Down	*NS P* < 0.1
RHOA	1.35	Down	*NS P* < 0.1
[1]*TFF1*	10.37	Down	*NS P* < 0.2
MAN2A1	1.42	Down	*NS P* < 0.2
ST3GAL5	2.00	Down	*NS P* < 0.2
MUC13	3.66	Down	*NS*
PDK4	1.65	Down	*NS*
GALNT10	2.74	Down	*NS*
GALNT7	1.68	Down	*NS*
MGAT2	2.09	Down	*NS*
MUC1	1.66	Down	*NS*
NEU1	1.54	Up	*NS*
CLIP1	2.37	Down	*NS*
LGALS2	1.27	Up	*NS*
OGT	1.43	Down	*NS*
AMIGO2	1.14	-	*NS*
ALG8	1.15	-	*NS*
GALK2	1.14	-	*NS*
SLC35A3	1.23	Down	*NS*
FPGT	1.07	-	*NS*
CGN	1.26	Down	*NS*
TJP3	1.01	-	*NS*
OCLN	1.66	Down	*NS*
EPHA2	1.07	-	*NS*
IL32	1.00	-	*NS*
IL8	1.53	Up	*NS*

[1]*TFF1* is not shown in Figure 3, and four further genes (*MGAT1, LGALS7, OSTC* and *LGALS7*) are only shown in Supplementary Figure 2.

(< 50) (*ST6GAL1*) to negligibly expressed (*ST6GAL2*) and all ST8SIAs (1-5) were also negligibly expressed. This is in line with the predominance of NeuAcα2-3Gal linkages over NeuAcα2-6Gal in HT29 cells[44] and the predominance of O-glycans over N-glycans. The overall effect to be expected is therefore one of increased sialyl Tn antigen, decreased sialyl Lewis x/a determinants on glycoproteins with potential effects on glycolipids. This may result in shorter glycan chains and may affect packing density because some GALNTs require recognition of neighbouring GalNAc residues, now increasingly masked by sialylation, to initiate new glycan chains.

Although a number of fucosyltransferases (*FUT1, FUT3, FUT4, FUT5, FUT6* and *FUT11)* were moderately expressed, only *FUT1* was upregulated significantly (1.2-fold) upon *H. pylori* infection (data not shown). This would suggest no major role for fucosylation in the E12 response to *H. pylori* infection. *FUCA1* is a lysosomal fucose hydrolase. Its strong expression and upregulation on infection (1.2-fold) might suggest net removal of surface expressed fucose residues.

Changes in O-glycosylation upon H. pylori infection

As stated above, E12 cells produce a copious adherent mucous layer of which the main components are O-glycans. The data presented here show expression of *MUC5AC, MUC1, MUC20* and *MUC13*. Only *MUC13* and *MUC20* were significantly downregulated (Figure 2B). *MUC5AC* was represented by two high signal probes on the array but neither showed any change upon *H. pylori* infection. (It is noted that *MUC5AC* appears as a significantly upregulated gene (1.2-fold) in Supplementary Table 3, however, this probe produced only a weak signal).

Mucin O-glycosylation initiates with the transfer of N-acetylgalactosamine (GalNAc) to a Ser/Thr residue on the protein catalysed by the polypeptide N-acetylgalactosamine transferases (GALNTs). Of 14 GALNTs represented on the array all 5 that were significant were downregulated upon *H. pylori* infection - *GALNT1* (1.5-fold), *GALNT2* (1.3-fold), *GALNT7* (1.4-fold), *GALNT10* (1.4-fold) and *GALNT12* (2.1-fold) (Figure 2C). *GALNT12* is believed to be involved in the initial glycosylation of MUC5AC and MUC1 but not of some other mucins[45]. It has different activities towards already glycosylated MUC5AC and MUC1 peptides suggesting that its activity may determine packing density of multiple O-linked glycans and that specifically glycan density may be reduced on *H. pylori* infection where *GALNT12* appears downregulated. Sialylation of the polypeptide-linked GalNAc by ST6GALNACs may also change the recognition of initiation sites by specific polypeptide GalNAc transferases. Changes in O-glycan initiation have been reported previously in serum proteins of patients with gastric carcinoma[46]. Differential expression of GalNAc transferases in gastric cancer cell lines and gastric carcinoma may be associated with occupancy of potential O-glycosylation sites in mucins[47,48]. GalNAc transferase expression has been reported to be variable among gastric cell lines with GALNTs 1, 2 and 3 showing ubiquitous expression while other GalNAc transferases show more restricted expression[49].

No genes coding enzymes that have the potential to produce core 1 to core 8 O-linked glycan structures were significantly regulated upon *H. pylori* infection. *C1GALT1* and *C1GALT1C1 (COSMC)* were expressed at moderate and high levels respectively based on signal intensities suggesting the likely presence of core 1 structures on E12 O-glycans. Extension of the core structures by B4GALTs and B3GNTs seemed likely with moderate expression of several B4GALTs and B3GNTs. The galactose transferases and N-acetylglucosamine transferases that were significantly downregulated were those more often associated with N-glycan (*B4GALT5,* 1.5-fold; *B4GALT1*, 1.2-fold) or glycosphingolipid synthesis (*B4GALT5*, *B4GALT1* and the strongly expressed *B3GNT5*, 1.5-fold). We detected downregulation of *B3GNT5* and *B3GALT5* whereas a gastric biopsy study showed upregulation of the same genes[50]. The downregulated *B3GNT2* (1.4-fold) is commonly involved in polylactosamine

synthesis so may be involved in N-glycans, O-glycans and/or glycosphingolipids. The other common form of non-mucin O-glycosylation in E12 cell glycoconjugates initiated by O-GlcNAc protein glycosyltransferase was significantly downregulated on *H. pylori* infection (*OGT*, 2.3-fold) according to microarray data (Supplementary Table 3).

Changes in N-glycosylation upon H. pylori infection

N-glycosylation can be split into the lipid-linked oligosaccharide (LLO) precursor synthesis process generating $Glc_3Man_9GlcNAc_2$ and the processes of trimming and extension. There was a general upregulation of the genes involved in the LLO step except for *ALG13* which instead showed downregulation. *ALG13* showed the largest fold change (1.8-fold, Table 2) of all the genes involved in LLO precursor synthesis. There was a general downregulation of the genes involved in trimming and extension. All mannosidases, most moderately expressed, were downregulated (Supplementary Table 3) and one, MAN2A2, was consequently confirmed by qPCR (below).

The significance of changes in N-glycosylation in an O-glycan-dominated environment is unclear. Also, whether the altered N-glycans are on mucins, other transmembrane glycoproteins, or both, cannot be determined from the available data. It has been suggested previously that LLO-precursor synthesis could be a constitutive process where precursors are available in excess and control is elsewhere[51]. *ALG13* may, however, play a key role because it showed the largest fold change of all the genes in the precursor synthesis pathway, it was *down*regulated unlike other genes in the LLO-precursor synthesis pathway and interestingly it adds on the first GlcNAc of the LLO-precursor. Downregulated mannosidases (*MAN2A1*, 1.9-fold; *MAN1A1*, 1.5-fold; *MAN2B2*, 1.3-fold) are suggestive of an overall reduction of trimming and an increased level of the $Man_9GlcNAc_2$ glycan. Overall the effects suggest shorter and less complex N-glycan chains following *H. pylori* infection.

Changes in availability of activated sugars upon H. pylori infection

Glycan synthesis could be limited by changes in the abundance and availability of activated sugars. The UDP-galactose (*SLC35A2*, 1.3-fold; *SLC35B1*, 1.5-fold) and GDP-fucose (*SLC35C2*, 1.3-fold) transporters were significantly upregulated while the CMP-sialic acid (*SLC35A1*, 1.3-fold) and UDP-GlcNAc (*SLC35A3*, 2.1-fold) transporters were downregulated upon infection (Supplementary Table 3). The UDP-Gal, UDP-GlcNAc and CMP-sialic acid transporters were all strongly expressed while the GDP-fucose transporter was moderately expressed. N-Acetylneuraminic acid synthase (*NANS*) was very strongly expressed and significantly upregulated on infection (Figure 2A). This suggests the availability of Neu5Ac, however, the downregulation of both *SLC35A1* and *CMAS* expression (though marginal) (Figure 2A) would possibly make a subsequent rise in activated Neu5Ac (CMP-Neu5Ac) levels seem less likely. Increased levels of sialidase (*NEU1*) may lead to cell shedding of sialylated epitopes with a compensatory switch on of Neu5Ac biosynthesis (Figure 2A). GO analysis had indicated the significance of hexose and, more specifically, galactose metabolism. All significant galactose metabolism enzymes were upregulated as were all significant mannose metabolism enzymes. All significant fucose metabolism enzymes were downregulated as were all sialic acid metabolic enzymes.

Modulation of lectin levels upon H. pylori infection

The two LMAN lectins *LMAN1* (1.5-fold down) and *LMAN2* (1.6-fold up) were expressed strongly and differentially (Supplementary Table 3). Both lectins are expressed in the endoplasmic reticulum and Golgi body and are believed to be involved in N-glycan sorting and recycling. Of the Gal binding lectins, *LGALS1* (1.4-fold) and *LGALS7* (2.3-fold) were both upregulated significantly, and *LGALS2* (1.6-fold) and *LGALS8* (1.4-fold) were both downregulated significantly, all being expressed at moderate levels (Supplementary Table 3). *LGALS7* is a myoepithelial cell marker, associated positively with cancer progression and metastasis, while *LGALS8* is involved in integrin-like cell interactions and is negatively associated with malignancy. The opposite regulation of *LGALS7* and *LGALS8*, therefore, is consistent with an altered cell phenotype. *LGALS3* and *LGALS4* were expressed very strongly (raw signal >5000) but not differentially upon infection according to microarray data. Where oligosaccharide chains are not terminated with sialic acid, the major exposed sugar is galactose. It might therefore be relevant that two Gal binding lectins (LGALs) were expressed very strongly (*LGALS3* and *LGALS4*) and four others were significantly regulated upon infection. Most other lectins (CLECs, collectins, selectins, siglecs) were either weakly or negligibly, but not differentially, expressed.

qRT-PCR validation of prediction changes in gene expression level

qRT-PCR validation was carried out on RNA from samples collected independently of the microarray samples and from several different infection experiments in an effort to identify variability between infections. qRT-PCR analysis was carried out on a selected set of genes. Initially two non-glycogenes (*PDK4*, *CLIP1*) were selected based on the maximum fold changes observed on the microarray. *PDK4* expression was confirmed as downregulated though with a smaller fold change. It was shown subsequently that *CLIP1* was not upregulated though initial microarray analysis had suggested upregulation. Only one (1558924_s_at) of six *CLIP1* probes on the array

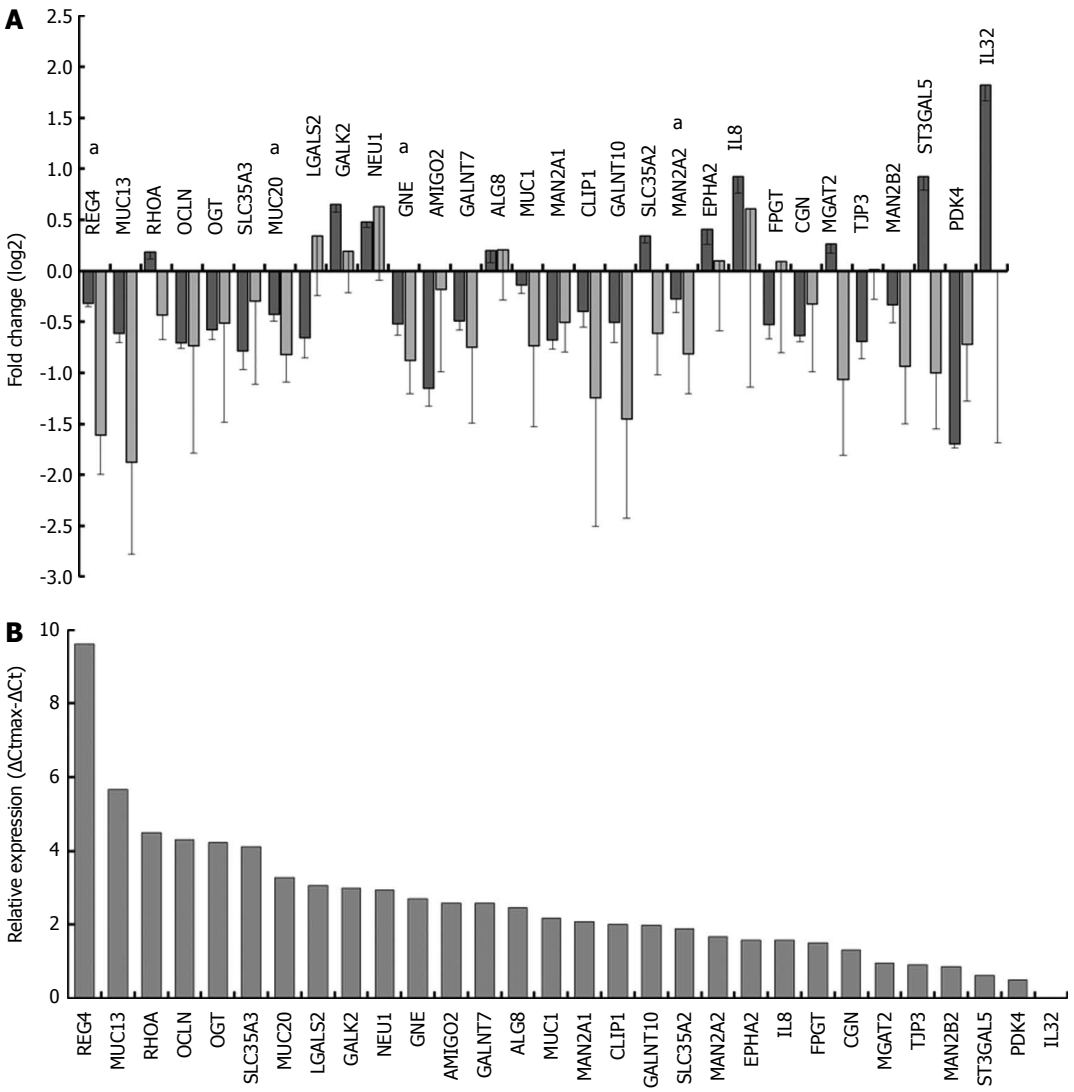


Figure 3 qRT-PCR validation of selected genes. Differential expression of selected genes upon *Helicobacter pylori* (*H. pylori*) infection of E12 cells was compared between microarray (dark grey) and qRT-PCR (light grey) analyses. qRT-PCR validation was carried out on RNAs from samples collected independently of the microarray samples. A: Microarray data (n = 3) was normalised across samples with GeneSpring. qRT-PCR data (n = 3) were normalised using geNorm and a panel of three housekeeping genes. Error bars show standard deviations determined using propagation of error rules; B: Relative expression of each gene in uninfected E12 cells was estimated by qRT-PCR using ΔCt determinations. ΔCtmax was the ΔCt for the lowest expressing gene (IL32) after correcting Ct values across samples with the housekeeping gene GAPDH. Further details are given in Materials and Methods. All genes were significantly differentially expressed in microarray analysis and genes marked with asterisks (a) were confirmed significant by qRT-PCR.

suggested upregulation: a probe producing a much higher intensity signal (201975_at) instead suggested 1.3-fold significant downregulation and it is this probe that correlated with the qRT-PCR analysis (Figure 3). In general *H. pylori*-infected samples showed qRT-PCR-corroborated upregulation of *IL8* and downregulation of *REG4* (Figure 3A), though the upregulation of *IL8* (and *IL32*) was highly variable across samples (see Supplementary Figure 2) resulting in an absence of significance. The stomach mucin *MUC1* showed no significant change in expression by either microarray analysis or qRT-PCR. This was true also for *MUC13*, a gene found at low levels in normal gastric mucosa but often expressed in metaplasia[52,53]. These two genes did however show consistent (not significant) downregulation in both microarray analysis and qRT-PCR. A third mucin gene, *MUC20*, was shown to be significantly downregulated (1.4-fold). *MUC5AC* showed no significant expression change based on microarray data (Figure 2B) or in a preliminary screen by qRT-PCR (data not shown). qRT-PCR did not corroborate regulation of a number of genes where the expected fold changes by microarray analysis were less than 2 (*LGALS2*, *ST3GAL5*, *MGAT2, FPGT* and *SLC35A2*).

Four genes (*REG4*, *MUC20*, *GNE* and *MAN2A2*) showed significant differential regulation by qRT-PCR (P < 0.05), and others approached significance (P < 0.1 - *MAN2B2, SLC35A2*, *RHOA*; P < 0.2 - *TFF1*, *ST3GAL5* and *PDK4*) (Table 3). The absence of significance in some cases was partly due to the small fold changes detected both by microarray and qRT-PCR analyses,

but it was also due to variability in the samples used for qRT-PCR. In several cases one of the three uninfected samples behaved differently from the other two. This was very striking for the interleukins *IL8* and *IL32*, and for *TFF1* which showed high downregulation (10.4-fold) on infection, but was not significant because one uninfected sample (3 in Supplementary Figure 2) showed much lower levels than the other two uninfected samples (1 and 2, Supplementary Figure 2). Hierarchical clustering (Pearson correlation with average linkage clustering) clearly shows the inconsistency across the qRT-PCR samples (Supplementary Figure 2) and how inconsistent genes cluster with other inconsistent genes suggesting a biological rather than a technical reason for this variability. This anomalous behaviour in the uninfected samples had also been seen in the microarray analysis. A small cluster of seven genes including all of the MUC genes assayed and *GALNT10*, suggests some common regulatory feature. *REG4*, *TFF1* and *ST3GAL5* also formed a tight cluster (Supplementary Figure 2).

DISCUSSION

A recent study of gastric cell lines has shown a wide range of responses to *H. pylori*[31]. Commonly used cell lines differ in their ability to polarise, to maintain a stomach-like epithelial morphology and differ in their pattern recognition receptors. *H. pylori* is, for the most part, a non-invasive bacterium that colonises the mucous layer surrounding the stomach and does not survive without this mucous layer. We have chosen for our study the HT29 derivative E12 cells because they produce an adherent mucous layer and the cells support *H. pylori* infection while the parent HT29 cells do not support *H. pylori* infection[36]. The main mucin components of the stomach mucous layer *in vivo* are the secreted gel-forming mucin, MUC5AC, and the cell surface mucin, MUC1. Other mucins include MUC4, MUC12, MUC13 and MUC6[54]. E12 cells have been shown by immunofluorescent methods to express high levels of MUC5AC and MUC1, and to a lesser extent MUC2[35]. The transcriptomic data presented here show expression of *MUC5AC*, *MUC13*, *MUC1* and *MUC20* with little or no expression of either *MUC2* or *MUC6*. E12 cells have therefore a mucin profile approximating that of the stomach and provide a good model for *H. pylori* infection studies.

The cellular response to pathogenic organisms is orchestrated by Toll-like receptors (TLRs). Strong evidence suggests that *H. pylori* infection is TLR2-mediated[55-57] yet there is some evidence that TLR4 is involved[58,59]. E12 cells however produce the hallmark response of IL-8 production upon *H. pylori* infection despite being TLR2 negative. The cellular response will also be determined by the receptor/adhesin characteristics of the host-pathogen interaction. Some *H. pylori* strains can bind to cells through the BabA adhesin which binds to the fucosylated Lewis b antigens on epithelial cell surface glycoconjugates[60,61]. Strain 26695, used throughout this study, possess an active *cag* pathogenicity island (PAI) associated with virulence[62] yet it has an inactive BabA adhesin[36]. Furthermore, fucosylated structures are relatively scarce on mucins secreted by E12 cells[36]. Therefore in this infection system the BabA adhesin interaction with Lewis antigens is not a factor in the IL-8 response to *H. pylori* and other *H. pylori* adhesin-host cell receptor combinations[63] are likely to play a role in mediating these responses.

Expression patterns of *H. pylori*-infected and uninfected E12 cells indicated variability especially in the uninfected E12 cells. The reasons for this are unclear. It might suggest that some E12 cells did not fully differentiate to form a fully polarised cell layer. However, TEER measurements were consistently high (about 600 Ω/cm^2) across all uninfected samples and could not differentiate E12 subpopulations. E12 cells, like the parent HT29 cells, are not a clonal population[64,65]. It has been shown that E12 cells show the occasional MUC2-expressing cell and that these cells can be expanded under the appropriate conditions[34]. It may therefore be that random samples contain differing proportions of cell types and so produce different expression profiles. It is likely that clonal cell lines would give more consistent results, however, we would argue that *in vivo* cells lining the stomach are not clonal, and therefore that non-clonal E12 cells may better reflect variation that is present *in vivo*.

Infection of the E12 cells was confirmed in this work by the presence of *cagA* RNA in exposed cells (data not shown). All significant expression changes on *H. pylori* infection were small with only 4%-5% being greater than 2-fold. This has been noted previously in similar experiments with *H. pylori* infection. The altered expression of glycosyltransferases and other glycosylation-related genes identified in this study is different in some specifics to that seen in related studies. This could be because E12 cells are derived from the colon where similar studies of *H. pylori* infection have used gastric cell lines[7,28,29]. But variability in gene expression response is also seen across gastric cell lines possibly as a result of differences in mucin presentation. We would therefore argue that E12 cells, with their gastric-like mucin composition, constitute a valid model of *H. pylori* infection. Although the expression of specific genes did not correspond with that seen in similar studies, gene ontology terms were in general agreement suggesting that, in the broader functional picture, there are strong similarities across the different *H. pylori* infection studies. Variability in gene response could equally be due to the choice of *H. pylori* strain.

One consistently downregulated gene was *REG4*, a 17 kD secreted C-type lectin, expressed in selected

enteroendocrine cells throughout the GIT and some goblet cells[66]. The functional role of REG4 is still unclear though it appears to be coexpressed with various enteroendocrine hormones. Although it is reportedly not expressed in normal stomach tissue, it is expressed, and has potential as a biomarker, in gastric carcinoma[67]. qPCR confirmed microarray data that expression of the *REG4* transcript was very high. Why this should be in E12 cells is unclear. It could be a manifestation of the colonic origins of HT29/E12 cells, or of the selection process for generating the E12 derivative, or it may be related to the high mucin production of this cell line. Another member of the REG family, REG3γ, has been shown to be intimately involved in the mucous layer protecting the underlying epithelial cells from bacterial colonisation[68] but there is no evidence yet that REG4 has any anti-bacterial activity.

The overall shortening of O-glycans and the decreased complexity of both N- and O-glycans that this study suggests follow *H. pylori* infection may be driven by the infecting bacteria in their efforts to ease access through the mucus to the underlying enterocytes. It could equally be a host cell response to reduce potential bacterial adhesion sites or a way to facilitate shedding of the mucous layer and the attached bacteria. The results presented from the E12 model will require confirmation by detailed oligosaccharide analysis. Alternative approaches, such as lectin histochemistry or direct biophysical analysis of released glycans from glycoproteins and glycolipids, may not be able to detect the responses seen at the transcriptomic level because of overlapping specificities of lectins. However, it is of interest that the results presented are consistent with the Rhesus Macaque *in vivo* model of *H. pylori* infection[6] and with the analysis of adherent mucins isolated from asymptomatic *H. pylori* infected patients[8].

In conclusion, E12 cells are a promising model of the stomach epithelium; the cells form tight junctions, they polarise, they produce a thick adherent layer of mainly MUC5AC and have a mucin composition similar to that of the stomach. The glycosyl transferase gene expression profiles suggested core 1 and core 2 extended structures on O-glycans, with little or no core 3-8 structures, which is consistent with mass spectroscopy studies of isolated E12 glycoproteins. This study has shown that *H. pylori* infection increased expression of some sialyltransferases which may lead to shortened O-glycan chains. Furthermore, differential changes to various ppGalNAc transferase transcripts (mainly downregulation) suggested effects on O-glycan packing density and O-glycan site occupancy. The enzyme profiles would also suggest a change in N-glycan structures upon infection to more high-mannose and less complex oligosaccharides. Such changes may not affect the overall glycocalyx since it is so O-glycan dominated but could affect specific N-glycoproteins. Many of the transferase enzyme transcripts that are regulated upon *H. pylori* infection have a noticeable preference for glycolipids which is consistent with the presence of Lewis b ligands on glycolipids rather than glycoproteins in E12 cells[36]. While the overall changes to glycosyltransferase transcripts upon infection were small, small changes in enzyme activities that determine physicochemical properties of the mucus and the range of exposed epitopes could lead to profound effects on the phenotype of these infected cells.

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COMMENTS

Background

Key pathogen-host cell interactions are likely mediated through the glycan components of surface glycoproteins and glycolipids. *Helicobacter pylori* (*H. pylori*) is protected in the harsh environment of the stomach by the mucus layer of the gastric epithelium. Here we have used a HT29-derivative cell line that secretes a thick adherent mucus layer. This study explores changes in host cell gene transcription, specifically the genes that code mucins, glycosyltransferases, glycosyl hydrolases and lectins that may be associated with *H. pylori* infection.

Research frontiers

H. pylori infections are currently effectively cleared by antibiotics, however, the occurrence of antibiotic resistant strains is increasing and it is inevitable that new treatments will need to be developed. Understanding the key interactions between host and pathogen will drive the development of new drugs possibly analogues of surface sugars such as sialic acid and fucose.

Innovations and breakthroughs

Different strains of *H. pylori* display different levels of virulence. Some strains of *H. pylori* bind *via* BabA adhesins to Lewis antigens on the host cells and the host response is mediated through the TLR2 receptor. Here they use a common virulent strain (26695) that does not present one of the typical adhesins (BabA) commonly involved in *H. pylori* adhesion.

Applications

Changes in glycan structure on host cells in response to *H. pylori* infection will be changes initiated by the host to fight off infection and changes in the host induced by the infecting bacteria. By understanding which enzymes are involved in these host and pathogen modified glycan structure changes, it may be possible to design specific inhibitors of these enzymes.

Terminology

Transepithelial electrical resistance is a measure of the integrity of a cell layer and indicates that tight junctions have formed between cells.

Peer-review

This is a well written manuscript that attempts to identify genes significantly differentially expressed upon *H. pylori* infected in the HT29 derivative cell line by using microarray analysis and qRT-PCR analysis.

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ORIGINAL ARTICLE

Basic Study

STAT3 deficiency prevents hepatocarcinogenesis and promotes biliary proliferation in thioacetamide-induced liver injury

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Abstract

AIM

To elucidate the role of STAT3 in hepatocarcinogenesis and biliary ductular proliferation following chronic liver injury.

METHODS

We investigated thioacetamide (TAA)-induced liver injury, compensatory hepatocyte proliferation, and hepatocellular carcinoma (HCC) development in hepatic STAT3-deficient mice. In addition, we evaluated TAA-induced biliary ductular proliferation and analyzed the activation of sex determining region Y-box9 (SOX9) and Yes-associated protein (YAP), which regulate the transdifferentiation of hepatocytes to cholangiocytes.

RESULTS

Both compensatory hepatocyte proliferation and HCC formation were significantly decreased in hepatic STAT3-deficient mice as compared with control mice. STAT3 deficiency resulted in augmentation of hepatic necrosis and fibrosis. On the other hand, biliary ductular proliferation increased in hepatic STAT3-deficient livers as compared with control livers. SOX9 and YAP were upregulated in hepatic STAT3-deficient hepatocytes.

CONCLUSION

STAT3 may regulate hepatocyte proliferation as well as transdifferentiation into cholangiocytes and serve as a therapeutic target for HCC inhibition and biliary regeneration.

Key words: Hepatocellular carcinoma; Ductular reaction; Signal transducer and activator of transcription 3; Yes-associated protein; Sex determining region Y-box9; Transdifferentiation

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Core tip: Cell transdifferentiation has been identified as one of the crucial sources of organ regeneration. In liver regeneration, both mature hepatocytes and cholangiocytes have been shown to transdifferentiate to each other to maintain hepatic homeostasis. Although STAT3 has been also shown to be implicated in cellular differentiation, the differentiative role of STAT3 in liver regeneration remains unknown. In this study, we found that hepatic-STAT3 deficiency promoted biliary ductular proliferation with upregulation of Yes-associated protein (YAP)/sex determining region Y-box9 (SOX9), crucial regulators of transdifferentiation, in liver injury of mice. This work suggests that STAT3 determines liver cell fate regulating YAP/SOX9 in liver regeneration.

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INTRODUCTION

Interleukin (IL)-6 family cytokines are indispensable to liver regeneration. The Janus kinase/signal transducers and activators of transcription (JAK/STAT3) pathway is thought to play the central role in signal transduction mediated by IL-6 family. Several target genes of STAT3 have been identified; these genes include *cyclin D, c-myc, bcl-xL*, and *mcl-1*, which are essential for cell proliferation and survival. In murine models of liver injury, ablation of STAT3 in liver results in enhanced liver injury and reduced liver regeneration[1]. These findings suggest the protective and proliferative roles of STAT3 in liver regeneration. In addition, STAT3 has been implicated in cellular differentiation, and its activation is associated with differentiation of T cells and macrophages[2]. Studies have demonstrated that STAT3 is essential for the maintenance of pluripotency of embryonic stem (ES) cells[3,4] and self-renewal of tumor-initiating cells in the liver[5]. However, the differentiative role of STAT3 in liver regeneration is questionable.

Both cholangiocytes and hepatocytes are derived from hepatoblasts generated in the foregut endoderm during liver development. The bipotential hepatobiliary stem/progenitor cells, such as small hepatocytes and oval cells, have been shown to exist in adult liver and are thought to contribute to liver regeneration[6]. On the other hand, previous reports have revealed direct transdifferentiation between hepatocytes and cholangiocytes in chronic liver damage[7,8]. Cholangiocytes transdifferentiate into hepatocytes and replenish massive hepatocyte loss[9]. In reverse, cholangiocytes were shown to be generated from transdifferentiating hepatocytes during biliary injury[10]. These evidences suggest that both mature cholangiocytes and hepatocytes play complimentary roles to promote liver regeneration through transdifferentiation. Sex determining region Y-box9 (SOX9), required for the normal differentiation of the biliary tract, is primarily expressed in mature cholangiocytes. However, weak expression of SOX9 was also observed in a small population of hepatocytes, which may display the potential to transdifferentiate into cholangiocytes[11]. Otherwise, cellular fate in the liver is regulated by Yes-associated protein (YAP), an important effector of Hippo pathway[12,13]. YAP depletion in the liver is characterized by cholangiocytes proliferation after biliary obstruction[14]. In contrast, YAP activation in hepatocytes results in their transdifferentiation into cholangiocytes, leading to over-

growth of atypical ductal cells - a process termed as atypical ductular reaction[12]. By interacting with TEAD transcription factor, YAP induces the expression of target genes, including *Notch2*[15]. NOTCH2 signaling, in turn, induces the expression of its target genes *SOX9*[12]. Therefore, YAP/SOX9 axis promotes transdifferentiation of hepatocytes into cholangiocytes. However, the mechanism underlying the regulation of YAP/SOX9 axis activation in hepatocytes needs to be elucidated.

To investigate if STAT3 is implicated in hepatocyte proliferation and differentiation in liver injury, we evaluated thioacetamide (TAA)-induced hepatocarcinogenesis and hepatocyte transdifferentiation in mouse with STAT3-deficient liver. STAT3-deficient liver showed profound ductular proliferation with upregulated YAP/SOX9 expression in hepatocytes. Our study indicates that STAT3 regulates transdifferentiation of hepatocytes into biliary cells.

MATERIALS AND METHODS

Mice

Mice were hosted in the cage whose dimension is 218 mm × 320 mm × 133 mm, in which recycled paper bedding and enrichment that stimulates natural instincts were put, and had free access to water and a standard rodent diet during the experimental period. All mice were maintained under specific pathogen-free conditions, and were monitored every other day. We observed any signs of abnormalities (*i.e.* marked weight loss or behavior change), but no mice became ill or died prior to the experimental end point. All mice experiments were conducted in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee. Albumin-Cre (Alb-Cre) mice on a C57/BL6 background expressing Cre recombinase under the control of the mouse albumin gene regulatory region and STAT3$^{flox/flox}$ mice on a C57/BL6 background were prepared previously described[16]. To generate hepatic-STAT3 deficient (*STAT3*$^{\Delta hep}$) mice, Alb-Cre mice were crossed with STAT3 $^{flox/flox}$ mice. 4-6 wk male mice weighing 15-20 g were used in this study. For modelling medium- and long-term Thioacetamide (TAA) responses such as inflammation, cirrhosis, and hepatocellular carcinoma (HCC), individual mice received drinking water with TAA (300 mg/L; Wako) for 16 wk (n = 3 for *STAT3*$^{\Delta hep}$ mice, n = 4 for control mice (wild-type mice or STAT3 $^{flox/flox}$ mice) and 30 wk (n = 6 for both *STAT3*$^{\Delta hep}$ and control mice) respectively. Mice were sacrificed by an overdose of diethyl ether.

Histological and immunohistochemical staining

Liver specimens were fixed in 10% buffered formalin and embedded in paraffin. Then, consecutive 4-μm sections were cut and stained with hematoxylin and eosin. Histopathological diagnosis was made according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer edited by the Liver Cancer Study Group of Japan. For evaluation of fibrosis, Azan staining was performed on paraffin-embedded tissue sections using standard methods. The area of fibrosis was quantified using WinRoof image software (version 6.1, Mitani Corporation, Fukui, Japan) and the results were presented as the ratio of the fibrotic area to the total area of the image. We performed immunohistochemical staining of the paraffin-embedded tissue slices using antibodies against; PCNA (PA5-27214; Thermo Fisher Scientific; dilution 1:100), Keratin19 (KRT19; a gift of Atsushi Miyajima; dilution 1:500), SOX9 (ab71762; Abcam, Cambridge, United Kingdom; dilution 1:500) and YAP (ab39361; Abcam, Cambridge, United Kingdom; dilution 1:100). The slices were soaked with 10mM citrate buffer (pH 6.0) and autoclaved at 121℃ for 5 min for antigen retrieval. Nonspecific protein staining was blocked by preincubation with Protein Block Serum-Free (DAKO). The slices were then exposed to each primary antibody or rabbit IgG as a negative control at 4℃ overnight. After washing, the sections were incubated with Envision secondary antibody labeled with horseradish peroxidase-polymer complexes (DAKO) and stained with DAB. The cellular nuclei were counterstained with hematoxylin.

Immunoblotting analysis

Isolated hepatocytes and liver tissues were homogenized with RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1 mmol/L EDTA (pH 8.0), 0.1 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 100 mmol/L PMSF, 1 mmol/L DTT, 2 × Protease Inhibitor Cocktail (Nacalai Tesque), and 2 × Halt phosphatase inhibitor cocktail (Pierce). Equal proteins (35 μg) were subjected to SDS-PAGE and subsequently transferred onto equilibrated polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, United States). Immunoblotting analysis was performed using antibodies against; SOX9 (ab71762; Abcam, Cambridge, United Kingdom) and YAP (ab39361; Abcam, Cambridge, United Kingdom), phospho (Y357)-YAP (ab62751; Abcam, Cambridge, United Kingdom), Alpha-1-Fetoprotein (AFP) (DAKO, Glostrup, Denmark), phospho(Y416)-Src family (#2101; Cell Signaling Technologies, Beverly, MA, United States), STAT3 (sc 482; Santa Cruz Biotechnology, CA, United States) and GAPDH (sc25779; Santa Cruz Biotechnology, CA, United States).

Quantitative real-time PCR

Total RNA was isolated using the TRIZOL Reagent I (Invitrogen, Carlsbad, CA, United States) and reverse transcription was performed using Superscript Ⅲ (Invitrogen). Real time PCR was carried out using SYBR

Green (Life Technologies) and TaqMan Assays (Life Technologies) with the following primers. *GAPDH* control forward: 5′-TGACGTGCCGCCTGGAGAAA-3′, *GAPDH* control reverse: 5′-AGTGTAGCCCAAGATGCCCTTCAG-3′; *SOX-9* forward: 5′-TGCAGCACAAGAAAGACCAC-3′, *SOX-9* reverse: 5′-TCTCAATGTTGGAGATGACG-3′; *KRT19* forward: 5′-GACTTCCTATAGCTATCGCC-3′, *KRT19* reverse: 5′-AGTAGGAGGCGAGACGATCA-3′; *AFP* forward: 5′-GGCCGACATTTTCATTGGACATT-3′, *AFP* reverse: 5′-TGGGGGAGGGGCATAGGTTTT-3′; *MMP2* forward: 5′-GATAACCTGGATGCCGTCGTG-3′, *MMP2* reverse: 5′-CTTCACGCTCTTGAGACTTTGGTTC-3′; *MMP9* forward: 5′-GCTGACTACGATAAGGACGGC-3′, *MMP9* reverse:5′-AGGAAGACGAAGTGAAGGGGAAGA-3′. TaqMan probes/primers for YAP (Mm01143263_m1), IL-6 (Mm00436767_m1), IL-33 (Mm00505403_m1) and Osteopontin (Mm00436767_m1) were purchased from Life Technologies. Expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The relative amount of specific mRNA was normalized to GAPDH.

Statistical analysis

Statistical significance was assessed using the Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant. The data were presented as mean ± SE.

RESULTS

Hepatic STAT3 deficiency enhanced necroinflammatory change

To examine the protective role of STAT3 in hepatocytes, we first examined whether hepatic-STAT3 deficiency promoted TAA-induced liver injury. $STAT3^{flox/flox}$ mice were crossed with Alb-Cre mice to generate hepatic-STAT3 deficient ($STAT3^{\Delta hep}$) mice (Figure 1A). $STAT3^{\Delta hep}$ and control mice were given water with TAA. Metabolites of TAA, which are covalently bound to proteins and lipids, caused hepatocytes necrosis and periportal infiltration of inflammatory cells. As shown Figure 1B, TAA-induced inflammatory infiltrates were augmented in $STAT3^{\Delta hep}$ mice compared to control mice. Moreover, TAA-induced necrotic changes of hepatocytes were also enhanced in $STAT3^{\Delta hep}$ mice (Figure 1B and C), indicating that hepatic-STAT3 deficiency promoted TAA-induced hepatocytes injury. As a consequence of enhanced inflammatory change, $STAT3^{\Delta hep}$ mice exhibited significantly severe liver fibrosis compared to control mice (Figure 1D and E).

Hepatic STAT3 deficiency suppressed hepatocyte proliferation and HCC development

To elucidate the proliferative role of STAT3, we next examined whether TAA-induced hepatocytes regeneration was attenuated in $STAT3^{\Delta hep}$ mice. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) showed compensatory proliferation of hepatocytes in TAA-induced liver injury (Figure 2A). PCNA positive hepatocytes were significantly decreased in $STAT3^{\Delta hep}$ mice (Figure 2A and B), indicative of the proliferative role of hepatic STAT3 in TAA-induced liver injury. Compensatory proliferation triggered by hepatocyte loss leads to not only liver regeneration but also development of HCC. Therefore, we next examined whether TAA-induced HCC development was inhibited in $STAT3^{\Delta hep}$ mice. TAA treatment for thirty-week developed liver tumors which were histologically consistent with well-differentiated HCC with no evident cholangiocellular carcinoma (Figure 2C). The liver tumor formations of $STAT3^{\Delta hep}$ mice were significantly lesser than those of control mice (Figure 2D). These findings suggested that STAT3 was required, at least in part, for compensatory hepatocyte proliferation leading to HCC development.

Hepatic STAT3 deficiency accelerated TAA-induced biliary ducts and ductular structure formation

Hepatic necroinflammatory change induces atypical ductular reaction, which is characterized by proliferation of ductular cells. TAA treatment caused the biliary duct/ductular structure formation especially around periportal area (Figure 3A). To confirm the characteristics of bile duct/ductular cells, we performed immunohistochemical staining for KRT19, a marker of mature cholangiocytes. Most of ducts/ductular cells were immunoreactive for KRT19 (Figure 3B). The number of bile duct/ductular structures around periportal area of $STAT3^{\Delta hep}$ mice was higher than that of control mice (Figure 3C left). The bile duct/ductular formations in centrilobular area were rarely observed in control mice, but those of $STAT3^{\Delta hep}$ mice were significantly increased (Figure 3C right). KRT19-positive bile ducts/ductular structures were significantly expanded at both periportal and centrilobular area in $STAT3^{\Delta hep}$ mice (Figure 3C and D).

STAT3 deficient hepatocytes showed upregulated SOX9 expression following TAA-induced liver injury

The formation of ducts/ductular structures has been thought to be attributable to both proliferation of mature cholangiocytes and biliary transdifferention of hepatocytes. To examine the origin of bile ducts/ductular structures, we next performed immunohistochemical staining for SOX9, because SOX9 is expressed in cholangiocytes and bipotential hepatobiliary cells[11]. As shown Figure 4A, the bile ducts/ductular structures were frequently composed of SOX9-positive cells. The number of SOX9-positive bile ducts/ductules of $STAT3^{\Delta hep}$ mice was higher than that of control mice (Figure 4B). Furthermore, immunoreactive intensity for SOX9 was increased in the bile ducts/ductular cells of $STAT3^{\Delta hep}$ mice compared to control mice. TAA treatment upregulated SOX9 expression in periportal hepatocytes. Interestingly, SOX9 expression was also

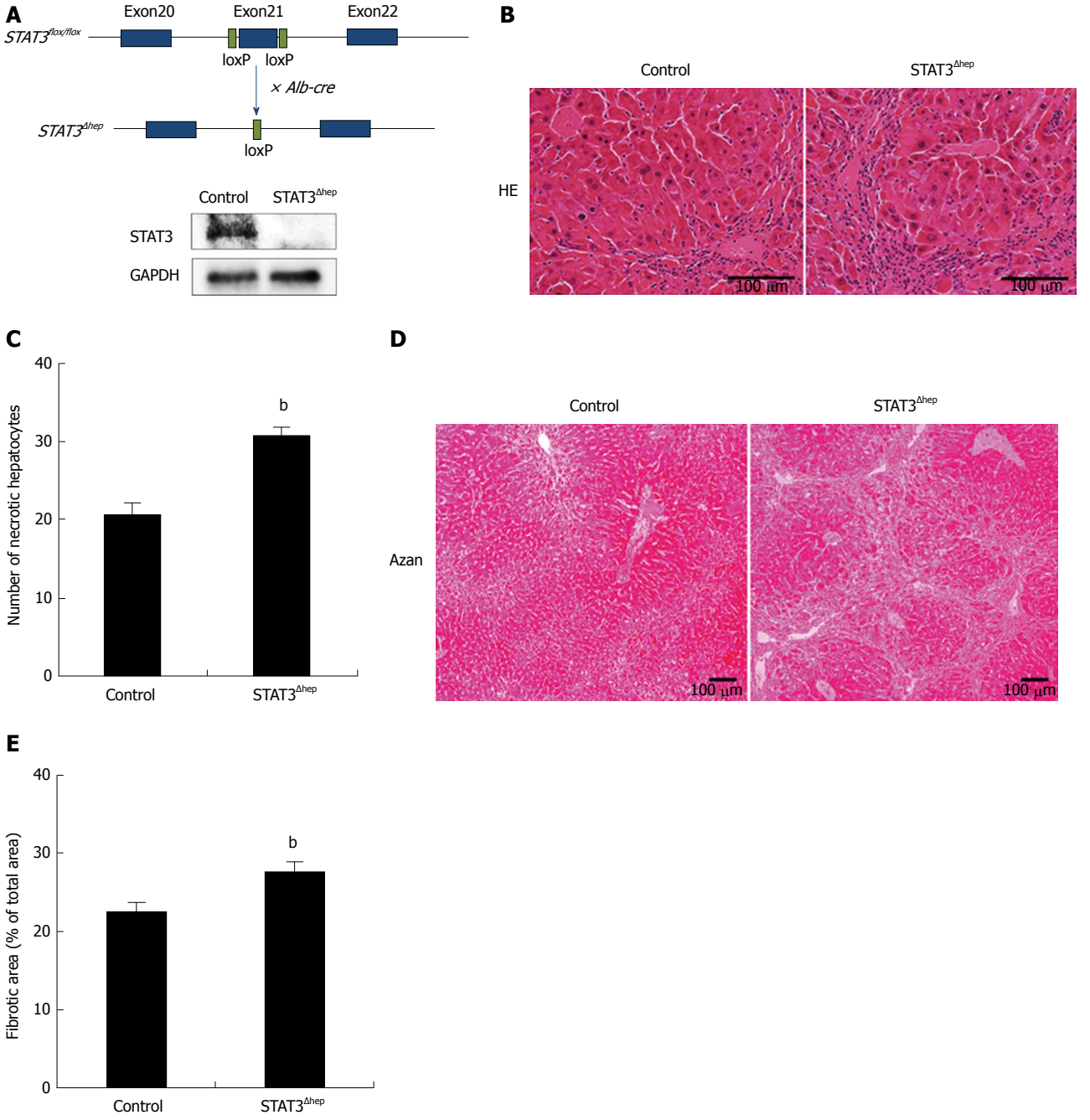


Figure 1 Establishment of hepatic-STAT3 deficient (*STAT3$^{\Delta hep}$*) mice. Hepatic-STAT3 deficiency enhanced thioacetamide-induced liver injury. A: The exon 21 of STAT3 was flanked by two identically oriented loxP sites (STAT3$^{flox/flox}$). To disrupt hepatic-STAT3 gene, STAT3$^{flox/flox}$ mice were crossed with mice carrying albumin promoter-driven Cre transgene (Alb-Cre). Deficiency of hepatic-STAT3 protein was confirmed by immunoblotting analysis. B: Representative hematoxylin and eosin (H&E) staining images of control and STAT3$^{\Delta hep}$ livers with TAA treatment for 16 wk. Hepatic-STAT3 deficiency accelerated inflammation cells infiltration and hepatocytes necrosis. C: Quantification of necrotic hepatocytes. Necrotic hepatocyte was identified with the eosinophilic/vacuolated cytoplasm and nuclear destruction such as karyolysis or Pyknosis. Values represent means ± SE of the mean. $^{b}P < 0.01$ ($P = 0.000269$) *vs* control. D: Representative Azan staining of control and STAT3Δhep livers with TAA treatment for 16 wk. E: Quantification of the ratio of fibrosis area to the total area in control and STAT3$^{\Delta hep}$ livers. Values represent means ± SE of the mean. $^{b}P < 0.01$ ($P = 0.00235$) *vs* control. TAA: Thioacetamide.

augmented in STAT3-deficient hepatocytes (Figure 4C).

YAP was activated in STAT3-deficient hepatocytes following TAA-induced liver injury

We next performed quantitative RT-PCR analysis to determine whether SOX9 expression was regulated at transcriptional level. TAA treatment upregulated the level of *SOX9* mRNA expression, which was significantly increased in the liver of *STAT3$^{\Delta hep}$* mice compared to that of control (TAA 16 wk) (Figure 5A). Immunoblot analysis confirmed higher levels of SOX9 expression in the liver of *STAT3$^{\Delta hep}$* mice (Figure 5B). In accordance with the marked bile duct/ductular cell proliferation, the liver of *STAT3$^{\Delta hep}$* mice showed robust up-regulation of KRT19 expression at transcriptional and protein level. Although IL-33 is a

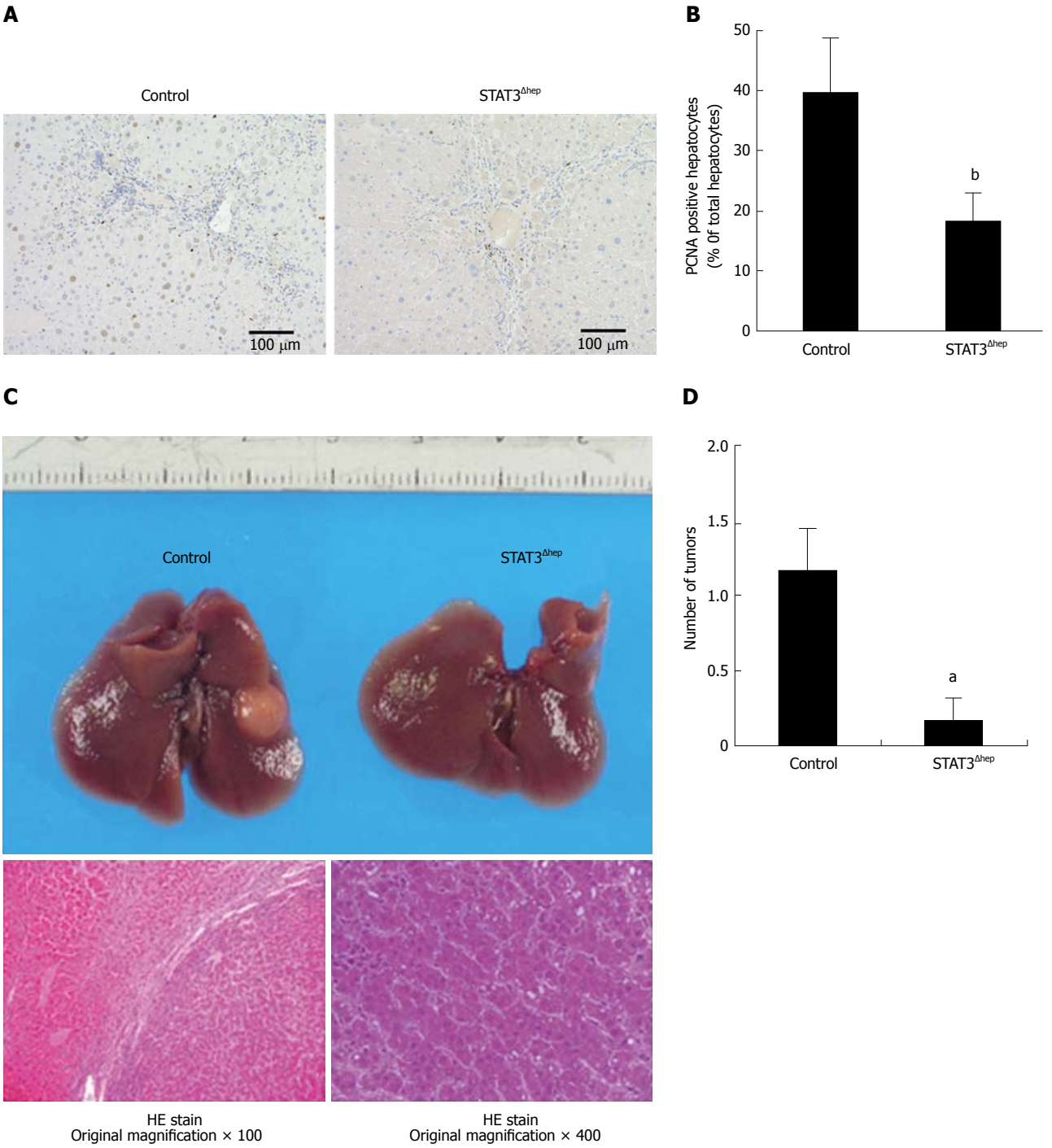


Figure 2 Hepatic STAT3 deficiency reduced hepatocyte proliferation and inhibited the development of hepatocellular carcinoma. A: Representative immunohistochemical staining for PCNA in livers of control and STAT3$^{\Delta hep}$ mice with TAA treatment for 16 wk. PCNA positive hepatocytes were decreased in STAT3$^{\Delta hep}$ mice. Original magnification × 200. B: The number of PCNA positive hepatocytes. The ratio of PCNA positive hepatocytes to all hepatocytes was quantified in three different samples per a group. Values represent means ± standard error of the mean. $^{b}P < 0.01$ ($P = 0.000232$) *vs* control. C: Gross liver appearance of control and STAT3$^{\Delta hep}$ mice with TAA treatment for 30 wk. Tumor formation with TAA treatment for 30 wk (upper panel). HE staining of representative sections of hepatocellular carcinoma. Original magnification × 100 (lower left panel) and × 400 (lower right panel). D: The number of HCC in control and STAT3$^{\Delta hep}$ mice. Values represent means ± SE of the mean ($n = 6$). $^{a}P < 0.05$ ($P = 0.0219$) *vs* control. TAA: Thioacetamide.

pivotal growth factor for cholangiocytes, *IL-33* mRNA was not upregulated in the liver of *STAT3*$^{\Delta hep}$ mice (Figure 5A). On the other hand, AFP expression of the liver of *STAT3*$^{\Delta hep}$ mice was decreased as compared to that of control mice (Figure 5A and B). Whereas YAP expression was restricted in normal liver, TAA treatment resulted in upregulation of YAP expression. *YAP* mRNA expression in the liver of *STAT3*$^{\Delta hep}$ mice was slightly higher than that of control mice. However, YAP protein expression was markedly increased in the liver of *STAT3*$^{\Delta hep}$ mice. Previously, it was shown that src-family kinase induced-YAP tyrosine (Y357) phosphorylation leads to its stabilization and nuclear localization, activating its transcriptional property[17].

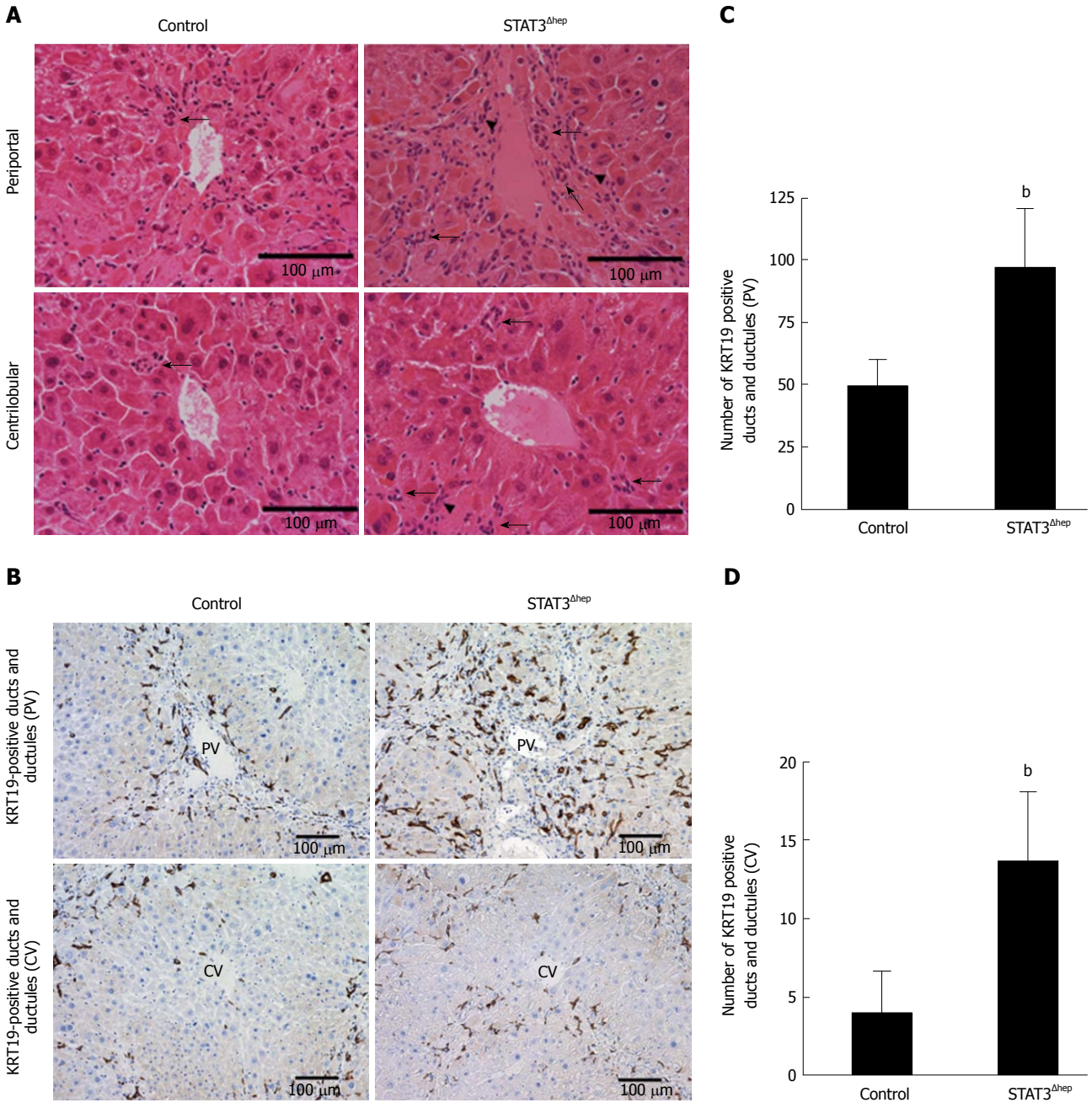


Figure 3 Increase in the formation of bile ducts and ductular structure in hepatic STAT3 deficient liver. A: Hematoxylin and eosin staining images of representative areas around portal vein (PV) and central vein (CV) of control and *STAT3*$^{\Delta hep}$ livers following treatment with TAA for 16 wk. TAA treatment caused bile ducts/ductular formation (arrow). B: Immunohistochemistry staining for keratin 19 (KRT19) in control and *STAT3*$^{\Delta hep}$ livers following TAA treatment for 16 wk. Original magnification × 200. C and D: The number of KRT19-positive bile ducts/ductules around PV and CV. To quantify the number of bile ducts/ductules around PV and CV, we observed three different samples per group using 20 × objective lens, imaged five randomly selected cross-sectional veins in the center of field, and calculated the average value after counting the bile duct/ductule number in the total area of the image. Values represent means ± SE of the mean. $^{b}P < 0.01$ [(C) $P = 0.00272$, (D) $P = 0.00187$] *vs* control. TAA: Thioacetamide.

YAP tyrosine (Y357) phosphorylation and src-kinase phosphorylation were enhanced in the liver of *STAT3*$^{\Delta hep}$ mice. Furthermore, immunohistochemical staining showed robust YAP expression in the nucleus of STAT3-deficient hepatocytes, indicating hepatocytes-transdifferentiation (Figure 5C and D). Taken together, profound proliferation of bile duct/ductular cells in the liver of STAT3$^{\Delta hep}$ mice may not be due to the proliferation of mature cholangiocytes but may be associated with transdifferentiation of hepatocytes into cholangiocytes.

DISCUSSION

In this study, we show that STAT3-deficient liver displayed reduced HCC development and promoted biliary ductular proliferation during TAA-induced liver injury. STAT3-deficient hepatocytes exhibited YAP activation and upregulation of SOX9 expression, indicating transdifferentiation of hepatocytes into

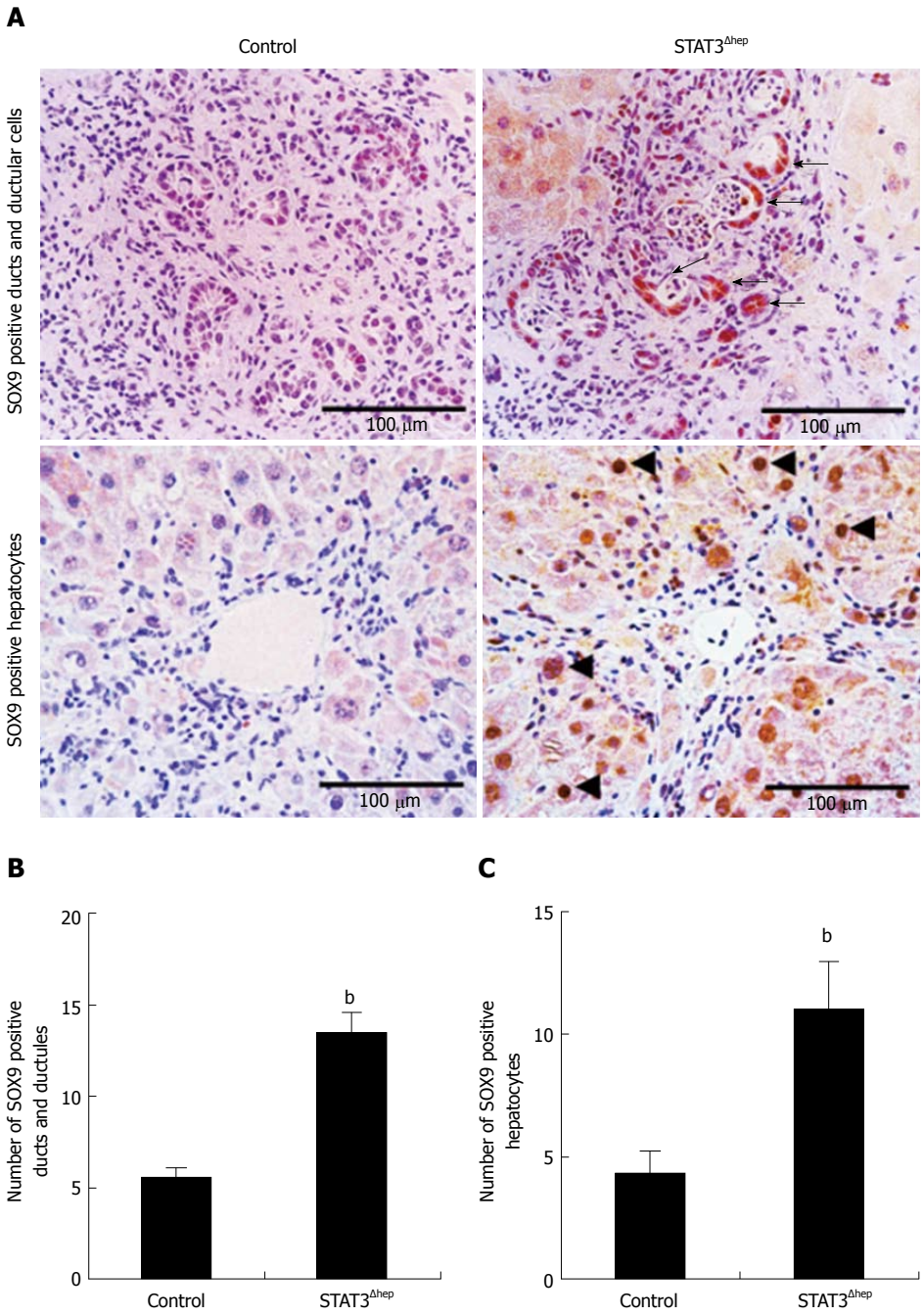


Figure 4 Hepatic STAT3 deficiency upregulated SOX9 expression in thioacetamide-induced liver injury. A: Immunohistochemistry staining for SOX9 in control and STAT3$^{\Delta hep}$ livers with TAA treatment for 16 wk. SOX9 expression was markedly enhanced in bile ducts/ductular structure (upper panel) and hepatocytes (lower panel) of STAT3Δhep livers. Original magnification × 200 (upper panel) and × 400 (lower panel). B and C: The number of SOX9 positive bile ducts/ductular structure and hepatocytes. For quantification of SOX9 positive bile ducts/ductular structure, three different samples per a group were observed using a 20 × objective lens and SOX9 positive ducts/ductular structure was counted at five PV areas. For quantification of SOX9 positive hepatocytes, three different samples per a group were observed using a 40 × objective lens and SOX9 positive hepatocytes was counted at five PV areas. Values represent means ± standard error of the mean. $^{b}P < 0.01$ [(B) $P = 0.0000103$, (C) $P = 0.00834$] *vs* control. TAA: Thioacetamide.

cholangiocytes. This study is the first report that STAT3 is implicated in both proliferation and differentiation of hepatocytes after liver injury.

IL-6 family cytokines are obviously essential for liver repair. IL-6-deficiency impairs liver regeneration and causes liver failure characterized with blunted DNA synthesis in hepatocytes but not in nonparenchymal cells[18]. The role of JAK/STAT3 pathway has been extensively evaluated and thought to be essential for IL-6-mediated liver repair, because STAT3 regulates many of genes associated with cell survival and proliferation. During liver regeneration after partial hepatectomy, hepatocytes-STAT3 activation was observed in periportal area[19], which might harbor putative stem cell nische[20]. Recently, periportal hepatocytes were shown to have extensive proliferative property for liver repair[11]. These reports suggested that STAT3 activation in periportal area was crucial for

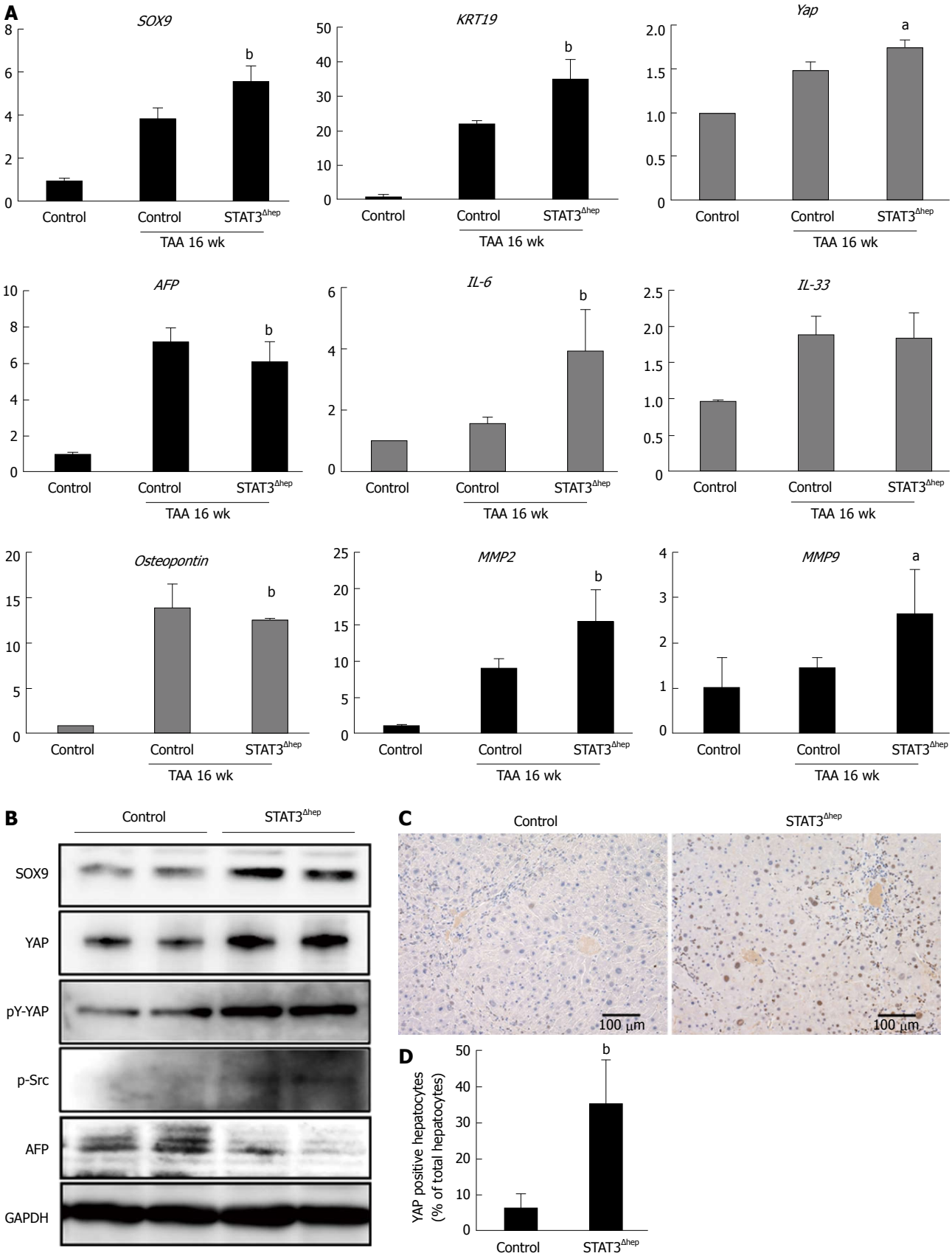


Figure 5 YAP activation in STAT3-deficient hepatocyte with thioacetamide treatment. A: Quantitative real-time RT-PCR analysis for *SOX-9*, *KRT 19*, *YAP*, *AFP*, *IL-6*, *IL-33*, *Osteopontin*, *MMP2*, and *MMP9* mRNA expression levels. The relative expression of each gene mRNA was normalized to GAPDH mRNA (n = 6). ^{a}P < 0.05 (*YAP*; P = 0.0134, *AFP*; P = 0.00347, *MMP9*; P = 0.00271) and ^{b}P < 0.01 (*SOX9*; P = 0.00274, *KRT19*; P = 0.00402, *IL-6*; P = 0.00147, *MMP2*; P = 0.00404) *vs* control (TAA treatment for 16 wk). B: Immunoblotting analysis for SOX-9, YAP, pY-YAP, p-Src and AFP protein expression levels. GAPDH served as loading control. C: Representative immunohistochemical staining for YAP in livers of control and STAT3$^{\Delta hep}$ mice with TAA treatment for 16 wk. Nuclear YAP was augmented in STAT3$^{\Delta hep}$ hepatocytes with TAA treatment for 16 wk. Original magnification × 200. D: The number of nuclear YAP positive hepatocytes. Ratio of nuclear YAP positive hepatocytes to all hepatocytes was quantified in three different samples per a group. Values represent means ± SE of the mean. ^{b}P < 0.01 (P = 0.00312) *vs* control. TAA: Thioacetamide.

liver regeneration. Indeed, TAA-induced liver damage was augmented in liver specific STAT3-deficient mice compared to control mice. However, hepatic STAT3 deficiency did not cause lethal liver injury after TAA treatment. Consistently, previous reports showed that hepatic STAT3 deficiency, unlike IL-6 deficiency, caused modest reduction of liver regeneration with no liver failure after partial hepatectomy[1]. These findings suggest that hepatic STAT3 plays a part of IL-6-mediated liver repair property. Because IL-6 family cytokines activate ERK/MAPK and PI3K/Akt pathways as well as JAK/STAT3 pathway through its receptor gp130, these pathways may lead to compensation of proliferation in STAT3-deficient hepatocytes.

Until recently, YAP has been shown to regulate cell proliferation in some organs including liver[21]. By binding to TEAD transcription factor in nucleus, YAP activates expression of multiple genes including *CTGF, CCND1* and *BCL2L1* responsible for cell proliferation, antiapoptosis and survival. In Hippo/Lats pathway, YAP serine (S127) phosphorylation results in cytoplasmic sequestration and inhibition of its transcriptional coactivator activity[22,23]. In contrast, src-family kinase induced-YAP tyrosine (Y357) phosphorylation leads to its stabilization and nuclear localization, activating its transcriptional property[17]. Recent report revealed that IL-6-mediated YAP tyrosine (Y357) phosphorylation via a gp130-src family kinase module promotes intestinal epithelial proliferation *in vivo*[24]. Therefore, YAP and STAT3 cooperatively might promote hepatocytes proliferation and survival for IL-6-mediated liver regeneration. Compensatory YAP activation probably due to IL-6 upregulation might restore the property of STAT3-deficient hepatocytes proliferation and survival. Recently IL-22 has also been shown to be critical for liver regeneration[25]. Although IL-22 does not interact gp130, IL-22 receptor bind SHP2 leading to activation of both src family kinase and STAT3[26]. YAP and STAT3 might be involved in not only IL-6-mediated but IL-22-mediated liver regeneration.

In addition to the role of cell proliferation and survival, YAP sustains undifferentiated state and pluripotency through regulating expression of stemness-associated genes such as *Oct4*, *Sox2* and *Nanog*[13]. YAP is overexpressed in cultured ES cells and may be required for self-renewal and suppression of differentiation[27]. YAP activation was shown to dedifferentiate mature hepatocytes into hepatobiliary progenitor cells[12]. SOX9-positive periportal hepatocytes display high regenerative property and were shown to self-renew and transdifferentiate into cholangiocytes[11]. It is interesting that SOX9 expression is upregulated by YAP activation through NOTCH pathway[12]. Therefore, activation of YAP/SOX9 axis is critical for hepatocyte dedifferentiation to accomplish liver repair. However, the regulation of YAP/SOX9 axis activation in hepatocytes is still unknown. In this study, we found that STAT3 deficiency enhanced YAP tyrosine (Y357) phosphorylation and SOX9 expression through Src activation during TAA-induced liver injury. These findings suggest that STAT3 may inhibit dedifferentiation and transdifferentiation of hepatocytes by preventing the activation of YAP/SOX9 axis.

Demetris *et al*[28] previously reported that KRT19 positive/AFP negative hepatocytes, called ductular hepatocytes, appeared to exhibit ductular reaction in submassive liver necrosis. The ductular hepatocytes were thought to contribute to biliary repair through transdifferentiation into cholangiocytes. As AFP synthesis and secretion were derived from differentiated hepatocytes in liver injury, TAA treatment upregulated AFP expression reflecting hepatocytes repopulation. The TAA treatment-induced AFP upregulation was significantly inhibited in hepatic-STAT3 deficient mice, whereas the expression of KRT19 was upregulated compared to control mice. Interestingly, AFP expression is suppressed by YAP in undifferentiated ES cells, and YAP inhibition results in AFP expression with differentiation of ES cell[27]. These findings suggest that YAP activation in STAT3-deficient hepatocytes might direct their transdifferentiation into cholangiocytes instead of their dedifferentiation into progenitor cells. An important question is whether YAP activation in STAT3-deficient hepatocytes contribute to development of liver cancer, as YAP has been found to be involved in cholangiocarcinoma[29]. However, hepatic-STAT3 deficient mice did not cause cholangiocarcinoma, suggesting that STAT3 deficiency-induced YAP activation is insufficient for oncogenic transformation at least in the model of TAA-induced liver injury. Otherwise, hepatic STAT3 depletion promoted fibrotic regeneration in TAA-induced liver injury model. Recent report showed that YAP expression was positively correlated with liver fibrosis in non-alcoholic steatohepatitis[30]. Because YAP has also been found to promote epithelial-mesenchymal transition[31], the possibility is not excluded that YAP activation might promote transdifferentiation of hepatocytes into myofibroblast leading to liver fibrosis.

In conclusion, STAT3 is not only involved in HCC development but also prevents biliary ductular formation in TAA-induced liver injury. Our study highlights hepatic STAT3 as a plausible target for biliary repair during liver injury.

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COMMENTS

Background

JAK/STAT3 pathway plays the central role in signal transduction mediated by

IL-6 family, indispensable cytokines for liver regeneration. Several target genes of STAT3 are essential for cell proliferation and survival. In murine models of liver injury, ablation of STAT3 in liver results in enhanced liver injury and reduced liver regeneration.

Research frontiers

Cholangiocytes transdifferentiate into hepatocytes and replenish massive hepatocyte loss. In addition, cholangiocytes are generated from transdifferentiating hepatocytes during biliary injury. STAT3 is implicated in cellular differentiation of some immune cells, although the role of STAT3 in transdifferentiation between hepatocytes and cholangiocytes has not been elucidated.

Innovations and breakthroughs

This study indicates that STAT3 regulates transdifferentiation of hepatocytes into biliary cells through Yes-associated protein (YAP)/Sex determining region Y-box9 (SOX9) axis activation.

Applications

Hepatic STAT3 is a plausible target for biliary repair during liver injury.

Terminology

YAP, an important effector of Hippo pathway which controls organ size in animals through the regulation of cell proliferation and apoptosis. A recent report revealed that YAP activation in hepatocytes results in their transdifferentiation into cholangiocytes. SOX9, one of transcription factors which is required for the normal differentiation of the biliary tract. It was recently reported that SOX9-positive periportal hepatocytes transdifferentiate into cholangiocytes and that SOX9 expression is upregulated by YAP activation. Transdifferentiation, a process in which one mature somatic cell converts into another mature somatic cell without going through an intermediate pluripotent or progenitor cell type.

Peer-review

The authors provided evidence showing that STAT3 may mediate carcinogen-induced hepatocellular carcinoma in mouse model. Hepatocyte-specific deletion of STAT3 reduced compensatory proliferation and cancer formation in response to TAA. Additional test indicated that deficiency of STAT3 enhanced YAP activation and SOX9 expression, which may contribute to the enhanced formation of biliary ductular structures. The manuscript was well-written and the results are straightforward.

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ORIGINAL ARTICLE

Basic Study

Performance verification and comparison of TianLong automatic hypersensitive hepatitis B virus DNA quantification system with Roche CAP/CTM system

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Institutional review board statement: The study was reviewed and approved by the 302 Military Hospital of China Medical Ethic Committee.

Institutional animal care and use committee statement: This study didn't involve animal subjects.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

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Abstract

AIM

To investigate and compare the analytical and clinical performance of TianLong automatic hypersensitive hepatitis B virus (HBV) DNA quantification system and Roche CAP/CTM system.

METHODS

Two hundred blood samples for HBV DNA testing, HBV-DNA negative samples and high-titer HBV-DNA mixture samples were collected and prepared. National standard materials for serum HBV and a worldwide HBV DNA panel were employed for performance verification. The analytical performance, such as limit of detection, limit of quantification, accuracy, precision, reproducibility, linearity, genotype coverage and cross-

contamination, was determined using the TianLong automatic hypersensitive HBV DNA quantification system (TL system). Correlation and Bland-Altman plot analyses were carried out to compare the clinical performance of the TL system assay and the CAP/CTM system.

RESULTS

The detection limit of the TL system was 10 IU/mL, and its limit of quantification was 30 IU/mL. The differences between the expected and tested concentrations of the national standards were less than ± 0.4 Log_{10} IU/mL, which showed high accuracy of the system. Results of the precision, reproducibility and linearity tests showed that the multiple test coefficient of variation (CV) of the same sample was less than 5% for 10^2-10^6 IU/mL; and for 30-10^8 IU/mL, the linear correlation coefficient r^2 = 0.99. The TL system detected HBV DNA (A-H) genotypes and there was no cross-contamination during the "checkerboard" test. When compared with the CAP/CTM assay, the two assays showed 100% consistency in both negative and positive sample results (15 negative samples and 185 positive samples). No statistical differences between the two assays in the HBV DNA quantification values were observed (P > 0.05). Correlation analysis indicated a significant correlation between the two assays, r^2 = 0.9774. The Bland-Altman plot analysis showed that 98.9% of the positive data were within the 95% acceptable range, and the maximum difference was -0.49.

CONCLUSION

The TL system has good analytical performance, and exhibits good agreement with the CAP/CTM system in clinical performance.

Key words: Analytical performance; Hepatitis B virus DNA quantification; Clinical performance; Hepatitis B virus; Real-time quantification PCR

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Core tip: The TianLong automatic hypersensitive hepatitis B virus DNA quantification system achieved a limit of detection of 10 IU/mL, limit of quantification of 30 IU/mL and good analytical performance in terms of accuracy, precision, reproducibility, linearity, genotype coverage and cross-contamination. In clinical performance, the TL system showed good correlation and agreement with the Roche CAP/CTM system.

Li M, Chen L, Liu LM, Li YL, Li BA, Li B, Mao YL, Xia LF, Wang T, Liu YN, Li Z, Guo TS. Performance verification and comparison of TianLong automatic hypersensitive hepatitis B virus DNA quantification system with Roche CAP/CTM system. *World J Gastroenterol* 2017; 23(37): 6845-6853 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6845.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6845

INTRODUCTION

Hepatitis B virus (HBV) infection is a worldwide disease, and over 2 billion people once have been infected with HBV and of these, 240 million have chronic hepatitis B[1,2]. Although there are effective vaccines and anti-viral medicine, HBV infection is still a major condition threatening the human health[3-5]. Globally, 30% of patients with liver cirrhosis and 45% with liver cancer are caused by HBV infection, respectively. Nationally, 500-600 million people in China have been infected with HBV, accounting for nearly 40% of the total national population[6-8]. Currently, China has approximately 93 million chronic HBV infected individuals, and 20 million of these patients have chronic hepatitis B[9,10]. An even higher percentage of patients with liver cirrhosis (60%) and hepatocellular cancer (HCC) (80%) were caused by HBV infection [11,12].

During the last decade, with the advent of new type interferon and antiviral drugs with a high genetic barrier to resistance, the antiviral treatment of chronic HBV infection has achieved significant progress[13-16]. HBV DNA quantification is one of the most important indicators used in HBV antiviral therapy, and can be used to determine whether the patient is suitable for antiviral therapy, monitor antiviral treatment response and virus resistance[17-19]. Use of hypersensitive HBV DNA quantification system to accurately detect HBV DNA in serum is the key factor in determining the curative effect and endpoints of hepatitis B antiviral therapy.

International associations for the study of liver diseases have required HBV DNA quantification detection, with hypersensitivity (a HBV detection kit should reach the sensitivity criterion of 10-15 IU/mL), wide linearity range (1-9 log_{10} IU/mL), and high specificity and good repeatability[20].

Many automatic HBV DNA detection systems are currently available in the Chinese market, including COBAS TaqMan HBV assay used in combination with COBAS AmpliPrep, abbreviated to CAP/CTM (Roche Molecular Diagnostics, Pleasonton, CA, United States), and Abbott Real Time HBV assay used in combination with Abbott m2000sp (Abbott Molecular, Des Plaines, IL, United States). However, due to their high cost and slow detection speed, these assays are not widely applied.

Most of the real-time HBV DNA qPCR detection kits in China have common shortcomings, such as poor specificity, low detection sensitivity, poor quantitative accuracy, narrow quantitative linear range and difficulty in detecting low or high viral load. To address these problems, the Chinese Food and Drug Administration (CFDA) released "The guideline principles for the technical review of hepatitis B virus DNA quantitative detection reagents registration" in

Table 1 Detail information of national standard materials

Art. No.	Standard Material No.	Batch No.	Standard material name	Reference Conc.
HS-0001	GBW (E) 090137	201511003	HBV DNA serum standard material	1.41×10^3 IU/mL
HS-0002	GBW (E) 090138	201512004	HBV DNA serum standard material	5.9×10^4 IU/mL
HS-0003	GBW (E) 090139	201511003	HBV DNA serum standard material	4.6×10^5 IU/mL

2013, which clearly put forward the requirements for HBV DNA detection. These principles will also guide the development direction of hypersensitive HBV DNA detection kits and prompt the rapid development of automatic hypersensitive HBV DNA quantitative detection systems.

The TianLong automatic hypersensitive HBV DNA quantification system (TL system) owns proprietary intellectual property rights, and has been approved by the CFDA. This study evaluates the analytical performance of the TL system and its clinical performance compared with the CAP/CTM system.

MATERIALS AND METHODS

Clinical samples

HBV-DNA positive samples: 200 residual blood samples were obtained from HBV infected patients in the clinical laboratory at 302 Military Hospital of China. These samples included HBV A genotype (4 cases), HBV B genotype (70 cases), HBV C genotype (66 cases), HBV D genotype (28 cases) and unknown genotype (32 cases).

HBV-DNA negative samples: These serum samples were obtained after immunology testing in the clinical laboratory at 302 Military Hospital of China, and were retested using the CAP/CTM system and confirmed to be negative.

High-titer HBV-DNA mixture samples: High-titer HBV-DNA samples were collected from the clinical laboratory at 302 Military Hospital of China. The high-titer HBV-DNA mixture samples were diluted 100 times and measured three times with the CAP/CTM system. The original concentration of the high-titer HBV-DNA mixture samples was calculated to be 2.5×10^8 IU/mL.

The clinical serum samples were tested for co-infections with HIV, HCV and HDV. All results were negative.

Standards samples (reference serum)

The worldwide HBV DNA performance panel (WWHD301, SeraCare, Milford, Massachusetts, United States) which contains HBV DNA (A-H) genotypes was used in this study. National standard materials for HBV serum were used to verify the assay performance including accuracy, limit of detection and limit of quantification. Details of the national standard materials are shown in Table 1.

TianLong automatic hypersensitive HBV DNA quantification system assay

The TL system consists of a PANA 9600E automatic nucleic acid workstation, Gentier 96E real-time quantitative PCR system (Xi'an TianLong Science and Technology, Xi'an, Shaanxi, China), nucleic acid extraction kit (magnetic bead method) and a HBV DNA quantitative detection kit (fluorescence qPCR method).

The experimental method for the TL system assay followed the manufacturer's instructions. Serum of 200 μL was used for HBV DNA extraction, and for PCR reaction assessment, 40 μL for each sample was prepared on the PANA 9600E automatic nucleic acid workstation. The Gentier 96E real-time quantitative PCR system was used for quantitative detection as follows: 50 ℃ for 2 min, 95 ℃ for 3 min, [94 ℃ for 15 s, 60 ℃ for 30 s (read fluorescence)] × 45 cycles. The data were automatically analyzed by software and the results were expressed in international units per milliliter (IU/mL). The TL system detected 96 samples (89 samples, 4 quantitative standards, a negative control, a weak positive control and a strong positive control) within 140 min (80 min for nucleic acid extraction and 60 min for real-time PCR detection).

Roche COBAS HBV v2.0 assay (CAP/CTM)

HBV DNA was extracted from 650 μL of serum by the Cobas AmpliPrep instrument according to the manufacturer's instructions. The Cobas TaqMan 48 analyzer was used for automated real-time PCR amplification and detection of PCR products according to the manufacturer's instructions. The data thus generated were analyzed with Amplilink software. HBV DNA levels were expressed in international units per milliliter (IU/mL).

Limit of detection

National standard materials for HBV serum [GBW (E) 090137, Batch No. 201511003] at a concentration of 1.41×10^3 IU/mL were diluted with HBV DNA negative serum to nominal concentrations of 30 IU/mL, 15 IU/mL, 10 IU/mL, 5 IU/mL, and 2.5 IU/mL. 25 replicates per run were tested with three different batches of reagents in three runs, and the test results were log transformed to verify whether the limit of detection of the TL system met the standard (10-15 IU/mL) recommended by the European Association for the

Table 2 Limit of detection test results

Sample Conc. (IU/ml)	1st Batch of reagent			2nd Batch of reagent			3rd Batch of reagent			Average detection rate
	NO. detected	NO. not detected	Detection rate	NO. detected	NO. not detected	Detection Rate	NO. detected	NO. not detected	Detection rate	
2.5	10	15	40%	12	13	48%	11	14	44%	44.0%
5	21	4	84%	20	5	80%	22	3	88%	84.0%
10	25	0	100%	24	1	96%	25	0	100%	98.7%
15	25	0	100%	25	0	100%	25	0	100%	100.0%
30	25	0	100%	25	0	100%	25	0	100%	100.0%

Study of Liver (EASL) [21].

Limit of quantification

National standard materials for HBV serum [GBW (E) 090137, Batch No. 201511003], at a concentration of 1.41×10^3 IU/mL were diluted with HBV DNA negative serum to nominal concentrations of 30 IU/mL, 20 IU/mL, and 10 IU/mL. 25 replicates per run were tested with three different batches of reagents in three runs, and the test results were log transformed to verify whether the limit of detection of the TL system met the standard (30 IU/mL) required in "The guideline principles for the technical review of hepatitis B virus DNA quantitative detection reagents registration".

Accuracy

National standard materials for HBV serum were used as samples and three replicates were tested at each concentration in three runs with three different batches of reagents. The sample results were log transformed to evaluate the accuracy of the TL system assay.

Comparison of clinical samples with CAP/CTM assay

A total of 200 clinical HBV positive samples with a uniform distribution of concentrations were tested with the TL system and the CAP/CTM system. The quantitative results from the two assays were counted and the Statistical Program for Social Sciences (SPSS 13.0 for Windows; SPSS, Chicago, IL, United States) was used to assess the agreement between the positive results obtained with the two assays.

Precision and reproducibility

High-titer HBV-DNA mixture samples were diluted with HBV DNA negative serum to nominal concentrations of 1×10^7 IU/mL, 1×10^4 IU/mL and 1×10^2 IU/mL. Twenty replicates were tested at each concentration with the same batch of reagent to assess the intra-assay precision. The inter-assay precision was evaluated by three tests with different batches of reagents, and five replicates at each concentration were tested per run. The inter-assay precision test was repeated over 4 d, in order to validate the reproducibility of the TL system assay. The results were log transformed for analysis.

Linearity

High-titer HBV-DNA mixture samples were diluted to nominal concentrations of 1×10^8 IU/mL, 1×10^7 IU/mL, 1×10^6 IU/mL, 1×10^5 IU/mL, 1×10^4 IU/mL, 1×10^3 IU/mL, 1×10^2 IU/mL, 60 IU/mL and 30 IU/mL. Triplicate measurements were made at each concentration level, the sample results were log transformed and then analyzed by Pearson's correlation analysis and linear regression analysis to assess the quantitative linear range of the TL system.

HBV genotypes coverage

The HBV DNA performance panel (WWHD301, Sera-Care, Milford, Massachusetts, United States) which contains all the genotypes of the HBV DNA (A-H) was used, triplicate measurements were continuously carried out for each HBV genotype for 3 days with the TL system to validate its capability of detecting all HBV DNA (A-H) genotypes.

Cross-contamination test

To assess the anti-cross contamination performance of the PANA 9600E automatic nucleic acid workstation, the "checkerboard" test was performed twice, using high-titer mixture samples interspersed with aliquots of HBV negative serum samples during DNA extraction. Each high-titer sample was included with HBV DNA negative plasma samples in the batch of 96 tests (48 positive samples and 48 negative samples) and the positions of positive samples and negative samples in the second round of testing were interchanged.

RESULTS

Limit of detection

The test results of the limit of detection are shown in Table 2, and a detection rate $\geqslant$ 95% was taken as an acceptable detection limit criterion. According to the test results, the average detection rates of the samples at five concentration levels were 44.0%, 84.0%, 98.7%, 100%, and 100%, respectively. Of these results, the detection rates for the 10 IU/mL samples were above 95% in all three tests. Therefore, the concentration of 10 IU/mL was determined as the detection limit of the TL system, which meets the

Table 3 Limit of quantification test results

Sample Conc. (IU/mL)	Test batch	Test result (Log_{10}IU/mL)			Accurate rate	
		Avg. value	CV	No. ≤ ± 0.4	per batch	Average
10	1	0.79	40.57%	19	76.00%	65.33%
	2	0.59	47.58%	13	52.00%	
	3	0.71	36.53%	17	68.00%	
20	1	1.45	14.11%	21	84.00%	82.67%
	2	1.45	16.65%	20	80.00%	
	3	1.36	20.55%	21	84.00%	
30	1	1.49	6.09%	25	100.00%	100.00%
	2	1.40	8.51%	25	100.00%	
	3	1.46	5.55%	25	100.00%	

Table 4 Accuracy test results

National standard materials for Hepatitis B virus serum		Expected	mean ± SD,	Absolute value of Log_{10} IU/mL difference
Standard material No.	Reference Conc.	Log_{10} IU/mL	Log_{10} IU/mL	
GBW (E) 090137	1.41×10^3 IU/mL	3.15	3.11 ± 0.02	0.04
GBW (E) 090138	5.9×10^4 IU/mL	4.77	4.80 ± 0.07	0.07
GBW (E) 090139	4.6×10^5 IU/mL	5.66	5.70 ± 0.05	0.05

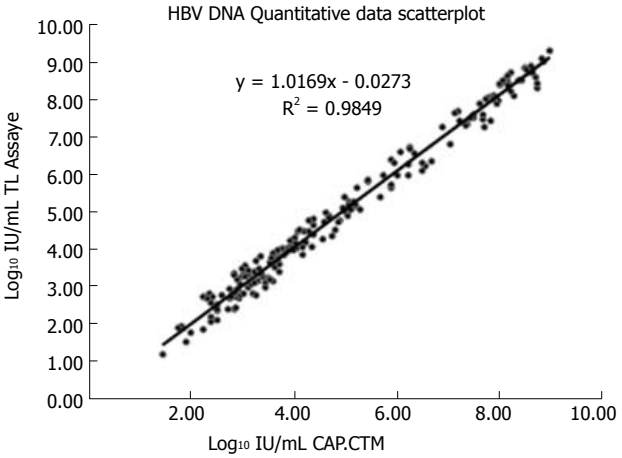


Figure 1 The correlation of measured hepatitis B virus DNA concentrations between the TL system assay and the CAP/CTM assay. HBV: Hepatitis B virus.

standard (10-15 IU/mL) recommended by the EASL.

Limit of quantification

The test results of the limit of quantification are shown in Table 3. Log_{10} IU/mL difference represents the log value difference between the detected and the expected concentrations. Taking ± 0.4 Log_{10} IU/mL as an acceptable value range in the detection results, this was converted using detected values of traceable national standard materials according to the National Center for Clinical Laboratories (NCCL) in China. When the detection accuracy rate of certain concentration was ⩾ 95%, this was considered the system quantification limit. According to the test results, all three test results for the 30 IU/mL samples showed 100% accuracy rate and the Log_{10} IU/mL difference values were between -0.22 Log_{10} IU/mL and 0.19 Log_{10} IU/mL. Therefore, the concentration of 30 IU/mL was determined as the quantification limit of the TL system, which meets the standard (30 IU/mL) required in "The guideline principles for the technical review of hepatitis B virus DNA quantitative detection reagents registration".

Accuracy

The accuracy test results are shown in Table 4. The Log_{10} IU/mL difference represents the log value difference between the detected and the expected concentrations, and when the Log_{10} IU/mL difference was less than ± 0.4, the test result was considered accurate. According to the test results, for three concentration levels of HBV national standard serum, the absolute values of Log_{10} IU/mL differences were 0.04, 0.07, and 0.05, respectively; therefore, the test results meet the accuracy requirement.

Comparison of clinical samples with CAP/CTM assay

In December 2015, 200 HBV samples were collected and used to compare the results of the TL system assay with the CAP/CTM assay. The results of the two assays showed 100% consistency for both negative and positive results (15 negative samples, 185 positive samples), and the concentrations of positive samples were uniformly distributed between 60 IU/mL and 8.7×10^9 IU/mL. The Statistical Program for Social Sciences (SPSS 13.0 for Windows; SPSS, Chicago, IL, United States) was used to assess the agreement between the positive results obtained with the two assays. The normality test results indicated that the concentration distribution did not conform to the normal distribution (P = 0.00, P < 0.05). The hypothesis test summary indicated that there were no statistical differences between the TL system and CAP/CTM assays (P = 0.817, P > 0.05). The scatter plot for the two assays is shown in Figure 1, with the linear equation y = 1.0286x - 0.0829 and correlation

Table 5 Precision and reproducibility test results

Sample Conc. (IU/mL)		1.0×10^7		1.0×10^4		1.0×10^2	
Tested Results Log_{10} (IU/mL)		Mean Value	CV	Mean Value	CV	Mean Value	CV
Intra assay precision[1]		6.97	0.23%	4.06	1.10%	2.29	3.10%
Inter assay precision	1st Day[2]	6.94	0.91%	4.19	1.82%	2.26	3.79%
	2nd Day[2]	6.95	1.04%	4.31	1.49%	2.41	3.25%
	3rd Day[2]	7.08	0.64%	4.31	1.67%	2.39	3.72%
	4th Day[2]	7.10	0.81%	4.32	1.63%	2.42	3.47%
Reproducibility[3]		7.02	1.36%	4.28	2.08%	2.37	4.41%

[1]n = 20; [2]n = 15; [3]n = 60 (15 tests/d × 4 d).

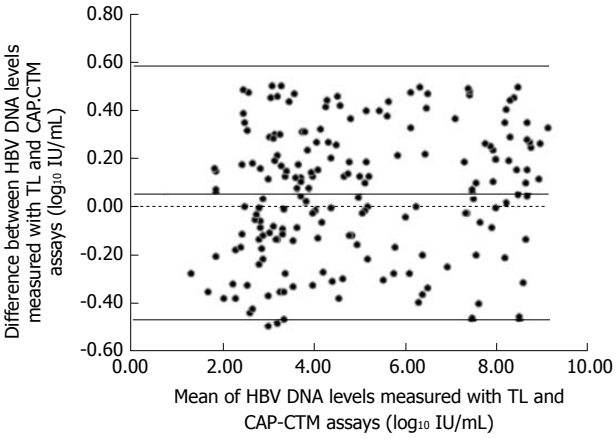


Figure 2 The difference of measured hepatitis B virus DNA concentrations between the TL system assay and the CAP/CTM assay. HBV: Hepatitis B virus.

coefficient r^2 = 0.9774, which indicated a significant correlation between the two assays. The Bland-Altman plot analysis was used to assess the difference between the positive results obtained with the two assays (Figure 2). The results showed that 98.9% (183/185) of the positive data were within the 95% acceptable range, the average difference value was 0.06, the maximum difference was -0.49, and 82.3% (153/185) of the difference values were between ± 0.4 Log_{10} IU/mL.

Precision and reproducibility

The precision and reproducibility test results are shown in Table 5. As shown in this Table, in the sample concentration range of 1.0×10^2 IU/mL to 1.0×10^7 IU/mL, the CV of intra-assay precision, inter-assay precision and reproducibility was all less than 5%.

Linearity

The linearity test results are shown in Figure 3. The linear regression analysis of eight concentration levels of HBV-DNA samples showed good linearity (the linear equation y=0.9848x - 0.0775 and correlation coefficient r^2 = 0.99).

HBV genotypes coverage

The HBV genotype coverage test results indicated that the TL system was capable of detecting all eight HBV DNA (A, B, C, D, E, F, G, H) genotypes as shown in Table 6. This almost covers all common genotypes worldwide, and meets the clinical requirements.

Cross-contamination

Following two rounds of "checkerboard" tests with the TL system assay, the detection rate of positive samples was 100%, and the detection rate of negative samples was 0. Therefore, throughout the entire test process, the negative samples were not contaminated.

DISCUSSION

HBV includes ten genotypes (A-I)[22-24]. According to its distribution in China, genotype B is the most frequent genotype in northern areas, genotype C is commonly seen in southern areas, mixed C/D genotypes are frequent in the Tibetan population in west China and genotype D is prevalent among Uighurs in Xinjiang, where genotype C is rare[25]. Infection with other HBV genotypes has not been reported in China. The 200 clinical HBV positive samples tested in this study were mainly composed of genotype B (70 cases), genotype C (66 cases) and genotype D (28 cases), genotype A (4 cases) and unknown genotypes. In order to verify the HBV genotype coverage using the TL system assay, the worldwide HBV DNA performance panel was used. The test results showed a 100% detection rate, which indicated that the TL system assay was capable of detecting not only the four genotypes in clinical samples, but also the eight HBV DNA (A, B, C, D, E, F, G, H) genotypes included in the worldwide HBV DNA performance panel.

In this study, the quantification limit test criterion adopted the analytical standard, which refers to the acceptable range ⩽ ± 0.4 Log_{10} IU/mL of external quality assessment used by the NCCL of China. The results of three rounds of 30 IU/mL quantification limit tests in this study complied with this criterion. However, there is another international frequently-used criterion that considers CV ⩽ 5% as the acceptable quantification limit test criterion. In this study, the CV in three rounds of 30 IU/mL quantification limit tests was 6.09%, 8.51%, and 5.55%, which failed to

Table 6 Hepatitis B virus genotypes coverage test Ct value

Hepatitis B virus DNA genotypes	Average tested Ct value			
	1st day	2nd day	3rd day	Negative
A	25.62	25.89	25.71	Undetected
B	25.58	25.78	25.78	Undetected
C	25.94	25.86	25.99	Undetected
D	22.61	22.78	22.78	Undetected
E	22.67	22.70	22.68	Undetected
F	22.68	22.72	22.71	Undetected
G	24.50	24.78	24.76	Undetected
H	24.81	24.80	24.85	Undetected

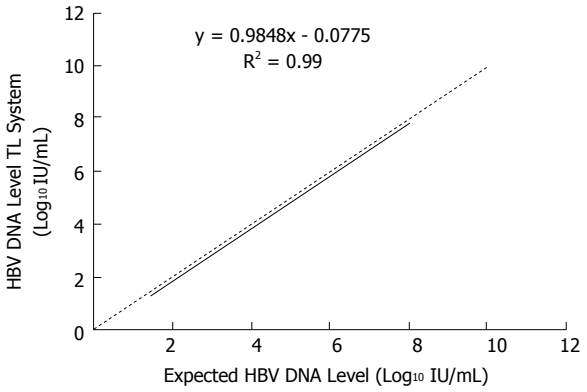


Figure 3 Linear correlation test standard curve. HBV: Hepatitis B virus.

meet this criterion. However, during the precision and reproducibility tests, the CV values for intra-assay and inter-assay tests of 1.0 × 10^2 IU/mL samples were both less than 5%. These findings indicate that the TL system assay still requires further optimization for the 30 IU/mL concentration test.

In this study, during the comparison test of 200 clinical samples, the upper limit of linearity range of both the TL assay and CAP/CTM assay was 1 × 10^8 IU/mL. Of 185 positive clinical samples, the test results of 23 samples obtained using both the TL and CAP/CTM assays exceeded the upper limit of linearity range. Four samples had the test results by the TL assay exceeded the upper limit of linearity range (8.08, 8.03, 8.08, 8.40), while the corresponding test results for the CAP/CTM assay were close to the upper limit of the linearity range (7.88, 7.80, 7.88, 8.00), but the Log_{10} IU/mL differences between the two assays were all less than ± 0.4. Following elimination of these 27 samples, the correlation analysis results (P = 0.575) showed no significant statistical differences between the two methods and good consistency. However, there were differences between the two assays in the detection of samples at a concentration over or close to the linearity range upper limit. In order to obtain more effective quantitative results, it is recommended that clinical samples are diluted and tested again.

In addition, this study also performed a cross-reaction test to analyze the effect of interfering substances on the TL system performance. However, we were unable to obtain the corresponding drug pharmacokinetic samples; thus, the HBV positive serum samples were adopted as the control group in the cross-reaction test, and these HBV positive serum samples were added to the interfering substances as the test group. The cross-reaction test results showed that the TL system was capable of detecting HBV positive serum samples containing four typical endogenous interfering substances (free hemoglobin, triglyceride, bilirubin and IgG) and four typical exogenous interfering substances (IFNα, lamivudine, adefovir dipivoxil, telbivudine) and the Log_{10} IU/mL differences between the corresponding samples from the test group and the control group were all less than ± 0.4. Therefore, it can be concluded that the TL system has anti-interference capability for both endogenous and exogenous interfering substances. For more precise evaluation of the anti-interference performance of the TL system, more tests should be carried out with drug pharmacokinetic samples and clinical samples containing interfering substances.

Qiu *et al*[26] reported a comparison of the Abbott and Da-an real-time PCR for the quantitation of serum HBV DNA. The Abbott assay had a higher sensitivity, shorter assay time, and wider dynamic range compared with the Da-an assay. However, the costs of the Abbott assay limited its routine use in clinical molecular laboratories in China. The clinical performance of the TL system was comparable to the CAP/CTM system, with reasonable costs.

There are some deficiencies in the TL system found by performance verification. First, although no negative samples were detected in the cross-contamination test, two amplification curves for negative samples showed an increased tendency at 43-45 cycles, but did not reach the threshold line. Thus, cleaning and maintenance processes are particularly important in the case of a long run time, and the possibility of accumulated contamination should be validated over this period. Second, the TL system assay adopts pre-packaged nucleic acid extraction kits (24T×4); thus, when the amount of test sample is not a multiple of 24, some nucleic acid extraction reagent is wasted and can increase costs, and this is one of the main deficiencies of the TL system which needs to be improved.

Only 200 μL of sample were required by the TL system, its limit of detection (10 IU/mL) meets the standard (10-15 IU/mL) recommended by the EASL, and its limit of quantification (30 IU/mL) also meets the standard required in "The guideline principles for the technical review of hepatitis B virus DNA quantitative detection reagents registration" issued by the CFDA. The differences between the expected and tested concentration values of national standards were less than ± 0.4 Log_{10} IU/mL, which demonstrated the high accuracy of the system. In addition, there were no statistically significant differences between the TL system assay and the CAP/CTM assay. This

study also assessed the precision, reproducibility, and linearity of the TL system, and the results showed that between 10^2 IU/mL and 10^6 IU/mL, the multiple test CV of the same sample was less than 5%; and between 30 IU/mL and 10^8 IU/mL, the linear correlation coefficient r^2 = 0.99. Furthermore, the worldwide HBV DNA performance panel, which contains HBV DNA genotypes (A-H), was used to validate the capability of the TL system to detect all eight genotypes. Finally, two rounds of "checkerboard" tests with 96 samples (48 strongly positive and 48 negative) were tested to validate the anti-cross-contamination performance of the TL system, and the results showed a 100% coincidence rate. In summary, the TL system has good analytical performance, clinical performance and stability.

COMMENTS

Background

Use of the hypersensitive hepatitis B virus (HBV) DNA quantification system to accurately detect HBV DNA in serum is important in hepatitis B antiviral therapy. International associations for the study of liver diseases have required HBV DNA quantification detection with hypersensitivity (limit of detection: 10-15 IU/mL), a wide linearity range (1-9 $\log_{10}$ IU/mL), high specificity and good repeatability. The CAP/CTM system is regarded as a worldwide standard for HBV DNA quantification. This study was conducted to validate and compare the automatic hypersensitive HBV DNA quantification system produced in China with the internationally accepted CAP/CTM system.

Research frontiers

The sensitivity, reproducibility and system automaticity for HBV DNA quantification have improved in recent years, and are important factors in determining the curative effect and endpoints of hepatitis B antiviral therapy.

Innovations and breakthroughs

This is the first study to evaluate the analytical performance of the TianLong automatic hypersensitive HBV DNA quantification system, which is the first CFDA approved system produced in China, and to compare its clinical performance with the CAP/CTM system. The TL system showed good analytical performance, and good correlation and quantitative agreement with the CAP/CTM system.

Applications

This study will be helpful in improving the performance of this system and its clinical applications.

Peer-review

The authors have evaluated the performance characteristics of the domestic TianLong HBV DNA Quantification System by testing 200 clinical HBV samples and compared the results with the international automatic HBV DNA detection systems including Roche CAP/CTM System. There was no statistically significant difference between TianLong and CAP/CTM systems. This study reports valuable results to validate the detection performance of TianLong HBV DNA Quantification System, which can pave the way to reduce cost of HBV DNA quantitative detection in the Chinese market.

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ORIGINAL ARTICLE

Case Control Study

Association of insertion-deletions polymorphisms with colorectal cancer risk and clinical features

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Abstract

AIM

To investigate the association between 16 insertion-deletions (INDEL) polymorphisms, colorectal cancer (CRC) risk and clinical features in an admixed population.

METHODS

One hundred and forty patients with CRC and 140 cancer-free subjects were examined. Genomic DNA was extracted from peripheral blood samples. Polymorphisms and genomic ancestry distribution were assayed by Multiplex-PCR reaction, separated by capillary electrophoresis on the ABI 3130 Genetic Analyzer instrument and analyzed on GeneMapper ID v3.2. Clinicopathological data were obtained by consulting the patients' clinical charts, intra-operative documentation, and pathology scoring.

RESULTS

Logistic regression analysis showed that polymorphism variations in *IL4* gene was associated with increased CRC risk, while *TYMS* and *UCP2* genes were associated with decreased risk. Reference to anatomical localization of tumor Del allele of *NFKB1* and *CASP8* were associated with more colon related incidents than rectosigmoid. In relation to the INDEL association with tumor node metastasis (TNM) stage risk, the Ins alleles of *ACE*, *HLAG* and *TP53* (6 bp INDEL) were associated with higher TNM stage. Furthermore, regarding INDEL association with relapse risk, the Ins alleles of *ACE*, *HLAG*, and *UGT1A1* were associated with early relapse risk, as well as the Del allele of *TYMS*. Regarding INDEL association with death risk before 10 years, the Ins allele of *SGSM3* and *UGT1A1* were associated with death risk.

CONCLUSION

The INDEL variations in *ACE*, *UCP2*, *TYMS*, *IL4*, *NFKB1*, *CASP8*, *TP53*, *HLAG*, *UGT1A1*, and *SGSM3* were associated with CRC risk and clinical features in an admixed population. These data suggest that this cancer panel might be useful as a complementary tool for better clinical management, and more studies need to be conducted to confirm these findings.

Key words: Colorectal cancer; Ins-del polymorphism; Admixed population; Potential biomarker; Diagnostic; Risk stratification; Prognostic; Clinical features

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Core tip: The insertion-deletions (INDEL) variations in *IL4* gene was associated with increased colorectal cancer (CRC) risk, while *TYMS* and *UP2* genes were associated with decreased risk. The Del-alleles of *NFKB1* and *CASP8* were associated with more colon related incidents than rectosigmoid. The Ins-alleles of *ACE*, *HLAG* and *TP53* were associated with higher TNM stage. The Ins-allele of *ACE*, *HLAG*, and *UGT1A1* were associated with early relapse risk, as well as the Del-allele of *TYMS*. The Ins-alleles of *SGSM3* and *UGT1A1* were associated with death risk. These data suggest that these INDEL might be useful as a complementary tool for better CRC clinical management.

Marques D, Ferreira-Costa LR, Ferreira-Costa LL, Correa RS, Borges AMP, Ito FR, Ramos CCO, Bortolin RH, Luchessi AD, Ribeiro-dos-Santos A, Santos S, Silbiger VN. Association of insertion-deletions polymorphisms with colorectal cancer risk and clinical features. *World J Gastroenterol* 2017; 23(37): 6854-6867 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6854.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6854

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer type in men and the second in women, considering 1477402 new cases in both sexes in 2015[1]. In that same year, Brazil was the tenth country with the highest CRC incidence, with 37167 new cases in both sexes[1], and making matters worse, its incidence and mortality continue to increase in the country.

Both genetic and environmental factors cause CRC[2], especially when combined[3,4]. Interestingly, these factors are ample and they vary pursuant to the cancer geographical regions[5]. However, inherited susceptibility is a major component of CRC predisposition, with an estimated 12%-35% risk attributed to genetic factors[6-8].

In relation to genetic factors, there are several mutations that might occur in human DNA, such as substitution, insertion, and deletion[9]. The second most abundant form of genetic variation in humans, after single nucleotide polymorphisms (SNPs), are the insertion-deletions (INDEL)[10]. INDELs are important because they are common genetic variations within genomes and among different ethnic groups[11,12], that may alter human traits and cause diseases[10,13], including CRC[14], by modifying the coding region[10,13] or mRNA stability[15]. The polymorphisms investigated in this study exhibit common features, given they are

all functional polymorphisms that alter the expression of genes participating in metabolic pathways associated with carcinogenesis. Also, these genes are associated with different types of cancer with high incidence in the Brazilian population, such as stomach and CRC.

Furthermore, allele frequency varies among different populations[16], and genomic ancestry distribution may influence cancer development[17,18] by affecting polymorphisms distribution[19,20]. Few studies have been evaluated INDEL association in CRC in admixed population, mainly in Brazil. Thus, the aim of this study was to determine the association between CRC risk and prognostic follow-up with 16 INDELs in genes involved in apoptosis signaling (*CASP8*), GTPase-activating (SGSM3), steroids metabolism (*CYP2E1*, *CYP19A1*, and *UGT1A1*), immune system (*HLAG*, *IL1A*, *IL4*, and *NFKB1*), MDM2-P53 pathway (*MDM2* and *TP53*), DNA replication and repair (*TYMS* and *XRCC1*) and angiogenesis (*UCP2* and *ACE*) in an admixed population from Rio Grande do Norte state (in the Northeast Region of Brazil).

MATERIALS AND METHODS

A statement of ethics

The protocol used in this study was approved by the Research Ethics Committee of Liga Norte Riograndense Contra o Câncer (Rio Grande do Norte, Brazil) by number 211/211/2011. Moreover, all participants signed a consent form prior to providing a blood sample.

Casuistic distinctions

The patients in the case group (n = 140) were diagnosed with CRC as primary cancer and treated in the Proctologist Clinic and Colorectal Surgery Department of Liga Norte Riograndense Contra o Câncer (Rio Grande do Norte, Brazil). The control subjects were cancer-free blood donors (n = 140) from the hemotherapy service (Hemovida, Rio Grande do Norte, Brazil) and were recruited in 2014.

Both Peripheral blood samples and questionnaire answers were collected from all subjects. The clinicopathological data were obtained by consulting the patients' clinical charts, intra-operative documentation, and pathology scoring. Furthermore, the CRC patients were followed up to 20 years by medical records.

Definitions

Alcohol consumption was classified as having the habit of alcohol consumption (Yes) or not having the habit of alcohol consumption (No). The subjects who have the habit of consuming alcohol were subcategorized according to consumption frequency (Eventually: $\leqslant$ 3 d per month; Frequently: > 3 d per month). The tobacco consumption was classified as have already smoked (Already) or not (Never). The subjects who have already smoked were subcategorized in Former (Stopped smoking for at least 1 year) and Current.

Tumor location was classified as rectosigmoid (sigmoid colon and rectum) and colon (ascending, transverse and descending colon) based on colonoscopy or on radiographic exam. The relapse records were obtained from histological or radiographic exams with subsequent clinical/radiological progression.

Overall survival was defined as the time from the date of surgery to the date of death or the date of the last follow-up of patients who were still alive. The relapse time was defined as the time from the date of surgery to the date of the first local. Patients with no local or distant relapse evidence at the date of death or the date of the last follow-up were censored.

DNA extraction and quantification

Genomic DNA was extracted by using a DNA extraction commercial kit, QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), and quantified with Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States).

Polymorphism selection

Recently, INDELs have been the focus of multiple investigations[21-25]. This type of polymorphism presents interesting features as genetic markers: (1) INDELs are spread throughout the human genome; (2) INDELs derive from a single event (they do not present homoplasy); (3) since the allele frequencies of many INDELs are significantly different in separated populations; (4) small INDELs can be analyzed using short amplicons, which improves the amplification of degraded DNA and facilitates multiplexing; and (5) INDELs can be easily genotyped with a simple dye-labeling electrophoretic approach. Furthermore, all these genes evaluated in the present study show potential activity in pathway and may contribute to the carcinogenesis process (Table 1). Their genetic variations could contribute to: (1) risk of developing CRC; (2) impact on treatment response; or (3) in prognosis.

Genotyping of polymorphism

Multiplex PCR was used to simultaneously amplify the 16 investigated markers, as shown in Supplementary Table 1. The amplification was performed on ABI Verity thermocycler (Life Technologies, Foster City, CA, United States). A single multiplex reaction used Master Mix QIAGEN Multiplex PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples were incubated at 95 ℃ for 15 min, followed by 35 cycles at 94 ℃ for 45 s, 60 ℃ for 90 s, and 72 ℃ for 1 min, with a final extension at 70 ℃ for 30 min.

For fragment analysis, we used capillary electrophoresis on the ABI 3130 Genetic Analyzer instrument (Life Technologies). 1.0 μL of PCR product was added to 8.5 μL of HI-DI deionized formamide (Life Technologies) and 0.5 μL of GeneScan 500 LIZ pattern size standard (Life Technologies). After data collection,

Table 1 Potential biological effects of insertion-deletions polymorphism selected in this study

Gene	dbSNP	Localization[1]	INDEL lenght	Region	Potential biological effect[84]				Potential impact on carcinogenesis
					mRNA splicing	mRNA stability	Gene expression	Protein function	
ACE	rs4646994	17:63488539	289	Intron	X	X		X	Angiogenesis, proliferation, progression and metastases[85]
CASP8	rs3834129	2:201232809	6	Promoter			X		Apoptosis[86-88]
SGSM3	rs56228771	22:40410092	4	3'-UTR		X	X		Proliferation and apoptosis [83,89-91]
CYP2E1	-	-	96	5'-Flanking			X		Metabolism of endo- and exogenous[92-97]
CYP19A1	rs11575899	15:51227749	3	Intron	X	X		X	Metabolism of endo- and exogenous[92-97]
HLAG	rs371194629	6:29830804	14	3'-UTR		X	X		Immune surveillance[98-102]
IL1A	rs3783553	2:112774138	4	3'-UTR		X	X		Induce chronic inflammation and proliferation[103,104]
IL4	rs79071878	5:132680584	70	Intron	X	X		X	Immune surveillance and proliferation[40,39,43,105,106]
MDM2	rs3730485	12:68807065	40	Promoter			X		Proliferation and apoptosis[107,108]
NFKB1	rs28362491	4:102500998	4	Promoter			X		Differentiation, proliferation and apoptosis [109,110]
TP53	rs17878362	17:7676372	16	Intron	X	X		X	Proliferation, apoptosis, repair, differentiation[111-114]
TP53	rs17880560	17:7668169	6	3'-Flanking		X	X		Proliferation, apoptosis, repair, differentiation[111-114]
TYMS	rs151264360	18:673444	6	3'-UTR		X	X		Differentiation, replication and repair[50,105]
UCP2	-	-	45	3'-UTR		X	X		Tumor aggressiveness and metastasis[56]
UGT1A1	rs8175347	2:233760235	2	3'-UTR		X	X		Metabolism of endo- and exogenous[92-97]
XRCC1	rs3213239	19:43576907	4	5'- Flanking			X		Repair[115-117]

[1]According to the single nucleotide polymorphism database (dbSNP); UTR: Untranslated region; INDEL: Insertion-deletions.

samples were analyzed on the GeneMapper ID v.3.7 software (Life Technologies).

Analysis of genetic ancestry

Genomic ancestry analysis was performed based on the method described by Santos *et al*[25] using 62 autosomal ancestry informative markers (AIMs). Two multiplex PCR reactions of 20 and 22 markers were performed and amplicons were analyzed by electrophoresis using the ABI Prism 3130 sequencer and GeneMapper ID v.3.2 software. The individual proportions of European, African, and Amerindian genetic ancestries were estimated using STRUCTURE v.2.3.3 software, assuming three parental populations (European, African, and Amerindian).

Statistical analysis

The categorical variables case and control participants were tested by the Chi-squared test. For ancestry index and age at diagnosis variables we used the Mann-Whitney test. Logistic regression analyses between the genotype model and CRC risk were performed by the SNPassoc package v.1.9-2, along with clinical features variables. The association between genotype and free-relapse survival time was evaluated by Kaplan-Meier plots, performed by the survival package v.2.41-3. Log-rank and Wilcoxon tests were used to examine the genetic effect on survival outcomes. The statistical power was estimated by 10000 simulations. All statistical analyses and plotting were performed with R package v.3.1.2[26]. Differences between groups were considered significant at $P < 0.05$.

RESULTS

Demographic characteristics

We analyzed 140 subjects with CRC and 140 cancer-free individuals. The demographic characteristics of participants were summarized in Table 2, which shows demographic features of the groups. Regarding genomic ancestry, significance was observed with the distribution of African ancestry ($P = 0.049$), Table 3. However, there was no difference between groups when an analysis of multinomial logistic regression was performed.

Distribution of genotypes associated with susceptibility to CRC

All INDEL polymorphisms are in Hardy-Weinberg equilibrium ($P > 0.05$). The genotypic and allelic frequencies of the subjects are presented in the Table 4. Genotypic frequency ($P = 0.01$) of *IL4* gene polymorphism was significantly different between case-controls, and higher frequency of Del allele were observed in cases than in controls.

The significant logistic regression analyses between case-controls are summarized in Table 5. Del allele polymorphism in *IL4* gene ($P = 0.0110$) was associated with an increased risk of CRC development, while Ins allele in *UCP2* ($P = 0.0210$) was decreased

CRC risk. Furthermore, the Del allele in the *TYMS* (P = 0.0120) gene was associated with decreased CRC risk.

Distribution of genotypes associated with prognostic follow-up in CRC

The baseline characteristics of CRC patients are summarized in Table 6. The follow-up time median was 5.28 years among 78 patients who had complete genotype and follow-up information. The 5-year free-relapse rate was 70% and the 10-year free-relapse rate was 66.4%. The 5-year survival rate was 91.4% and the 10-year survival rate was 87.9%.

We also evaluated the genetic impact in the clinical features. The Del allele in *NFKB1* and *CASP8* were associated with more incidents to colon than rectosigmoid (Table 7). In relation to the INDEL association with TNM stage risk, the Ins alleles of *ACE*, *HLAG* and *TP53* (6 bp INDEL) were associated with a higher TNM stage (Table 8). Regarding the INDEL association with relapse risk, the Ins alleles of *ACE*, *HLAG*, and *UGT1A1* were associated with relapse risk, as well as the Del allele of *TYMS* (Table 9). Moreover, these findings corroborate those observed in the free-relapse survival curve (Figure 1). Regarding INDEL association with death risk, the Ins alleles of *SGSM3* and *UGT1A1* were associated with death risk (Table 10).

DISCUSSION

Despite the effective strategies for prevention, early detection, and treatment[27-32], there are ethnic differences in the CRC incidence and survival[33,34], specifically in individuals with African American ancestry, who have higher CRC incidence and lower 5-years survival rates than other ethnic groups[33-38].

In this work, we evaluated the association between 16 INDEL [*ACE, CASP8, SGSM3, CYP19A1, CYP2E1, HLAG, IL1A, MDM2, NFKB1, TP53* (16 and 6 bp), *TYMS, UCP2, XRCC1*, *IL4* and *UGT1A1*] and the risk of developing CRC in a Brazilian population, as well as their clinical features. We found significant association between three investigated INDEL polymorphisms and CRC risk, two associated with anatomical localization, three associated with TNM stage, four associated with early relapse risk, and two associated with death risk before 10 years.

Variations in the IL-4 activity or in the IL-4 receptor due to mutations have been associated with cell proliferation and might affect signal transduction pathways in cancer[39]. We evaluated INDEL of 70 bp in intron 3 of the *IL4* gene (rs79071878), a variation which may influence the production of this cytokine. The higher IL-4 production may result in diminished cell-mediated immune response, and escape from immune surveillance in the tumor cells. The cell-mediated immune response may be inhibited by downregulating the expression of Th1 cytokines, decreasing the CD8+ T-cell response in the tumor microenvironment[39-41]. Furthermore, this INDEL has been associated with gastric cancer[39] and other immune diseases[42,43]. However, this is the first study indicating an association between this *IL4* polymorphism and the risk of developing CRC. Our results indicate that the Del allele in *IL4* was associated with the risk of developing CRC.

The *TYMS* gene plays an essential role in the biosynthesis of the DNA-component thymidylate (dTTP) and is required for DNA replication and repair[44]. The insertion of 6 bp in the 3'-UTR of *TYMS* primary transcript (rs151264360) may significantly influence gene expression as shown by using a luciferase assay[15]. Mandola *et al*[15] observed that a Del allele might decrease the TYMS mRNA stability, and the TYMS protein expression. Moreover, Rahman *et al*[45] showed in vitro that TYMS overexpression might induce the transformation of mammalian cells into a malignant phenotype. Studies indicate that this INDEL is associated with many cancers[46-49], especially colorectal[48]. These results suggested that this INDEL variation might decrease CRC risk, as showed in the present work. However, this finding diverges from data from Mexico[50], in which association was not observed. On the other hand, our results showed that this Del allele was associated with an increase relapse risk.

Uncoupling proteins (UCPs) are a family of mitochondrial proteins, which were originally reported to play essential roles in reducing the reactive oxygen species[51,52]. *UCP2* plays a role in carcinogenesis in various tissues, including colon cancer, and regulates the responsiveness of carcinomas to chemotherapy[53-56]. Adaptive mechanisms of cancer cells include resistance to tumor growth inhibition and evasion of apoptosis, and cellular events that are appreciably affected by oxidative stress[57,58]. The UCP2 expression level is significantly higher in colon cancer tissue than in its adjacent tissue and *UCP2* may play a role in intestinal epithelial cells from benign to malignant transformation[59]. However, the role of *UCP2* in development of colon cancer is unclear. INDEL polymorphism may regulate *UCP2* mRNA stability via post-transcriptional modification of UCP2 protein expression[60,61]. Indeed, in the present study was observed that INDEL polymorphism might be associated with colorectal cancer. However, this is the first study indicating an association between this *UCP2* polymorphism and the risk of developing CRC.

The renin-angiotensin system (RAS), which regulates systemic blood pressure, also exerts local effects on cell proliferation, apoptosis, inflammation and angiogenesis in different tissues[62]. In addition, there is evidence linking the RAS with tumorigenesis and tumor angiogenesis[63]. The polymorphisms in the various components of the RAS that may possess clinical relevance[62], and the most common polymorphism in the gene encoding angiotensin converting enzyme (*ACE*) is INDEL of a 287-bp fragment in intron 16 and is responsible for the inter-individual variation in the

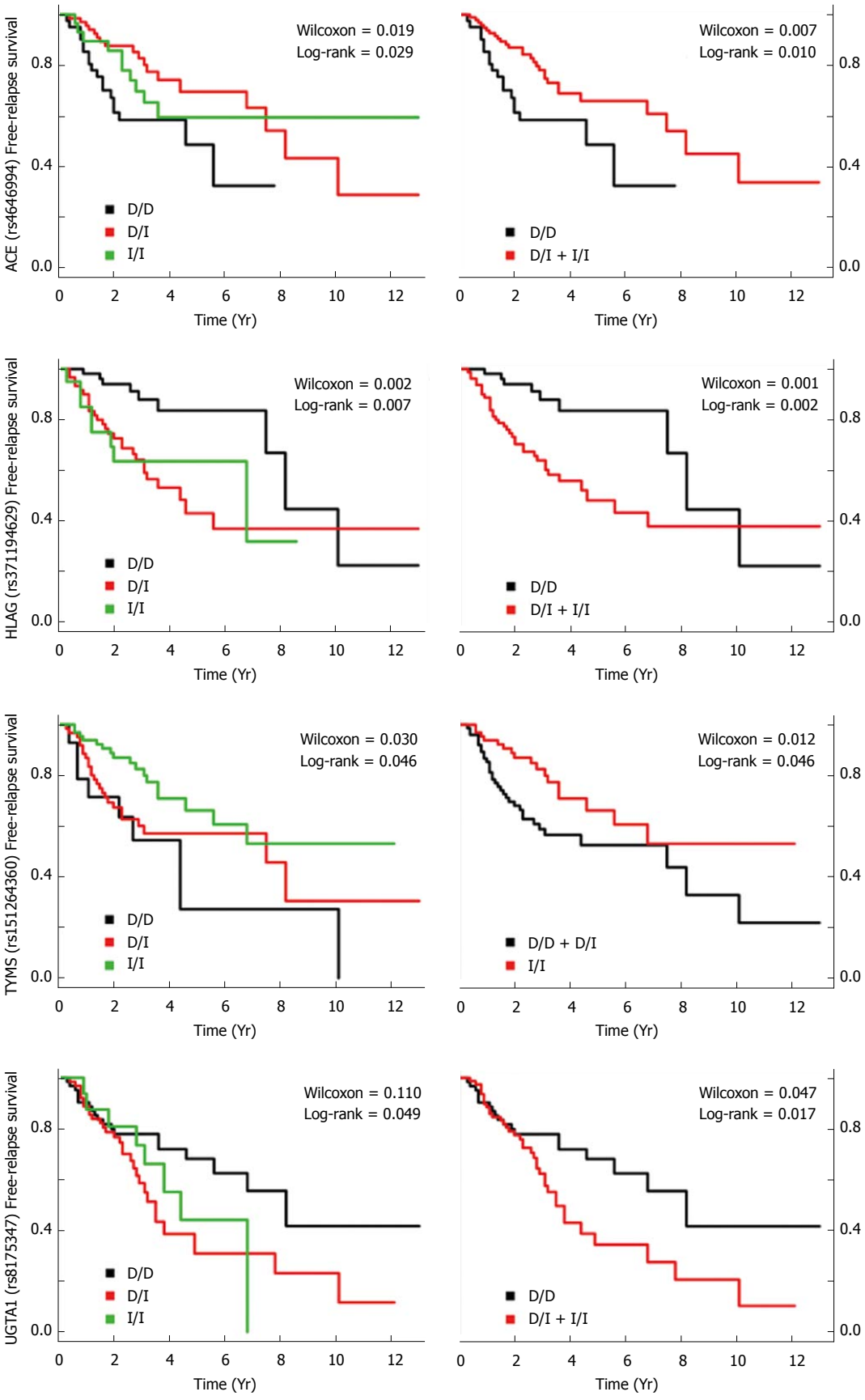


Figure 1 Free-relapse survival of patients with colorectal cancer related with significant insertion-deletions. Logistic regression adjusted for confounders. The analyses and graphic were performed by survival packages, in *R* statistical software.

Table 2 Participant demographic and clinical characteristics and their stratification by case and control groups

Characteristic	Total, *n* = 280	Cases, *n* = 140	Controls, *n* = 140	*P* value
Age (yr)	48 (21-93)	59 (23-93)	37 (21-81)	< 0.001
< 45	136 (48.7)	23 (16.5)	113 (80.7)	
⩾ 45	144 (51.3)	116 (83.5)	27 (19.3)	
Gender				< 0.001
Male	172 (61.6)	62 (44.6)	110 (78.6)	
Female	108 (38.4)	78 (55.4)	30 (21.4)	
Alcohol consumption				< 0.001
No	180 (65.1)	118 (85.5)	62 (44.9)	
Yes	96 (34.9)	20 (14.5)	76 (55.1)	
Eventually	56 (20.4)	11 (8.0)	45 (32.6)	
Frequently	40 (14.5)	9 (6.5)	31 (22.5)	
Tobacco consumption				< 0.001
Never	182 (65.4)	68 (48.9)	114 (82.0)	
Already	96 (24.6)	71 (48.1)	25 (18.0)	
Former	66 (23.8)	55 (39.6)	11 (7.9)	
Current	30 (10.8)	16 (11.5)	14 (10.1)	

Categorized data are presented by absolute numbers of individuals (percentage) and analyzed by Chi-square test. Continuous data are presented by mean (min-max) and analyzed by Mann-Whitney test.

Table 3 Genetic ancestry distribution between case and control groups

Genetic ancestry (%)	Total, *n* = 280	Cases, *n* = 140	Controls, *n* = 140	OR (95%CI)	*P* value
European	65.3 ± 15.5	64.2 ± 15.6	66.4 ± 15.3		0.243
95-80	50 (17.9)	21 (15.1)	29 (20.7)	1.0 (Reference)	
80-70	70 (25.1)	33 (23.7)	37 (26.4)	1.23 (0.59-2.56)	0.577
70-60	67 (24.0)	38 (27.3)	29 (20.7)	1.81 (0.86-3.80)	0.117
60-50	47 (16.8)	22 (15.8)	25 (17.9)	1.21 (0.54-2.71)	0.634
50-40	26 (9.3)	14 (10.1)	12 (8.6)	1.61 (0.62-4.18)	0.327
40-30	11 (3.9)	6 (4.3)	5 (3.6)	1.66 (0.45-6.16)	0.451
30-20	7 (2.5)	5 (3.6)	2 (1.4)	3.45 (0.61-19.54)	0.161
20-10	1 (0.4)	-	1 (0.7)	-	
Amerindian	16.2 ± 10.1	16.0 ± 10.3	16.3 ± 9.9		0.645
02-10	90 (32.3)	45 (32.4)	45 (32.1)	1.0 (Reference)	
10-20	113 (40.5)	60 (43.2)	53 (37.9)	1.13 (0.65-1.97)	0.661
20-30	47 (16.8)	18 (12.9)	29 (20.7)	0.62 (0.30-1.27)	0.193
30-40	21 (7.5)	11 (7.9)	10 (7.1)	1.10 (0.42-2.85)	0.844
40-50	7 (2.5)	4 (2.9)	2 (2.1)	2.00 (0.35-11.47)	0.437
50-60	1 (0.4)	1 (0.7)	-	-	
African	18.6 ± 12.0	19.8 ± 12.3	17.3 ± 11.7		0.049
02-10	81 (29.0)	36 (25.9)	45 (32.1)	1.0 (Reference)	
10-20	89 (31.9)	41 (29.5)	48 (34.3)	1.07 (0.58-1.95)	0.832
20-30	66 (23.7)	38 (27.3)	28 (20.0)	1.70 (0.88-3.27)	0.114
30-40	28 (10.0)	15 (10.8)	13 (9.3)	1.44 (0.61-3.42)	0.405
40-50	8 (2.9)	5 (3.6)	3 (2.1)	2.08 (0.47-9.31)	0.337
50-60	5 (1.8)	3 (2.2)	2 (1.4)	1.87 (0.30-11.83)	0.504
60-70	2 (0.7)	1 (0.7)	1 (0.7)	1.25 (0.07-20.68)	0.876

Categorized data are presented by absolute numbers of individuals (percentage) and analyzed by Chi-square test. Continuous data are presented by mean ± standard variation and analyzed by Mann-Whitney test.

ACE levels in blood and tissues[64]. The insertion allele in this gene was associated with ACE levels, the rate of disease progression, shorter TTF, and lower circulating levels of ACE[62,65]. This INDEL has been associated with cancer risk susceptibility[66-68], including CRC[65,68], and with response to bevacizumab[62]. Our results indicate that Ins allele was not associated with CRC risk development, as showed by Yang *et al*'s meta-analysis[69] and Liu *et al*[70] case-control study (241 cases and 299 control, China). On the other hand, our results also showed that this INDEL was also associated with TNM stage risk and relapse risk.

The *HLAG* is an important immunomodulatory molecule related to several mechanisms of tolerance[71]. Since the discovery of the HLA-G protein expression in cancer[72], several pieces of evidence have supported a considerable role for HLA-G in tumor cell escape from immuno-surveillance and antitumor immune responses[73]. The 14 bp INDEL (rs371194629) has been suggested to have functional significance. The Ins allele has been shown to be associated with alternative splicing, resulting in deletion of 92 bp in

Table 4 Genotype and allele frequency in percentage of patients with colorectal cancer and controls

Gene	dbSNP	Case/control (*n* = 140/140)						
		Genotype frequency				Allele frequency		HWE
		II	ID	DD	*P* value	I	D	*P* value
ACE	rs4646994	21.0/14.3	49.3/54.3	29.7/31.4	0.335	45.7/41.4	54.3/58.6	0.161
CASP8	rs3834129	34.5/30.0	46.0/46.4	19.4/23.6	0.605	57.6/53.2	42.4/46.8	0.424
SGSM3	rs56228771	7.2/3.6	30.2/36.4	62.6/60.0	0.274	22.3/21.8	77.7/78.2	0.415
CYP19A1	rs11575899	35.8/37.9	49.6/50.0	14.6/12.1	0.82	60.3/62.9	39.7/37.1	0.402
CYP2E1	-	0.7/0.7	17.3/12.9	82.0/86.4	0.588	9.4/7.1	90.6/92.6	0.716
HLAG	rs371194629	14.7/13.6	43.4/50.0	41.9/36.4	0.538	36.4/38.6	63.6/61.4	0.514
IL1A	rs3783553	46.0/52.1	38.8/37.9	15.1/10.0	0.368	65.5/71.1	34.5/28.9	0.348
IL4	rs79071878	48.9/60.0	40.3/37.1	10.8/2.9	0.017	69.1/78.6	30.9/21.4	0.223
MDM2	rs3730485	50.4/50.7	43.2/37.1	6.5/12.1	0.219	71.9/69.3	28.1/30.7	0.132
NFKB1	rs28362491	38.1/40.7	46.0/42.1	15.8/17.1	0.806	61.2/61.8	38.8/38.2	0.203
TP53	rs17878362	3.6/1.4	27.3/32.1	69.1/66.4	0.383	17.3/17.5	82.7/82.5	0.181
TP53	rs17880560	7.2/7.1	39.6/32.9	53.2/60.0	0.489	27.0/23.6	73.0/76.4	0.297
TYMS	rs151264360	46.0/42.9	43.9/42.1	10.1/15.0	0.459	68.0/63.9	32.0/36.1	0.308
UCP2	-	6.6/9.3	35.3/44.3	58.1/46.4	0.149	24.3/31.4	75.7/68.6	0.745
UGT1A1	rs8175347	11.5/10.8	44.6/46.0	43.9/43.2	0.785	33.8/33.8	66.2/66.2	0.735
XRCC1	rs3213239	41.7/42.9	47.5/45.0	10.8/12.1	0.894	65.5/65.4	34.5/34.6	0.941

Genotype frequencies are presented as the percentage of patients with colorectal cancer/percentage of controls, and analysis by Chi-square test. dbSNP: Register of genetic variation on NCBI database; bp: Base pairs of DNA sequence; HWE: Hardy-Weinberg Equilibrium.

Table 5 The logistic regression analyses between case-control and insertion-deletions polymorphism

Gene	Model	OR (95%CI)	*P*-value
IL4	Ins/Ins *vs* Del/Ins + Del/Del	2.26 (1.20-4.31)	0.0110
TYMS	Ins/Ins + Del/Ins *vs* Del/Del	0.26 (0.08-0.75)	0.0120
UCP2	Del/Del *vs* Del/Ins + Ins/Ins	0.48 (0.25-0.90)	0.0210

Adjusted by age at diagnosis, gender, alcohol consumption, tobacco consumption and ancestry distribution. The Supplementary Table 2 shows the genotype frequency and all logistic regression.

Table 7 The significant insertion-deletions associations with anatomic localization

Gene	Model	OR (95%CI)	*P* value
CASP8	Ins/Ins *vs* Del/Ins + Del/Del	0.28 (0.08-0.97)	0.0303
NFKB1	Ins/Ins *vs* Del/Ins+Del/Del	0.31 (0.10-0.93)	0.0276

Logistic regression adjusted for confounders. The Supplementary Table 3 shows the genotype frequency and all logistic regression.

Table 8 The significant insertion-deletions associations with tumor node metastasis stage risks

Gene	Model	OR (95%CI)	*P* value
ACE	Del/Del *vs* Del/Ins + Ins/Ins	2.82 (1.26-6.31)	0.0092
HLAG	Del/Del + Del/Ins *vs* Ins/Ins	2.74 (1.01-7.42)	0.0416
TP53 06 bp	Del/Del *vs* Del/Ins + Ins/Ins	2.50 (1.23-5.06)	0.0099

Logistic regression adjusted for confounders. The Supplementary Table 3 shows the genotype frequency and all logistic regression.

Table 6 Clinical characteristics of patients with colorectal cancer at diagnosis and follow-up

Characteristics	Cases (*n* = 140)
Tumor localization	
Colon	25 (17.9)
Rectosigmoid	115 (82.1)
Tumor grade	
G1, G2	130 (92.9)
G3, G4	10 (7.1)
Depth of invasion	
T1, T2	37 (26.6)
T3, T4	89 (64.0)
Tx	13 (9.4)
Lymph node involvement	
N0	77 (55.4)
N1, N2	47 (33.8)
Nx	15 (10.8)
Distant metastasis	
M0	109 (78.4)
M1	13 (9.4)
Mx	17 (12.2)
AJCC stage	
Stage Ⅰ	31 (22.3)
Stage Ⅱ	43 (30.9)
Stage Ⅲ	43 (30.9)
Stage Ⅳ	15 (10.8)
Unknown	7 (5.1)
Relapse, Yes	48 (34.5)
Death, Yes	18 (12.9)

Categorized data are presented by absolute numbers (percentage) and continuous data are presented as median (min-max). Tumors were classified according to the guidelines of the American Joint Committee on Cancer (AJCC) staging system.

exon 5 from mature mRNA, which then leads to low levels of soluble HLA-G (sHLA-G)[74]. Furthermore, our results indicated that the Ins allele was associated with a higher TNM stage and relapse up to 5 years. These findings suggest that low levels of sHLA-G might influence in poor prognostics.

UGT1 is a family of membrane-bound enzymes involved in the inactivation and elimination of lipophilic molecules through glucorination. Moreover, variants

Table 9 The significant insertion-deletions associations with relapse risks

Gene	Model	Time of follow-up	OR (95%CI)	*P* value
ACE	Del/Del *vs* Del/Ins + Ins/Ins	2 yr	0.32 (0.13-0.77)	0.0113
ACE	Del/Del *vs* Del/Ins + Ins/Ins	3 yr	0.37 (0.15-0.91)	0.0298
HLAG	Del/Del *vs* Del/Ins + Ins/Ins	2 yr	2.75 (1.07-7.08)	0.0281
HLAG	Del/Del *vs* Del/Ins + Ins/Ins	4 yr	2.83 (1.07-7.52)	0.0332
HLAG	Del/Del *vs* Del/Ins + Ins/Ins	5 yr	3.47 (1.20-9.99)	0.0194
TYMS	Ins/Ins *vs* Del/Ins + Del/Del	2 yr	3.35 (1.36-8.28)	0.0058
TYMS	Ins/Ins *vs* Del/Ins + Del/Del	3 yr	3.42 (1.41-8.28)	0.0046
UGT1A1	Del/Del *vs* Del/Ins + Ins/Ins	4 yr	3.23 (1.27-8.22)	0.0116
UGT1A1	Del/Del *vs* Del/Ins + Ins/Ins	5 yr	3.50 (1.24-9.84)	0.0145

Logistic regression adjusted for confounders. The Supplementary Table 4 shows the genotype frequency of all insertion-deletions polymorphism.

Table 10 The significant insertion-deletions associations with death risks

Gene	Model	Time of follow-up	OR (95%CI)	*P* value
SGSM3	Del/Del *vs* Del/Ins + Ins/Ins	6 yr	3.61 (1.01-12.92)	0.0487
SGSM3	Del/Del *vs* Del/Ins + Ins/Ins	7 yr	4.60 (1.16-18.23)	0.0260
UGT1A1	Del/Del *vs* Del/Ins + Ins/Ins	6 yr	5.30 (1.43-19.73)	0.0084
UGT1A1	Del/Del *vs* Del/Ins + Ins/Ins	7 yr	4.64 (1.19-18.10)	0.0202
UGT1A1	Del/Del *vs* Del/Ins + Ins/Ins	8 yr	6.50 (1.47-28.80)	0.0091

Logistic regression adjusted for confounders. The Supplementary Table 5 shows the genotype frequency of all insertion-deletions polymorphism.

in this gene have been shown to be useful tools to identifying patients more likely to experience severe toxicity related to irinotecan-containg regimens[75]. In particular, INDEL variants in *UGT1A1* (rs8175347) were associated with significantly decreased glucuronidation activity, which results in reduced SN-38 clearance[76] and an increased risk of these toxicities in patients homozygous for the Ins allele[75,77-80]. Our results showed that the Ins allele in UGT1A1 was associated with early relapse risk, as well as with death risk prior to 8 years. This genetic variation may identify patients who might benefit from increased irinotecan dosing, as observed by Chen *et al*[75].

The *SGSM3* belongs to a novel protein family consisting of three members and appears to be associated with small G-protein coupled receptor signal transduction pathways, and could control cellular functions by a Ras-mediated signaling pathway[81]. Studies have linked Rab dysfunction to various human diseases including cancer[82,83], and our results have shown that the Ins allele might also be associated with death risk prior to 8 years.

The aims of this study were to determine the association between CRC risk and the clinical features with 16 INDEL in genes involved with carcinogenesis pathways in an admixed population from Brazil. Although we have achieved our goal, there are limitations regarding sample number. We suggest, therefore, that an extensive study should be conducted in the Brazilian population to confirm the findings, as well as in other admixed populations.

In summary, the present work indicates that polymorphisms in *ACE* (rs4646994), *TYMS* (rs151264360), *UCP2* (45 bp), *IL4* (rs79071878), *NFKB1* (rs28362491), *CASP8* (rs3834129), *TP53* (rs17880560), *HLAG* (rs371194629), *UGT1A1* (rs3213239), and *SGSM3* (rs56228771) genes were associated with CRC risk and clinical features in an admixed population. These data suggest that this cancer panel might be useful as a complementary tool for better clinical management, and more studies need to be conducted to confirm these findings.

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COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer type in men and the second in women. Despite the effective strategies for prevention, early detection, and treatment, there are ethnic differences in CRC incidence and survival. These variances occur specifically in African Americans, who have higher CRC incidence and lower survival rates than other ethnic groups. Thus, the present study evaluated the association between 16 insertion-deletions (INDEL) polymorphisms with colorectal cancer risk in an admixture population, as well with clinical features.

Research frontiers

The second most abundant form of genetic variation in humans, after single nucleotide polymorphisms (SNPs), are the INDEL. The INDEL understanding is important because they are common genetic variations within genomes, and they may alter human traits and cause diseases, including colorectal cancer, by modifying the coding region or mRNA stability. One of the challenges for genetic polymorphism association studies is the lack of knowledge regarding the frequency of the polymorphism in the targeted population, mainly in admixed populations (*e.g.* Brazil).

Innovations and breakthroughs

This is the first case-control study to evaluate the association between these 16 INDEL polymorphisms with colorectal risk, clinical features and prognostic follow-up in an admixture population, adopting the methodology that can be easily used to perform multiplexing assays.

Applications

This pilot study is design and findings could be used to determine sample size for a larger randomized controlled study aiming to test the impact of these INDEL polymorphism panel in colorectal risk, clinical features and prognostic follow-up.

Terminology

Ancestry informative marker - In population genetics, an ancestry informative marker (AIM) is a polymorphism that exhibits substantially different frequencies between populations from different geographical regions. A set of many AIMs can be used to estimate the proportion of ancestry of an individual derived from each geographical region.

Peer-review

This is an interesting study aiming to determine the association between CRC risk, and the clinical features with 16 INDEL in genes involved with carcinogenesis pathways in an admixed population from Brazil. The overall structure of the manuscript is complete and conforms to the academic rules.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hospital readmissions in decompensated cirrhotics: Factors pointing toward a prevention strategy

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Abstract

AIM

To reduce readmissions and improve patient outcomes in cirrhotic patients through better understanding of readmission predictors.

METHODS

We performed a single-center retrospective study of patients admitted with decompensated cirrhosis from January 1, 2011 to December 31, 2013 (n = 222). Primary outcomes were time to first readmission and 30-d readmission rate due to complications of cirrhosis. Clinical and demographic data were collected to help describe predictors of readmission, along with care coordination measures such as post-discharge status and outpatient follow-up. Univariate and multivariate

analyses were performed to describe variables associated with readmission.

RESULTS

One hundred thirty-two patients (59.4%) were readmitted at least once during the study period. Median time to first and second readmissions were 54 and 93 d, respectively. Thirty and 90-d readmission rates were 20.7 and 30.1 percent, respectively. Predictors of 30-d readmission included education level, hepatic encephalopathy at index, ALT more than upper normal limit and Medicare coverage. There were no statistically significant differences in readmission rates when stratified by discharge disposition, outpatient follow-up provider or time to first outpatient visit.

CONCLUSION

Readmissions are challenging aspect of care for cirrhotic patients and risk continues beyond 30 d. More initiatives are needed to develop enhanced, longitudinal post-discharge systems.

Key words: Chronic disease; Re-hospitalizations; Liver disease; Readmissions; Cirrhosis; Chronic care

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Core tip: To reduce readmissions and improve patient outcomes, we conducted a retrospective cohort study of 222 decompensated cirrhotics admitted to a single institution and followed longitudinally for readmission. Nearly 60% were readmitted during the study, with hepatic encephalopathy as the most common cause of readmission, with social factors, education level and insurance, also affecting readmission rates. We also found that readmission risk in this population continues well beyond 30 d, with 30% of patients being readmitted at 90 d, calling for continued, coordinated care after hospitalization.

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INTRODUCTION

Cirrhosis is the second most common digestive disease cause of death and 12th overall cause of death in the United States[1]. Cirrhosis and complications of cirrhosis account for around 40000 deaths in the United States annually, similar to diabetes and slightly more than kidney diseases, with a mortality rate of 25.7 deaths per 100000 people[2,3]. In addition to high mortality, cirrhosis and its complications are the cause of many high-cost hospitalizations. One study in 2002 showed that cirrhosis accounts for 150000 hospitalizations in the US, with an estimated single hospital admission cost of $15000 and a total estimated cost of $4 billion annually[2]. A more recent study found that 37% of patients with decompensated cirrhosis were readmitted within one month of discharge at a cost of roughly $20000 per admission[4]. The additional burdens of disease, including lost days of work, diminished quality of life, and health care costs are difficult to measure.

In the current value-based healthcare landscape, hospitals will be increasingly held financially accountable for readmissions under value-based care programs. Moreover, no evidence-based systems have been developed to reduce readmission rates in most chronic disease conditions. Providers and health systems face challenges, as the current body of literature lacks data on effective chronic disease care management for cirrhosis, and our current episodic system of health care delivery is insufficient to tackle this issue. Factors associated with readmissions in decompensated cirrhosis in previous works include Model for End-Stage Liver Disease (MELD), diabetes, nosocomial infections, BMI and hepatic encephalopathy[5-7].

Our institution has sought to improve the continuity of care for high-risk patients through post-discharge phone calls, as well as through care management programs for chronic conditions such as heart disease and diabetes. We hope to extend such programs to patients with cirrhosis to improve clinical outcomes and reduce readmissions[8]. We performed this study to better understand and define the predictors of readmission, as well as time to first readmission, in a cohort of patients with decompensated cirrhosis, to better target future programs aimed at reducing readmissions and improving patient outcomes.

MATERIALS AND METHODS

Selection of cohort

We utilized an internal research patient data registry (RPDR), which is a database consisting of 1 billion records encompassing 4.5 million patients from 1988 to the present, to identify our cohort. Data come from multiple sources within 5 major Boston area hospitals, including billing registries and electronic medical records, and contain patient encounters, laboratory test results, imaging, and other clinical data. We searched this database to identify potential subjects by any of the International Classification of Diseases 9 (ICD-9) diagnosis groups or common procedure terminology codes associated with cirrhosis and complications of cirrhosis (Table 1).

Patients over the age of 18 who were hospitalized

Table 1 ICD-9 codes used to identify patients

Bleeding from esophageal varices	456
Esophageal varices in diseases classified elsewhere	456.2
Spontaneous bacterial peritonitis	567.23
Alcoholic fatty liver	571
Alcoholic cirrhosis of liver	571.2
Laennec's cirrhosis	571.2
Cirrhosis of liver nos	571.5
Cirrhosis of liver without mention of alcohol	571.5
Macro nodular cirrhosis	571.5
Micro nodular cirrhosis	571.5
Portal cirrhosis NOS	571.5
Posthepatitic cirrhosis	571.5
Post necrotic cirrhosis	571.5
Cholangitic cirrhosis	571.6
Cholestatic cirrhosis	571.6
Hepatic encephalopathy	572.2
Portal hypertension	572.3
Hepatorenal syndrome	572.4
Other sequelae of chronic liver disease	572.8
Hepatopulmonary syndrome	573.5
Ascites	789.5

due to one of the complications of cirrhosis between January 1, 2011 - December 31, 2013 were included in the initial study cohort. Complications of cirrhosis from Table 1 were grouped into the following categories for analysis: (1) Hepatic encephalopathy (HE), defined as altered mental status due to chronic liver disease and improved after treatment with lactulose; (2) Gastrointestinal bleeding (GIB), defined as a clinically significant bleeding and varices, gastric antral vascular ectasia and portal hypertensive gastropathy seen on endoscopy; (3) Spontaneous bacterial peritonitis (SBP), defined as infection of preexisting ascitic fluid and more than 250 polymorphonuclear cells per high power field and/or monomicrobial culture in the ascitic fluid without evidence for an intra-abdominal secondary source such as a perforated viscus; (4) Volume related, defined as ascites or hepatic hydrothorax requiring paracentesis during the hospitalization. Acute renal failure or hyponatremia was also included in this category; and (5) Other complications defined as hepatorenal syndrome, hepatopulmonary syndrome, hepatocellular carcinoma (HCC) and portal vein thrombosis.

Emergency Department (ED) visits or hospitalizations due to any diagnosis other than complications of cirrhosis were not considered as a readmission. We did not include alcohol-related admissions, such as alcohol withdrawal, as admissions due to complications of cirrhosis. Also, in our institution many patients receive regular large volume paracentesis for ascites in ED visits. These brief encounters were not considered an elective readmission due to decompensation.

Exclusion criteria were advanced extrahepatic malignancy, end stage renal failure, end stage heart failure, patients with prior liver transplant, patients who had no further encounters within our health care system, and patients who died or were placed on hospice within one month from the index hospitalization.

Institutional structure

This study was conducted at a single academic transplant center. Our outpatient liver program includes 7 hepatologists, 31 gastroenterologists not specifically trained in hepatology, and 2 nurse practitioners, as well as an advanced liver fellow. Inpatients patients with liver disease can be admitted to our dedicated liver service but are most often admitted to a general medicine hospitalist service with hepatology or gastroenterology consultation. A majority of our patients are local and live within an hour's drive or less from the hospital, though patients on the transplant list may come from other parts of New England, such as Maine and New Hampshire, and are seen more regularly as outpatients by local primary care physicians (PCPs) and gastroenterologists.

Collection of baseline data

Medical records for patients meeting the above inclusion criteria were individually reviewed. Baseline variables collected from chart review and RPDR data included age, gender, race, education, health insurance, marital status, MELD scores, laboratory test results, co-morbidities, medications, date of index and subsequent admissions, date of follow-up visits by a PCP or a gastroenterologist, post-discharge setting, smoking history and alcohol use. Admission and discharge MELD scores were calculated based on Mayo Clinic MELD calculator, using creatinine, total bilirubin, and international normalized ratio (INR) extracted closest to the admission and discharge time, respectively[9]. The Charlson Co-morbidity Index (CCI) was also calculated for each patient at index admission[10].

Follow-up time and study outcomes

Patients were followed from the index admission until readmission, death, discharge with hospice care, liver transplantation, loss to follow-up or December 31, 2013, whichever came first. All patients were followed post-discharge for a minimum of 90 d and all readmissions were reviewed. The primary outcomes were time to first readmission and 30-d readmission rate for cirrhosis or a complication of cirrhosis. The secondary outcome was the 90-d readmission rate.

Statistical analysis

Univariate analyses were performed using chi-square or proportional hazard ratio to find predictors of 30-d readmission and time to first readmission. Clinically relevant candidate variables with $P < 0.15$ were used

Table 2 Baseline characteristics of cohort (n = 222) n (%)

Demographics	Number of patients
Male	149 (67.2)
White	168 (76)
Married	82 (36.9)
Age	mean ± SD: 56.7 (11.7)
Insurance	
Private insurance	81 (36.5)
Medicaid or mass health	74 (33.3)
Medicare	62 (27.9)
No Insurance (self-pay/free care)	5 (2.3)
Education	
High school/equivalent or less	127 (57.2)
Some college and above	60 (27)
Not recorded	35 (15.8)
Clinical History	
Alcohol use	
Former heavy use	110 (49.5)
No significant history	65 (29.3)
Current heavy use	47 (21.2)
Smoking	
Never smoked	82 (36.7)
Former smoker	77 (34.7)
Current smoker	63 (28.4)
Etiology of cirrhosis	
Alcohol	83 (37.4)
HCV or HBV (HCV, n = 46)	49 (22.1)
Combination of etiologies (mainly HCV + alcohol)	40 (18)
NASH	29 (13)
Cryptogenic or other etiologies	21 (9.5)
Co-morbidities (Top 5)	
Mental health (Depression or bipolar disorder)	68 (30.2)
Diabetes mellitus	65 (29.8)
Coronary artery disease	27 (12)
Chronic kidney disease	27 (12)
Hepatocellular carcinoma	24 (10.6)

HCV: Hepatitis C virus; HBV: Hepatitis B virus.

Table 3 Hospitalization characteristics n (%)

Index hospitalization	
Complication of cirrhosis at index admission	Number of patients
Volume-related	64 (28.8)
Hepatic encephalopathy	61 (27.5)
Variceal bleeding	54 (24.3)
Spontaneous bacterial peritonitis	27 (12.2)
Other or > 1 complication	16 (7.2)
	mean ± SD
Length of stay	7.2 (7.8)
MELD at admission	13.3 (7.7)
MELD at discharge	12.2 (7.5)
Readmissions	Number of patients
Readmitted with the same complication	88 (49.4)
Readmitted in one month	46 (20.7)
Cause of readmission	
Hepatic encephalopathy	49 (35.5)
Volume-related	38 (27.5)
Variceal bleeding	31 (22.5)
Other or > 1 complication	12 (8.7)
Spontaneous bacterial peritonitis	8 (5.8)

MELD: Model for end-stage liver disease.

in Cox proportional hazards regression (for time to first readmission) and logistic regression (for 30 and 90 d readmission rates) models. Due to co-linearity between reason for index admission and medications at discharge, medications at discharge was excluded from the final models. Two-sided P values ≤ 0.05 were considered significant. All statistical analyses were performed using SAS v.9.3. The statistical methods and results of this study were reviewed by biostatistician Han Lee, PhD of Massachusetts General Hospital and Harvard Medical School.

RESULTS

From January 1, 2011 to December 31, 2013, 222 patients were admitted to our center due to one of the complications of cirrhosis and did not meet any of the exclusion criteria. The median time from hospital discharge to end of the study period was 273 d. Baseline characteristics of the study population are presented in Table 2.

One hundred thirty-two of these 222 patients (59.4%) were readmitted at least once during the study period. The median time to first readmission, our primary outcome, was 54 d. Thirty and 90-d readmission rates were 20.7 and 30.1 percent, respectively. The most common cause of index admission was one of the volume related conditions (28.8%) while the most common cause of readmission was hepatic encephalopathy (35.5%) (Table 3). The average length of stay for the index hospitalization was 7.2 d, and 88 patients (49.4%) were readmitted with the same complication of cirrhosis.

Post-discharge conditions are summarized in Table 4 based on the post-discharge care setting. Patients discharged to a skilled nursing facility, those who left the hospital against medical advice, and homeless patients who were discharged to shelters had more 30-d readmissions but the results were not statistically significant (P < 0.12).

One hundred sixty five patients out of the 222 index patients (74.3 %) had a follow-up outpatient visit in our institution at some point after index admission (Table 4). The rest of the patients were not seen at our center after discharge and may have had local follow-up. Of those 165 patients seen at our institution, 83 (37.4%) were seen only by a gastroenterologist, 22 (9.9%) only by a PCP and 31 (14%) both by a gastroenterologist and a PCP. The remaining 29 patients had an outpatient visit scheduled post-discharge but were readmitted before the scheduled visit. Among the patients who had a post-discharge follow-up visit at our institution, there was no significant difference in 30-d readmission rates based on the visit by a PCP versus a gastroenterologist

Table 4 Post-discharge follow-up *n* (%)

	Number of patients	30-d readmissions	*P* value
Post-discharge status			
Home	120 (54.3)	19 (15.9)	$P < 0.12$
VNA	60 (27.2)	14 (23.3)	
SNF/rehab or LTAC	30 (13.5)	8 (26.7)	
Eloped	5 (2.3)	0	
Shelter	5 (2.3)	0	
Mental Institute	1 (0.4)	1(100)	
Follow-up outpatient visits			
Gastroenterologist	83 (37.4)	10 (12.1)	$P = 0.9$
PCP	22 (9.9)	2 (9.1)	
Both	31 (14)	4 (12.9)	
Readmitted before visit	29 (13.1)	29 (100)	
Total	165 (74.3)	45 (27.3)	
First post-discharge outpatient follow-up (PCP and/or GI)			
< 2 d	35 (21.2)	31 (60.0)	$P = 0.1$
2-14 d	66 (40.0)	13 (19.7)	
> 14 d	64 (38.8)	3 (4.7)	
Time to visit (d)	Mean ± SD		
Time to first visit by GI	22.6 (43.7)		
Time to first visit by PCP	31.9 (35)		
Time to first visit by PCP or GI	24.7 (33.8)		

PCP: Primary care physician; GI: Gastrointestinal.

Table 5 Predictors of time to readmission

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Education level (ref = Some college and above)[1]	1.00	0.02	1.00	
Finished high school and less	1.64 (1.02-2.46)	0.02	1.56 (0.98-2.46)	0.05
MELD category (ref = MELD < 15)	1.00	0.007	1.00	
15 ≤ MELD < 25	1.45 (0.97-2.15)	0.06	1.52 (1.00-2.31)	0.04
MELD ≥ 25	2.51 (1.33-4.75)	0.004	2.51 (1.24-5.11)	0.01
Etiology of CLD (ref = Alcohol)	1.00	0.03	1.00	
HCV	1.74 (1.10-2.76)	0.03	1.73 (1.06-2.82)	0.02
NASH/Cryptogenic	1.25 (0.76-2.05)	0.36	1.81 (1.02-3.18)	0.04
Other/Combination of etiologies	1.94 (1.20-3.11)	0.006	2.02 (1.24-3.29)	0.004
History of HCC	1.85 (1.10-3.08)	0.01	1.67 (0.95-2.93)	0.07
HE	1.51 (1.04-2.16)	0.01	1.61 (1.11-2.40)	0.01

[1]Education variable was adjusted for unknown status. HCC: Hepatocellular carcinoma; MELD: Model for End-Stage Liver Disease; HCV: Hepatitis C virus.

or both ($P = 0.9$).

We further analyzed 30 d readmission rates stratified by time until first post-discharge follow-up visit: < 2 d, 2-14 d, and > 14 d. Thirty five patients (21%) were seen in an office visit within two days after index discharge, 66 (40%) between 2 and 14 d, and 64 (38.8%) after 14 d, but there was no statistically significant difference in the rate of readmission amongst these groups ($P = 0.1$). The average time between discharge and the first outpatient follow-up appointment was also calculated at 22.6 d for patients seen by a GI specialist, 31.9 d for those who saw a PCP and 24.7 d for those who saw both.

In the univariate time-to-event analysis, the following covariates were associated with time to readmission ($P < 0.05$): education level, etiology of cirrhosis, MELD score, diagnosis of HE at index admission, history of HCC, and history of depression or bipolar disorder (Table 5). In the multivariate analysis, lower education level, any cirrhosis etiology other than alcohol, higher MELD score, HE at index admission and history of HCC were associated with shorter time to readmission (Table 5). Kaplan-Meier curves for time to readmission stratified by MELD score and etiology of cirrhosis are shown in Figures 1 and 2, respectively. In both the univariate and multivariate analysis of predictors for 30-d readmissions, patients with lower education, HE at index, ALT more than upper limit of normal (ULN) and Medicare patients had higher 30-d readmission rates ($P < 0.05$) (Table 6). The c-statistic for this test was 0.75.

DISCUSSION

Hospital readmissions are costly and represent opportunities for improvements in quality of care,

Table 6 Predictors of 30-d readmission

Variable	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Insurance (ref =Medicaid/No insurance)	1		1	
Medicare[1]	1.8 (1.00 -3.94)	0.05	3.42 (1.3-9.00)	0.01
Education (ref = Some college and above)	1.00		1	
Finished high school and less[2]	3.56 (1.40-9.00)	0.002	2.62 (1.01-7.12)	0.05
ALT > ULN at discharge	2.52 (1.29-4.89)	0.006	2.29 (1.09-5.11)	0.03
Hepatic encephalopathy	2.17 (1.10-4.29)	0.01	2.34 (1.09-4.80)	0.03

[1]Insurance variable was adjusted for patients with private insurance; [2]Education variable was adjusted for unknown status. ULN: Upper limit of normal.

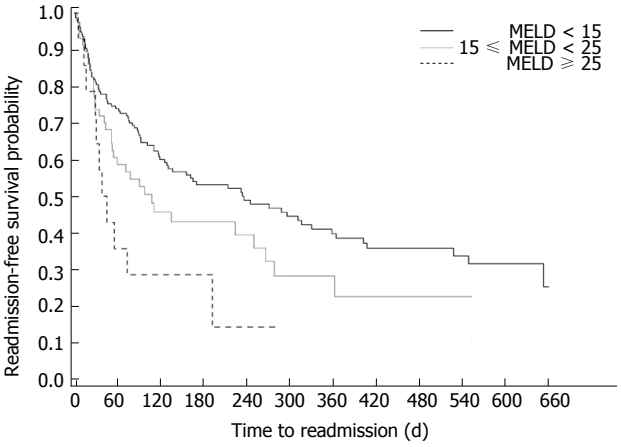


Figure 1 Kaplan-Meier curve for time to readmission stratified by MELD score.

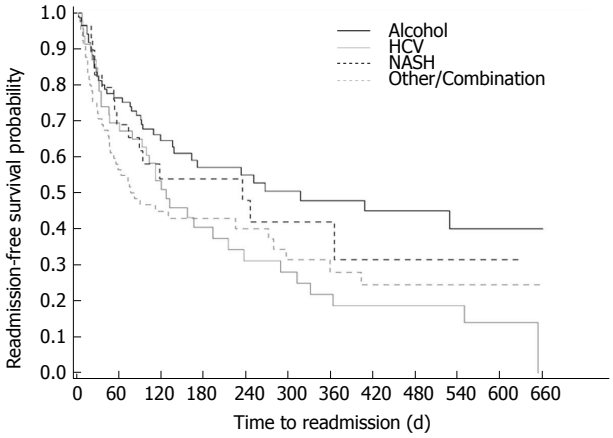


Figure 2 Kaplan-Meier curve for time to readmission stratified by etiology of cirrhosis.

communication, and coordination. Readmissions are viewed as potentially avoidable and the large payers, such as the United States Department of Health and Human Services Center for Medicare and Medicaid Services Readmission Prevention Program penalizes hospitals for higher than expected readmissions for heart failure, myocardial infarction and pneumonia. As healthcare moves towards value-based payment methods, primary care and specialists alike must prepare to better organize care for the highest risk and highest cost patients. Understanding the attributes of patients and causes for readmission is the foundation for designing strategies to mitigate the economic losses and prevent clinical morbidity. Additionally, while the current 30-d readmission threshold is appropriate for discrete admission events, such as surgery, applying this model to a chronic disease population may not capture true readmission risk or opportunity for improvement. By studying our patients longitudinally, we showed that about 20% of our patients were readmitted within 30 d, but that rate rose to 30% at 90 d. We also found that lower education level, MELD score ⩾ 15, chronic liver disease due to more than one etiology, HE at index admission, and history of HCC are associated with shorter time to readmission with the median time to first readmission of 54 d.

Our data is consistent with previous studies which have shown similar 30-d and 90-d readmission results[4,7,11-13]. We also found that Medicare patients, patients with lower education levels, higher ALT levels at discharge, and HE at the time of presentation are associated with higher 30-d admission rates. We also found that MELD score, which is a measure of hepatic and renal function combined, is associated with more 30-d readmission and shorter time to first readmission rates. Prior studies have also demonstrated that patients with higher MELD score are more prone to 30-d readmission[4,11] or more first hospital admission in patients who had not been admitted the previous year[14].

As seen in other recent studies, hepatic encephalopathy was a predictor for readmission, based on both 30-d and time to readmission analyses[7,15,16]. The one-year mortality for cirrhotic patients with hepatic encephalopathy is very high, approaching 60%-80% in recent studies[17,18]. HE is an expensive disease, costing approximately $63000 per case and is responsible for approximately 7.2 billion dollars

in direct costs annually[19,20]. Thirty-four out of 62 (54.9%) of our patients who presented first with hepatic encephalopathy were readmitted at least once with the same diagnosis in one year. HE is not simply a marker of disease severity; forty four (71%) of our index patients who presented with HE had MELD score below 15. This is an opportunity for focused management, as we hypothesize that HE patients are more prone to readmission due to the functional impact of encephalopathy.

Careful discharge planning and expedited outpatient follow-up may help prevent readmissions, though further study is needed for cirrhosis patients. In our study, patients discharged to skilled nursing facility, those who left the hospital against medical advice and homeless patients who were discharged to shelters had higher 30-d readmission rates, but the results were not statistically significant. While not statistically significant, it is worth noting that the majority of our patients (54.3%) were discharged home without any support services, and only 27.2% of our patient cohort had VNA services after their index admission, pointing to an opportunity for further outreach once the patient leaves the hospital. As many cirrhotic patients have HE, compromising their decision making, coordinating medications and returning to their outpatient clinics for follow-up more challenging. As health systems design interventions for these complex patients, the role of home health care should be considered, with home visits for patients in our catchment area from nurses and/or allied health professionals who help patients organize their care once they've left the hospital.

Early outpatient follow-up by primary care and/or specialists may be another opportunity to improve outcomes but not necessarily reduce readmissions. A recent study conducted by the VA found that early (within 7 d of discharge) outpatient follow-up in patients with cirrhosis slightly increased the rate of 30-d readmissions, but significantly lowered the risk of mortality[21]. A study in Denmark showed that one-hour rehabilitation clinic within 1-3 wk after discharge from HE, reduced alcohol consumption and improved survival, but did not diminish the subsequent hospital admissions[22]. While our own numbers on outpatient follow-up were small, we did not show that relatively early medical follow-up (14 d or less) prevented readmissions. Follow up in two or three weeks for cirrhotics may not be sufficient. As the average time to a post-discharge visit ranged from 22.6-31.9 d, and 29 patients were readmitted before they had an outpatient visit, there is clearly room to improve access to post-discharge care, potentially harnessing alternate care models such as home health services, RN-led clinics and virtual visits.

Due to our use of clinical records, rather than insurance claims data, we were not able to directly gather data about hospitalizations outside our health care system. Also, our inclusion criteria only searched for admissions due to complications of cirrhosis, although (many) patients were hospitalized electively or non-electively for existing co-morbidities other than complications of cirrhosis. Our study is subject to selection bias given that patients followed at tertiary care centers tend to be more medically complex.

In summary, we showed that readmission risk for patients with cirrhosis continues well beyond 30 d and may be attributable to serious chronic disease and compromised coping skills. We found that lower education level, MELD score $\geqslant$ 15, chronic liver disease due to more than one etiology, HE at index admission, and history of HCC are associated with shorter time to readmission with the median time to first readmission of 54 d. As health systems and insurers increasingly support the adoption of value-based payment methods and patient-centered care, providers and health systems leaders must work proactively to identify high-cost and high-risk patient populations that may benefit from integrated care systems to help proactively manage chronically ill patients[23,24]. We believe that patients with cirrhosis are a population that will benefit by a systematic multidisciplinary outreach program which has the potential for preventing readmissions, improving care and saving money. Future studies should explore systems of care to define post-discharge best practices for continued care once the patient leaves the hospital environment.

COMMENTS

Background

Hospital readmissions are gaining increasing scrutiny as a measure of value-based health care as they directly impact both quality and cost of care. Cirrhosis and complications of cirrhosis account for around 40000 deaths in the United States annually and also account for 150000 hospitalizations in the US, with an estimated total estimated cost of $4 billion annually. A recent study found that 37% of patients with decompensated cirrhosis were readmitted within one month of discharge at a cost of roughly $20000 per admission.

Research frontiers

In the current value-based healthcare landscape, hospitals will be increasingly held financially accountable for readmissions under value-based care programs. Moreover, no evidence-based systems have been developed to reduce readmission rates in most chronic disease conditions, such as cirrhosis.

Innovations and breakthroughs

Factors associated with readmissions in decompensated cirrhosis in previous works include MELD, diabetes, nosocomial infections, BMI and hepatic encephalopathy. Early outpatient follow-up by primary care and/or specialists may be another opportunity to improve outcomes but not necessarily reduce readmissions. A recent study found that early (within 7 d of discharge) outpatient follow-up in patients with cirrhosis slightly increased the rate of 30-d

readmissions, but significantly lowered the risk of mortality. While our own numbers on outpatient follow-up were small, we did not show that relatively early medical follow-up (14 d or less) prevented readmissions. Follow up in two or three weeks for cirrhotics may not be sufficient, as the average time to a post-discharge visit ranged from 22.6-31.9 d, and 29 patients were readmitted before they had an outpatient visit; there is clearly room to improve access to post-discharge care, potentially harnessing alternate care models such as home health services, RN-led clinics and virtual visits.

Applications

Authors' institution has sought to improve the continuity of care for high-risk patients through post-discharge phone calls, as well as through care management programs for chronic conditions such as heart disease and diabetes, and they hope to extend such programs to patients with cirrhosis to improve clinical outcomes and reduce readmissions. Authors performed this study to better understand and define the predictors of readmission, as well as time to first readmission, in a cohort of patients with decompensated cirrhosis, to better target future programs aimed at reducing readmissions and improving patient outcomes.

Terminology

Value-based payments are a strategy increasingly adopted by payers with the goal of shifting from volume-based payment, *e.g.*, "fee-for service" payments, to those based on improved clinical outcomes.

Peer-review

For chronic disease populations, current 30-d readmission thresholds may not capture true readmission risk or opportunities for improvement. This paper found that lower education level, MELD score ≥ 15, chronic liver disease due to more than one etiology, HE at index admission and history of HCC are associated with shorter time to readmission with the median time to first readmission of 54 d. Designing post-admission continuity of care interventions with these factors in mind would improve clinical practice and patient outcomes.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Measurement of biological age may help to assess the risk of colorectal adenoma in screening colonoscopy

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Author contributions: Kim BJ designed research; Kim BJ and Kim SJ performed research; Kang H analyzed data; Kim SJ wrote the paper.

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Informed consent statement: Informed consent was waived by the IRB because the study used anonymous clinical data.

Conflict-of-interest statement: The authors declare no conflict of interest in this study.

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Abstract

AIM

To investigate the clinical utility of biological age (BA) measurement in screening colonoscopy for the detection of colorectal adenomas in the average-risk population.

METHODS

A consecutive series of asymptomatic subjects aged ⩾ 30 years who underwent colonoscopy in routine check-ups were enrolled. Colorectal adenoma was classified according to size, number, and location. BAs were calculated using the MEDIAGE™ Biological Age Measurement System.

RESULTS

A total of 2696 subjects were investigated (1876 men and 820 women). The mean chronological age (CA) was 46.0 years and the mean BA was 44.7 years. Metabolic syndrome (MS) was diagnosed in 218 subjects (8.1%). The prevalence of overall colorectal adenoma was 23.1% (622/2,696). When the subjects were divided into four groups based on BA (⩽ 39 years; 40-49 years; 50-59 years; ⩾ 60 years), the prevalence of colorectal adenoma was increased as BA increased (P < 0.001). Colorectal adenoma located in the proximal colon was more prevalent in the BA-dominant group (BA-CA ⩾ 5

years) than the CA-dominant group (CA-BA ⩾ 5 years) (P = 0.034). When the subjects were categorized into four groups according to MS and age gap between BA and CA, the incidence of colorectal adenoma increased with MS and BA-dominance (P < 0.05).

CONCLUSION

Measurement of BA may help to assess the risk of colorectal adenoma in screening colonoscopy.

Key words: Chronological age; Biological age; Colorectal adenoma; Age gap

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Core tip: The present study was conducted on the basis of the hypothesis that colorectal adenoma recognized as a premalignant lesion of colorectal cancer is associated with the biological aging process, suggesting that biological age (BA) represents a biological activity in human. Thus, we intended to assess the risk of colorectal adenomas in the average-risk population from the biological perspective. As a result, the present study demonstrated that the prevalence of colorectal adenoma correlated well with BA, and was more common in the distal colon in BA -dominance.

Kim SJ, Kim BJ, Kang H. Measurement of biological age may help to assess the risk of colorectal adenoma in screening colonoscopy. *World J Gastroenterol* 2017; 23(37): 6877-6883 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6877.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6877

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer mortality in the world[1,2]. Colonoscopy is effective for detecting adenomatous polyps, and colonoscopy with polypectomy has been shown to significantly reduce the expected incidence of CRC by 76%–90% in multiple cohort studies[3,4].

The guidelines of the American Society of Gastrointestinal Endoscopy for colorectal cancer screening and surveillance recommend that screening colonoscopy should begin at the age of 50 for average-risk individuals[5,6]. The current cutoff age is based mainly on the fact that the incidence of CRC begins to rise during the 6th decade of life[7]. However, given the long lead time associated with adenoma-to-carcinoma progression, the increased number of CRCs diagnosed in this age group may reflect the end result of adenomas present in individuals under 50 years old[6]. In fact, nearly 1 in 15 of CRCs develops in adults under 50 years of age[8]. Therefore, reasonable guidelines for performing screening colonoscopy for colorectal cancer should be proposed for the average-risk population.

Aging is a highly individual process, and has a high degree of inter-individual and between-individual difference[9]. Chronological age (CA) is the age as determined by the passage of time since birth. However, biological age (BA) is determined by physiology rather than chronology[10]. As CA does not accurately reflect the functional state of the body, it does not provide an accurate indication of the aging process[11,12]. CA and BA may differ significantly, since both genetics and the environment contribute to BA[10]. Individuals might be healthier, more vital, and biologically younger than might be expected from their CA, and vice versa[13].

If subjects with an average risk of colon cancer undergo colonoscopy according to BA, colon cancers may be prevented by colonoscopy with polypectomy for colorectal adenomas and premalignant lesions at the optimal age of life. Thus, it is necessary to stratify the risks and identify high-risk groups in a specialized manner. In addition, there might be a rationale by which to recommend screening colonoscopy for early cancer detection and prevention besides CA determination.

To date, no worldwide studies have been conducted to estimate the clinical application of BA as a new indicator in CRC screening. Hence, we intended to assess the risk for colorectal adenomas in the average-risk population from the biological perspective. Therefore, the aim of the present study was to investigate the clinical utility of BA measurement in screening colonoscopy for the detection of colorectal adenomas in the average-risk population.

MATERIALS AND METHODS

Subjects

We conducted this study on a consecutive series of asymptomatic subjects ⩾ 30 years of age who underwent colonoscopy during a routine check-up at the Health Promotion Center of Bundang Jesaeng Hospital in Gyungki-do, South Korea between August 2011 and August 2012. The exclusion criteria were a history of colonic disease, such as CRC, polyps, and inflammatory bowel disease, and a history of colorectal surgery or colonoscopy within the previous 10 years. Information regarding age, sex, medical history of diabetes mellitus and hypertension, and family history of colon cancer was collected from a standardized questionnaire. The study was approved by the Institutional Review Board of the Bundang Jesaeng Hospital in Korea (IMG 15-04).

Measurement of BA

BAs were calculated as described previously using models for predicting BA (MEDIAGE™ Biological Age Measurement System, MEDIAGE Research Center, Gyeonggi-do, South Korea)[14,15]. This measurement

system has been used as an adjunctive measure for easily predicting individual differences in health and aging status with the use of clinical parameters that are commonly used at many health check-up centers in South Korea.

Test battery parameter

We collected clinical profiles such as physical, biochemical, and hormonal parameters for the calculation of BA. A profile of physical parameters included height, weight, waist circumference, lean body mass, body fat measurement, body mass index, blood pressure, forced expiratory volume 1, and forced vital capacity. Waist circumference was measured at the high point of the iliac crest during minimal respiration. Blood samples were drawn from the antecubital space in the morning. Biochemical parameters included complete blood count with differential count, erythrocyte sedimentation rate, fasting blood glucose, liver function test, lipid profile, blood urea nitrogen, creatinine, bone turnover markers such as osteocalcin and deoxypyridinoline, and tumor markers such as prostate specific antigen, and total antioxidant status were measured using Randox TAS assay kits (Randox, United States) based on the reaction between metmyoglobin and hydrogen peroxide. Lastly, the hormonal parameters included thyroid-stimulating hormone, temporary threshold shift, sex hormone-binding globulin, follicle-stimulating hormone, insulin-like growth factor-1, and dehydroepiandrosterone sulphate.

Metabolic syndrome

Based on the ATP Ⅲ-WPRO (Regional Office for the Western Pacific Region of WHO) definition according to the WPRO criteria, metabolic syndrome (MS) was defined as the presence of three or more of the following[16]: (1) abdominal obesity, waist circumference > 90 cm in men and > 80 cm in women; (2) hypertriglyceridemia, ≥ 150 mg/dL; (3) low high-density lipoprotein cholesterol level, < 40 mg/dL in men and < 50 mg/dL in women; (4) high blood pressure, ≥ 130 mm Hg systolic or ≥ 85 mm Hg diastolic; and (5) high serum fasting glucose level, ≥ 110 mg/dL.

Colonoscopy

We performed colonoscopies that reached the cecum after bowel preparation with 4 L of Colyte powder (TAEJOON PHARM Co., Ltd., South Korea). The Colyte powder was composed of polyethylene glycol (3350.29 g), anhydrous sodium sulfate (2.85 g), sodium hydrogen carbonate (0.84 g), sodium chloride (0.73 g), and potassium chloride (0.37 g). We examined colonoscopic features, including the size, location, number, and histologic findings of polyps. Polyp size was assessed using open colonoscope biopsy forceps (MTW-Endoskopie Manufaktur, Wesel, Germany). Adenoma size was classified into < 10 and ≥ 10 mm (the largest size was used for multiple adenomas). The location of colorectal adenomas was divided into three categories: the proximal colon, including the cecum, ascending colon, and transverse colon; the distal colon, including the splenic flexure, descending colon, sigmoid colon, and rectum; and both sides of the colon. The number of adenomas was classified as either single or multiple (≥ 2). Histological findings were classified according to their premalignant potential: colorectal adenomas included tubular, villous, or serrated adenomas; and controls had normal colonoscopic findings and non-polypoid benign lesions such as non-specific colitis or histologically confirmed hyperplastic polyps. In addition, advanced adenomas were defined as ≥ 1 cm in estimated diameter, containing ≥ 25% villous features, and/or high-grade dysplasia.

Statistical analysis

For continuous variables, data distribution was first evaluated for normality using the Shapiro-Wilk test. As all the variables did not pass not pass normality test, data were analyzed using Mann-Whitney's *U* tests and presented as medians (P_{25}–P_{75}). Descriptive variables were analyzed using χ^2 analyses or Fisher's exact tests, as appropriate, and $P < 0.05$ were considered statistically significant. Statistical analyses were conducted using SPSS 21.0 (IBM Corp., Armonk, NY, United States).

RESULTS

We identified a total of 2699 subjects in the study period. Among them, three with colorectal carcinoma were excluded. Thus, the study population consisted of 2696 subjects. The demographic and clinical characteristics of the enrolled subjects are summarized in Table 1.

The subjects comprised 1876 men (69.6%) and 820 women (30.4%). The median age of the subjects in CA was 46.0 years, whereas the median age og the subjects in BA was 44.7 years old.

MS was diagnosed in 218 subjects (8.1%). When the subjects were divided into four groups based on BA (≤ 39 years; 40-49 years; 50-59 years; ≥ 60 years), the prevalence of MS was increased with increasing BA ($P < 0.001$). Interestingly, the gradient of prevalence of MS in BA was higher than that of CA.

The prevalence of overall colorectal adenoma was 23.1% (622/2696). The colonoscopic findings of the subjects according to location, size, and number are summarized in Table 2.

When the subjects were divided into four groups based on BA or CA (≤ 39 years; 40-49 years; 50-59 years; ≥ 60 years), the prevalence of colorectal adenoma was increased as BA and CA increased ($P < 0.001$) (Figure 1).

Clinical and colonoscopic characteristics were evaluated in accordance with the age gap between CA and BA. The prevalence of colorectal adenoma in

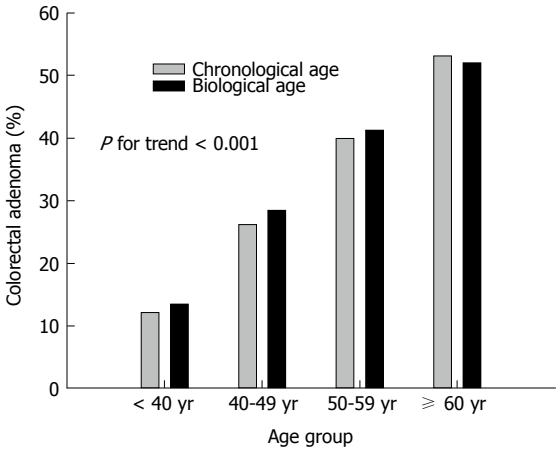


Figure 1 Prevalence of colorectal adenoma according to chronological age and biological age. When the subjects were divided into four groups (≤39 yr; 40-49 yr; 50-59 yr; ≥ 60 yr), the prevalence of colorectal adenoma increased with increasing biological age ($P < 0.001$).

Table 2 Characteristics of colorectal adenoma in subjects *n* (%)

	All subjects (*n* = 2696)
Colorectal adenoma	
Yes	622 (23.1)
No	2074 (76.9)
Location	
Proximal	489 (18.1)
Distal	244 (9.1)
Both	53 (2.0)
Size (mm)	
< 10	714 (26.5)
≥ 10	73 (2.7)
Number	
1	484 (18.0)
≥ 2	138 (5.1)

dominant age groups was assessed. The subjects were divided into two groups based on an age gap between CA and BA of more than 5 years: BA-dominant group (BA-CA ≥ 5 years) and CA-dominant group (CA-BA ≥ 5 years). The incidences of MS as well as high blood pressure, fasting glucose level, and HbA1c in the BA-dominant group were significantly higher than those in the CA-dominant group (Table 3). As shown in Table 4, colorectal adenoma located in the distal colon was more prevalent in the BA-dominant group than the CA-dominant group ($P = 0.034$).

The prevalence of colorectal adenoma according to MS and age gap was evaluated. We categorized subjects into four groups according to MS and age gap between BA and CA. As a result, the incidence of colorectal adenoma increased with MS and BA-dominance: The incidence of colorectal adenoma was 21.5%, 22.8%, 34.5%, and 39.2% for the first group (MS^- and BA < CA, $n = 368/1711$), the second group (MS^- and BA ≥ CA, $n = 175/767$), third group (MS^+ and BA ≥ CA, $n = 31/79$), and the fourth group (MS^+ and BA < CA, $n = 48/139$) ($P < 0.05$) (Figure 2).

Table 1 Baseline characteristics of the subjects

	All subjects (*n* = 2696)	
	Chronological age	Biological age
Age [median (P_{25}-P_{75}), yr]	46.0 (39.0-54.0)	44.7 (38.2-53.1)
Gender		
Male	1876	(69.6)
Female	820	(30.4)
High blood pressure		
Yes (%)	544	(20.2)
No (%)	2152	(79.8)
Total cholesterol (mg/dL)	193.00	(172.00-217.75)
LDL cholesterol (mg/dL)	118.00	(99.00-141.00)
HDL cholesterol (mg/dL)	53.00	(45.0-64.00)
Triglyceride (mg/dL)	98.00	(66.00-152.00)
Fasting glucose (mg/dL)	85.00	(79.00-92.00)
HbA1c	5.40	(5.20-5.70)
CEA	1.55	(1.00-2.38)
Metabolic syndrome		
Yes	218	(8.1)
No	2478	(91.9)

Data expressed as median (P_{25}-P_{75}), or absolute number (%). High blood pressure, ≥ 130/85 mmHg or documented use of antihypertensive therapy. LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA1c: Hemoglobin A1c; CEA: Carcinoembryonic antigen.

DISCUSSION

The present study was conducted on the basis of the hypothesis that colorectal adenoma recognized as a premalignant lesion of colorectal cancer is associated with the biological aging process, suggesting that BA represents a biological activity in human. Thus, we intended to assess the risk of colorectal adenomas in the average-risk population from the biological perspective. As a result, the present study demonstrated that the prevalence of colorectal adenoma correlated well with BA, and was more common in the distal colon in BA-dominance. Furthermore, the incidence of MS correlated more precisely with BA compared with CA.

Aging in humans refers to a multidimensional process of physical, psychological, and social changes that occur from birth[14]. Aging is usually assessed in terms of the CA, which is defined as the time elapsed since birth. However, CA fails as an accurate indicator of the aging process[17]. BA estimates the functional status of an individual with respect to their chronological peers on the basis of how well they function in comparison with others of the same CA[18]. Different individual rates of aging lead to differences between CA and BA, and individual values of BA can therefore vary widely at any given CA and are ultimately expected to correspond to the inter-individual variations in longevity and timing and/or magnitude of sequelae of the aging process[19,20]. It has been proposed that BA may serve as an indicator of an individual's general health status, remaining healthy life span, and active life expectancy[21]. Considering that BA corresponds well with the aging process, BA may be a good indicator for starting screening colonoscopy at the most biologically

Table 3 Clinical characteristics between biological age-dominant and chronological age-dominant groups

	BA-dominant group (n = 28)	CA-dominant group (n = 76)	P value
Age (yr, mean ± SD)	42.00 (35.25-49.75)	52.00 (43.25-59.00)	0.001
Gender			0.013
Male	25 (89.3)	49 (64.5)	
Female	3 (10.3)	27 (35.5)	
High blood pressure			< 0.001
Yes (%)	11 (39.3)	6 (7.9)	
No (%)	17 (60.7)	70 (92.1)	
Fasting glucose (mg/dL)	88.00 (82.00-97.50)	81.00 (75.25-87.00)	< 0.001
HbA1c	5.55 (5.30-5.80)	5.30 (5.20-5.50)	0.001
CEA	1.91 (0.97-3.20)	1.67 (1.03-2.57)	0.858
Metabolic syndrome			< 0.001
Yes	7 (25.0)	1 (1.3)	
No	21 (75.0)	75 (98.7)	

High blood pressure systolic blood pressure/diastolic blood pressure > 140/90 mmHg. Data expressed as median (P_{25}-P_{75}), or absolute number (%). BA: Biological age; CA: Chronological age; HbA1c: Hemoglobin A1c; CEA: Carcinoembryonic antigen.

Table 4 Colorectal adenoma between biological age-dominant and chronological age-dominant groups n (%)

	BA-dominant group (n = 28)	CA-dominant group (n = 76)	P value
Colorectal adenoma			0.386
Yes	3 (10.7)	15 (19.7)	
No	25 (89.3)	61 (80.3)	
Location			0.034
Proximal	0 (0.0)	18 (23.7)	
Distal	4 (14.3)	6 (7.9)	
Both	1 (3.6)	1 (1.3)	
Size (mm)			0.088
< 10	3 (10.7)	23 (30.3)	
⩾ 10	2 (7.1)	2 (2.6)	
Number			0.433
1	2 (7.1)	13 (17.1)	
⩾ 2	1 (3.6)	2 (2.6)	

BA: Biological age; CA: Chronological age.



Figure 2 The prevalence of colorectal adenoma according to metabolic syndrome and age gap. The incidence of colorectal adenoma increased for the first group (MS- and BA < CA), the second group (MS- and BA ⩾ CA), the third group (MS+ and BA ⩾ CA), and the fourth group (MS+ and BA < CA) (P < 0.05). MS: Metabolic syndrome; BA: Biological age; CA: Chronological age.

optimal time in an individual's life. If so, CRC may eventually be preventable using colonoscopy with polypectomy for premalignant lesions.

Many previous studies have revealed that colorectal adenoma is associated with MS. The clinical characteristics of MS, such as obesity, dyslipidemia, and insulin resistance, have been linked to an increased risk of CRC in several studies[16,22]. In this study, the subjects with MS had more colorectal adenomas compared to those without MS (44.9% *vs* 27.8%, P < 0.001). In particular, the prevalence of colorectal adenoma was increased along with MS and BA-dominance (P < 0.001) (Figure 2).

In this study, the prevalence of MS increased proportionally with BA compared with CA. This suggests that MS is related to BA more than to CA because the metabolic change is associated with aging. Soprano *et al*[23]. reported that MS can be considered an age-related disease by identifying a correlation between MS and aging. In addition, they highlighted aging-related dysfunctions that contribute to the onset of several metabolic diseases[23]. Therefore, physiological disorders such as MS should be measured based on BA rather than CA.

In this study, a CA-dominant subject was defined as an individual younger than their CA, showing that they are healthier than expected. On the other hand, a BA-dominant subject was defined as an individual older than their CA, showing that they are less healthy than expected. The prevalence of MS was higher in the BA-dominant group than in the CA-dominant group in this study. Therefore, this result also suggests the clinical application of BA to various metabolic diseases related with age.

In this study, the prevalence of colorectal adenoma increased with BA. As shown in Figure 1, the prevalence of colorectal adenoma rose sharply along with BA distribution rather than CA distribution with increasing age. The prevalence of colorectal adenoma was higher in the BA-dominant group than in the CA-

dominant group, although the difference between the two groups was not statistically significant. Interestingly, colorectal adenomas in the distal colon were more common in the BA-dominant group compared with the CA-dominant group ($P = 0.034$).

Based on these results, BA deserves attention as an alternative indicator for screening for various cancers including CRC. Interestingly, the prevalence of colorectal adenoma in subjects aged < 50 years was as high as 19.9% (329/2696) in this study. Hence, the commencement of screening colonoscopy should be determined based on BA and physiological criteria rather than indiscriminately at 50 years of CA. This strategy will help in identifying those at risks of CRC at an early stage (before the age of 50 years).

This study was limited because the study population, who voluntarily attended health screening, may have been individuals more concerned about their health than average and trying harder to maintain their health than members of the general population. As a result, the age gap between BA and CA was not as large as expected. Therefore, further studies targeting members of the general population are needed in the future.

The present study highlights the important implications for the future planning of CRC screening in that the higher prevalence of BA-dominance was observed in subjects with colorectal adenoma. It should be emphasized that long-term prevention of both colorectal adenoma along with the biological aging process is of clinical importance.

In conclusion, measurement of BA may help to assess the risk of colorectal adenoma in screening colonoscopy. In particular, careful colonoscopic examination in the distal colon should be performed in BA-dominant individuals.

COMMENTS

Background

Current guidelines for colorectal cancer screening are based on chronological age (CA) of 50 years. However, a significant number of colorectal cancers have been found even under 50 years of age. Therefore, screening colonoscopy at a more reasonable age may be necessary from the biological perspective.

Research frontiers

The authors investigated the clinical utility of biological age (BA) measurement in screening colonoscopy for the detection of colorectal adenomas in the average-risk population.

Innovations and breakthroughs

The present study demonstrated that the prevalence of colorectal adenoma correlated well with BA, and was more common in the distal colon in BA-dominance. Furthermore, the incidence of metabolic syndrome correlated more precisely with BA compared with CA.

Applications

Measurement of BA may help to assess the risk of colorectal adenoma in screening colonoscopy. In particular, careful colonoscopic examination in the distal colon should be performed in BA-dominant individuals.

Terminology

BA is determined by physiology rather than chronology. BA estimates the functional status of an individual with respect to their chronological peers on the basis of how well they function in comparison with others of the same CA.

Peer-review

The author aimed to investigate the clinical utility of BA measurement in screening colonoscopy for the detection of colorectal adenomas in the average-risk population. The present paper is indeed interesting.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic factors of response to endoscopic treatment in painful chronic pancreatitis

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Data sharing statement: The technical appendix, statistical code and dataset of the manuscript are available from the corresponding author at office@doctortantau.ro. Participants gave informed consent for data sharing.

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Abstract

AIM

To evaluate the endoscopic treatment efficacy and

prognostic factors of long-term response to treatment for painful chronic pancreatitis.

METHODS

This retrospective analysis identified 168 patients with painful chronic pancreatitis hospitalized during January 2010-January 2015 in a Romanian tertiary referral center. Data on demographics, medical history, alcohol consumption, smoking habit, clinical parameters, type and number of endoscopic procedures and hospital admissions number were collected from the medical charts and analyzed. The absence or substantial reduction of pain (mild pain) at the end of the follow-up associated with the technical success of endotherapy was considered as clinical success.

RESULTS

Among the 168 patients with painful chronic pancreatitis admitted to our department during the study period, 39 (23.21%) had optimal response to the medical therapy. One hundred and twenty-nine patients required endoscopic treatment. The median follow-up period was 15 mo (range, 0-60 mo). Overall, technical success of endotherapy was achieved in 105 patients (81.39%). More than two-thirds of patients (82.78%) had substantial improvement of pain after the endoscopic treatment, including frequency and severity of the pain attacks. Patients younger than 40 years had significantly more successful endoscopic procedures (P = 0.041). Clinical success was higher in non-smoking patients (P = 0.003). The hospital admission rate was higher in patients with recognized alcohol consumption (P = 0.03) and in smokers (P = 0.027). The number and location of pancreatic stones and locations of strictures did not significantly influence the technical success (P > 0.05) or the clinical success (P > 0.05).

CONCLUSION

Younger age than 40 years can be considered an important factor positively influencing endoscopic treatment outcome in patients with painful chronic pancreatitis.

Key words: Pain; Chronic pancreatitis; Endoscopic procedures; Alcohol; Smoking; Hospital admission; Technical success; Clinical success

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Core tip: We evaluated the endoscopic treatment efficacy and prognostic factors of long-term response in painful chronic pancreatitis. Technical and clinical success was achieved in more than two-thirds of patients. Patients younger than 40 years had significantly more successful endoscopic procedures. Non-smoking patients had greater clinical success. Also, the hospital admission rate was higher in patients with recognized alcohol consumption and in smokers. Number and location of pancreatic stones and locations of strictures did not significantly influence the technical or clinical success. Younger age can be considered an important factor that positively influences the endoscopic treatment outcome in these patients.

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INTRODUCTION

Patients with chronic pancreatitis (CP) often present with severe abdominal pain, resistant to common analgesics. The exact pathophysiology of CP is still unknown, being a debated subject[1]. Increased intrapancreatic pressure within the parenchyma and/or pancreatic duct causing tissue ischemia (due to pancreatic duct strictures and stones), inflammation of the pancreas, and pancreatic and extrapancreatic complications[1] (*i.e.,* pseudocysts, bile duct/duodenal strictures and peptic ulcers) are factors suggested as causing pain in CP.

The endoscopic treatment of painful CP remains a clinical challenge. The goals of endotherapy are to drain the pancreatic duct[2], to reduce the frequency and the severity of the pain, and to resolve the local complications of the disease. Unfortunately, few data are available on the long-term efficacy of this endoscopic treatment. Most of the authors of the related literature consider that endoscopic retrograde cholangiopancreatography (ERCP) should be used as a first-line treatment in patients unresponsive to medical therapy. The efficacy of ERCP is comparable to that surgery, with a higher success rate in properly selected patients and a low morbidity rate. Moreover, it can be performed repeatedly and independently of patient age. In some cases it may be considered as a "bridge" to subsequent surgery[3-5].

Several studies have identified the factors associated with a good long-term outcome of endoscopic treatment in painful CP, including cephalic intraductal stones, complete pancreatic clearance, absence of ductal stricture, and recent onset of disease[6-9]. Also, cessation of alcohol consumption and of smoking is associated with a good outcome[7,10]. Higher rates of clinical success have been associated with a short interval between the CP onset and endoscopic therapy and older age at the onset of disease[7]. Different literature data have evidenced better results of surgery *vs* endoscopic treatment in terms of pain control[11,12]. Regarding the short-term outcome, no significant differences have been found between endoscopic treatment and surgery in terms of hospitalization rate and medical costs. Instead, the hospitalization rate

and its duration were found to be significantly higher in patients with endoscopic treatment, regarding the long-term outcome compared to surgery[13].

In this study, we considered our case experience from a referral tertiary center in Romania to evaluate the endoscopic treatment efficacy and prognostic factors of long-term response to treatment in patients with painful CP. Our intention was to determine whether ERCP maintains merits in the management of pain in CP and if there are some new clinical or endoscopical factors which might help to achieve long-term results.

MATERIALS AND METHODS

Patients

We retrospectively searched the medical database of our hospital (a tertiary referral center; Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania) to identify all patients with painful CP hospitalized for treatment in our Gastroenterology Department during January 2010-January 2015 (n = 168). Patients with good response to medical therapy and without required endotherapy were excluded from the study (n = 39). All patients who underwent endotherapy with treatment intention were included in the study (n = 129).

Data on demographics, medical history, clinical data (*i.e.,* abdominal pain pattern and severity, diarrhea, diabetes, jaundice), hospital admissions number, type and number of endoscopic procedures and complications of treatment were collected from the medical charts and analyzed. Alcohol consumption was defined as a daily intake of > 20 g of ethanol for both women and men. Cigarette smoking was defined as smoking at least 5 pack-years[7].

The first parameter taken into account was abdominal pain relief in patients with pancreatic drainage. The pain character and intensity was quantified based on patients' medical records at study enrollment and at end of follow-up. No standardized instruments for pain quantifying, quality of life or psychosocial impact evaluation were used. In the medical records, the severity of pain was qualified as severe, moderate and/or mild with continuous or intermittent pattern.

Endoscopic treatment

All ERCPs and endoscopic procedures were performed by the same experienced endoscopist physician (> 600 ERCPs/year). According to the initial pancreatography findings, the patients with pancreatic drainage were divided into three different groups: patients with pancreatic strictures; patients with pancreatic intraductal stones; and patients with pancreatic strictures and intraductal stones. An efficient pancreatic sphincterotomy and/or successful stent placement across the strictures and/or intraductal stones removal was considered a technical success. Otherwise, the endoscopic treatment was considered as a failure. Also, we considered any form of surgical drainage performed during the follow-up period as indicating failure of the endoscopic treatment. All instances of early complications (in first 24 h post-ERCP) were also noted.

In patients with intention to treat, the endoscopic treatment was considered as complete if no further ERCP session was required and if no pancreatic stents were *in situ* at the cut-off date (January 2015). Otherwise, endotherapy was considered as ongoing. The absence of significant reduction of pain (absent or mild pain) at the end of follow-up associated with the technical success of endotherapy was considered a clinical success. Treatment of concomitant bile duct strictures (n = 35) and pseudocysts (n = 28) were also noted. Considering biliary drainage, clinical success was noted in the case of jaundice improvement with the bile duct stricture waist disappearing.

According to the European Society of Gastrointestinal Endoscopy guidelines, in patients with pseudocysts, technical success was considered where at least one stent from a pancreatic pseudocyst to the digestive lumen was able to be inserted[6] or by resolution of the fluid collection. Short-term clinical success was considered in the case of complete relief of the initial symptoms with a decrease in pseudocyst diameter of at least 30%-50% at 1 mo[6]. For large ductal stones in the pancreatic head or body, one or multiple sessions of extracorporeal shock wave lithotripsy (ESWL) were performed. A third-generation electromagnetic lithotripter (Dornier MedTech, Weissling, Germany) was used, with the patient under general anesthesia.

Statistical analysis

Qualitative data is presented as counts and percentages. Quantitative data is presented as means and standard deviations for normally distributed data, and as medians and standard deviations and interquartile ranges or ranges for skewed distributions. Associations between qualitative variables were assessed with the chi-square test or Fisher's exact test. Comparisons between two groups of quantitative skewed data were performed with the Wilcoxon rank sum test, and comparisons between three groups with the Kruskal-Wallis test, followed by nonparametric post hoc tests.

For all statistical tests performed, we used the two-tailed P-value and an 0.05 level of significance. All analyses were computed with R environment for statistical computing and graphics version 3.2.3 (http://www.R-project.org/).

RESULTS

Clinical characteristics of patients

From January 2010 to January 2015, 168 patients with painful CP were admitted to our department.

Table 1 Demographic, etiological and clinical characteristics of patients at study entry, [*n* = 129, *n* (%)]

Sex as male/female	106 (82.17)/23 (17.83)
Age (mean ± SD, yr)	51.55 ± 11.34)
Etiology	
Alcoholic	102 (79.07)
Autoimmune	2 (1.55)
Genetic	2 (1.55)
Idiopathic	13 (10.08)
Metabolic	3 (2.33)
Pancreas divisum	7 (5.43)
Pain pattern: continuous/intermittent	22 (17.05)/107 (82.95)
Pain intensity: severe/moderate	57 (44.19)/72 (55.81)
Diabetes	41 (31.78)
Diarrhea	63 (48.84)

Thirty-nine patients (23.21%) had optimal response to the medical therapy (*i.e.,* paracetamol, non-steroidal anti-inflammatory drugs, tramadol, co-medication for neuropathic pain, and pancreatic enzyme substitution) and they were excluded from the study. Thus, 129 patients were enrolled in the study. The mean age was 51.55 years (range, 15-82 years). One hundred and six were men (82.17%) and 23 were women (17.83%). Demographical, etiological and clinical data are shown in Table 1. Seventy-five patients (58.14%) were alcohol drinkers and 47 patients (36.43%) were smokers at study enrollment. The median follow-up period was 15 mo (range, 0-60 mo).

Endoscopic pancreatic drainage

During the study period a total of 265 ERCPs were performed, with a median of 2.8 procedures/patient. At the initial pancreatography, 89 patients (68.99%) had only pancreatic duct strictures, 17 patients had only intraductal pancreatic stones (13.18%) and 23 patients (17.83%) had both intraductal stones and strictures. Seven patients (5.43%) had pancreas divisum and were treated *via* the minor papilla.

Overall, technical success was achieved in 105 patients (81.39%). At the end of study, endoscopic treatment was completed in 103 patients and ongoing in 2 patients. Endotherapy failure was noted in 24 patients (18.6%). In 7 of these patients (29.16%) the endoscopic procedures could not be performed due to severe stenosis of the pancreatic duct and/or giant intraductal stones. The number and size of the intraductal pancreatic stones and the location of ductal stenosis did not significantly influence the clinical and technical success (Tables 2 and 3).

Early complications post-ERCP appeared in 7 patients (6.67%) who had technical success and in 2 patients (8.33%) who had failure of the endoscopic treatment. Acute pancreatitis post-ERCP was noted in 8 patients (6.2%) and post-sphincterotomy bleeding in 1 patient (0.77%). All complications were successfully managed conservatively.

Patients with at least one session of endoscopic treatment (*n* = 122): There were no statistically significant differences regarding the number of hospital admissions, the number of ERCP/patients and the number of endoscopic procedures between patients with technical success (86.06%) *vs* failure of endotherapy (13.93%) (Table 4). More than two-thirds of the patients with at least one session of endoscopic treatment (*n* = 101, 82.78%) had clinical success after the endoscopic treatment, including improvements in frequency and severity of pain attacks (Table 5).

Patients with pancreatic strictures alone at initial pancreatography (*n* = 85): In 23 patients (27.05%), a pancreatic sphincterotomy was the only sufficient procedure. Sixty-two patients (72.94%) required multiple procedures. Multiple pancreatic restenting sessions were performed in 6 patients (7.05%). The clinical success rate was 81.17% (*n* = 69) (Table 6).

Patients with pancreatic intraductal stones alone at initial pancreatography (*n* = 17): Two patients (11.76%) required initial ESWL followed by endoscopic stone extraction. To achieve stone clearance, multiple sessions of stone extraction were performed in all patients. The clinical success rate was 76.47% (*n* = 13) (Table 6).

Patients with pancreatic strictures and intraductal stones at initial pancreatography (*n* = 20): Multiple procedures were performed in all patients. Six patients (30%) required pancreatic restenting, five of who (25%) required multiple stone extraction sessions. Two patients still had pancreatic plastic stents in place at the end of the study period. The clinical success rate was 95% (*n* = 19) (Table 6). There were significant differences regarding the number of ERCP/patient between the group of patients with pancreatic strictures and those with strictures and stones ($P < 0.001$) and in terms of procedures/patient in all three groups ($P < 0.001$) (Table 6). No statistically significant differences were found regarding the mean age and severity of initial pain, technical success ($P = 0.995$) or clinical success ($P = 0.899$). However, the technical success was significantly higher in patients younger than 40-years-old ($P = 0.041$).

No statistically significant association was found between the continuation of alcohol drinking and clinical success ($P = 0.066$). In contrast, non-smoking patients had a higher rate of clinical success than their smoking counterparts ($P = 0.003$). Considered separately, patients without any alcohol consumption or smoking had a better prognosis than patients who had one or both addictions ($P = 0.007$) (Table 7). Alcohol drinkers (*n* = 75) had a higher hospital admission rate (4 *vs* 3, $P = 0.03$) than patients who were not alcohol drinkers (*n* = 54). The number of

Table 2 Technical and clinical success according to the initial pancreatography *n* (%)

	n	Technical success	Clinical success
All patients	129	105 (81.39)	101 (78.29)
Only pancreatic stones	17	14 (82.35)	13 (76.47)
Cephalic	16		
Non-cephalic	1		
Cephalic and non-cephalic	0		
Only pancreatic strictures	89	71 (79.77)	69 (81.18)
Cephalic	82		
Non-cephalic	5		
Cephalic and non-cephalic	2		
Pancreatic strictures + stones	23	20 (86.95)	19 (95)
Cephalic stones + cephalic strictures	19		
Cephalic stones + cephalic and non-cephalic strictures	2		
Cephalic and non-cephalic stones + cephalic strictures	2		

Table 3 Clinical and technical success according to number and size of stones *n* (%)

	Technical success				Clinical success			
	Yes[1]	No[2]	OR (95%CI)	*P*-value	Yes[1]	No[2]	OR (95%CI)	*P* value
Stone number, median (IQR)	2 (1-2)	1 (1-1.75)	0.28 (0.004-5.96)	0.39	2 (1-2)	1 (1-1)	0.36 (0.03-3.64)	0.098
Stone size, *n*	34	6			32	5		
5-10 mm, *n* = 14	12 (80)	3 (20)		0.073	11 (78.57)	3 (21.43)		0.346
< 5 mm, *n* = 23	22 (91.67)	2 (8.33)			21 (91)	2 (8.7)		
< 10 mm, *n* = 1	-	1			-	-		

[1]Technical or clinical success achieved; [2]Failure of endoscopic treatment and no pain improvement. IQR: Interquartile range.

Table 4 Hospital admissions and procedures in patients with endoscopic treatment (technical success *vs* failure of endoscopic treatment) (*n* = 122)

	Technical success		
	Yes, *n* = 105	No, *n* = 17	*P* value
Hospital admissions	3 (3-5)	3 (3-5)	0.728
ERCP/patient	2 (1-3)	2 (1-2)	0.605
Procedures/patient	3 (1-4)	2 (0-3)	0.055

Data are presented as median (IQR). IQR: Interquartile range; ERCP: Endoscopic retrograde cholangiopancreatography.

endoscopic procedures/patients was higher (3 *vs* 2) in alcohol drinkers, but not significantly (P = 0.249). In smokers (n = 47), the hospital admission rate was higher (4 *vs* 3, P = 0.027) than for patients who were non-smokers (n = 82) and the endoscopic procedures required was also greater (3 *vs* 2, P = 0.622). Newly diagnosed diabetes appeared in 14 patients (13.46%) with clinical success during follow-up.

Biliary drainage

During the follow-up period, 35 patients (28.68%) developed biliary stricture with jaundice. Biliary drainage was achieved using a single plastic biliary stent in 21 patients (60%) and simultaneous biliary plastic stents in 2 patients (5.71%). Multiple biliary stent sessions were performed in 12 patients (34.29%). Covered self-expending metallic stents (SEMS) were used in 2 patients (5.71%) who were deemed at high surgical risk. Biliary drainage was successful in 26 patients (74.29%) and failed in 9 patients (25.71%).

Pancreatic pseudocysts

Pancreatic pseudocysts were identified in 28 patients (22.95%). Transpapillary drainage was performed in 17 cases (60.71%) and transmural drainage in 9 cases (32.14%). In 1 patient (3.57%) the endoscopic approach could not be achieved due to the severe stenosis of the pancreatic duct and surgical drainage was indicated. Technical success was achieved in 96.43% cases (n = 27). Short-term clinical success was achieved in 26 patients (96.3%). Endoscopic ultrasound drainage was indicated in 1 patient because of an increase in the size of the pseudocyst and remarkable collateral circulation in the portal hypertension context.

Surgery

In 24 patients (18.6%) the endoscopic treatment failed and some form of surgery was performed during follow-up. In 7 patients, no endoscopic procedures were performed. The endoscopic treatment was converted to surgery in 17 patients (13.93%).

Table 5 Pain evolution in patients with endoscopic treatment [*n* = 122, *n* (%)]

	Technical success			
	Yes, *n* = 105	No, *n* = 17	OR (95%CI)	*P* value
Pain reduction	104 (99.05)	13 (76.47)	30.3 (2.7-1572.8)	0.001
Final pain				
Absent	52 (49.52)	1 (5.88)	Absent *vs* others:	
Mild	49 (46.67)	5 (29.415)	15.4 (2.2-668.6)	< 0.001
Moderate	4 (3.81)	11 (64.71)	Absent + mild *vs* moderate + severe: 42.8 (9.5-245.0)	
Severe	-	-		

Table 6 Endoscopic drainage (endoscopic retrograde cholangiopancreatography and procedures/patients) according to initial pancreatography changes (*n* = 122)

	Stones, *n* = 17	Strictures, *n* = 85	Strictures + stones, *n* = 20	*P* value
ERCP/patient[1]	2 (1-3)	2 (1-2)[c]	2 (2-3)[c]	< 0.001
Procedures/patient[1]	3 (2-5)[a]	2 (1-3)[c]	4 (3-4)[ac]	< 0.001
Only one procedure	0	23	0	< 0.001
Multiple procedures	17	62	20	< 0.001
Clinical success	13	69	19	0.221

[1]Data are presented as median (IQR). [a]*P* < 0.05, *vs* the groups with Stones to the group Strictures + Stones had a; [c]*P* < 0.05, *vs* the groups with Strictures to the group Strictures + Stones had a. IQR: Interquartile range; ERCP: Endoscopic retrograde cholangiopancreatography.

DISCUSSION

Through this retrospective study, we obtained results of the endoscopic approach of painful CP patients in a tertiary referral center. Technical success was achieved in 81.39% of patients with significant improvement of pain experienced by more than two-thirds of the patients, including frequency and severity of pain attacks. About 50% of the patients were pain-free at the end of follow-up. We found that age under 40 years, no smoking, no alcohol consumption and multiple ERCP sessions are independent factors associated with a good response to the endoscopic treatment.

Few data are available on the long-term efficacy of endoscopic treatment in painful CP[4,6-8,14,15]. Endoscopy societies recommend endoscopic drainage as first-line therapy in painful obstructive CP[6,16]. Our study was conducted on a large group of patients with a median long-term follow-up and describes the new approach to the position of endotherapy in treatment of patients suffering from CP.

The aim of endotherapy is to decompress the main pancreatic duct by complete stone clearance and ductal drainage[17]. The cause of pain in CP is multifactorial, explaining why symptom relief can be unsatisfying even if technical success is achieved[16]. Surgery is indicated in patients with persistent pain that does not respond to medical and/or endoscopic treatment[16], in patients with a heavy stone burden (especially in the body/tail of the pancreas with pancreatic ductal dilatation and/or strictures[11,17,18]) or in the case of a suspected pancreatic inflammatory mass[16]. Endotherapy should also be considered in patients at high surgical risk[19].

There are several studies with good results regarding the long-term outcome after endoscopic treatment in painful CP[4,7-8,14-15] with a median rate of endotherapy success of 83%[6]. In our series, most patients required multiple procedures to obtain the goal of endoscopic therapy. In these patients, the pancreatography changes were more severe than those in patients with only a pancreatic sphincterotomy. There is one small series with a median of three stents for refractory pancreatic strictures showing good results after a mean follow-up of 38 mo[20], and three prospective series of the use of SEMS[21,22]. The use of multiple large plastic stents and expandable metal mesh stents in the pancreatic duct is not yet considered standard of care[16]. In a selected center with experience, ESWL of large pancreatic stones can lead to ductal clearance, as effective and more cost effective than the ERCP combination[9,23]. Our center does not dispose of equipment for ESWL, and 2 patients with large intraductal stones with diameter > 15 mm, impacted upstream of a tight ductal stenosis benefited from this procedure in another center prior to endoscopic ductal clearance.

We found that the numbers of procedures and sessions of ERCP was statistically higher in patients with stones and strictures than in patients with stones or strictures alone. But, we did not find any correlation between the size and number of stones or the number and location of stenosis with technical or clinical success. Data showed that the cephalic stones[9], complete pancreatic stone clearance and absence of main pancreatic duct stricture are the factors independently associated with long-term (⩾ 2 years) pain relief following endoscopic therapy[6,24,25]. A single stricture located in the pancreatic head is the ideal situation for endoscopic treatment compared to an isolated stricture in the tail or multiple strictures in the pancreas body[24].

There are few data regarding the correlation between age of patient and technical or clinical success of endotherapy[25]. Using a cut-off of 40 years for age, we noticed that younger patients had a better response to endoscopic treatment (*P* = 0.041). As far as we know, our study is the first described in the literature to evaluate the association of younger age and endoscopic technical success in patients with painful CP. We must emphasize that endoscopic treatment should be initiated earlier in young patients with painful CP in order to achieve better results.

Alcohol and smoking are independent risk factors of CP. Alcohol abuse causes pancreatic fibrosis by stellate cell activation[26] and is an important factor for mortality in CP. Alcohol abstinence may help in providing pain relief[16]. Smoking is an independent

Table 7 Smoking and alcohol drinking influence on clinical success and technical success [*n* = 122, *n* (%)]

	Technical success					Clinical success				
	Yes	No	OR (95%CI) *vs* none	*P* value	*P* value overall	Yes	No	OR (95%CI) *vs* none	*P* value	*P* value over all
Smoker only, *n* = 11	8 (66.67)	3 (33.3)	0.16 (0.02-1.15)	0.036		7 (63.64)	4 (36.36)	0.05 (0.001-0.59)	0.006	
Alcohol only, *n* = 36	31 (77.5)	5 (22.5)	0.27 (0.04-1.20)	0.064		30 (83.33)	6 (16.67)	0.13 (0.003-1.17)	0.048	
					0.103					0.007
Non-smoker and non-alcohol, *n* = 40	39 (92.8)	1 (7.14)	-	-		39 (97.5)	1 (2.5)	-	-	
Smoker and alcohol, *n* = 35	27 (77.1)	8 (22.8)	0.26 (0.04-1.23)	0.099		25 (71.43)	10 (28.5)	0.066 (0.001-0.52)	0.002	

risk factor for the development and progression of pancreatitis[10,27] and carcinogenesis[28]. It has been shown that abstinence not only slows the disease progression but also prevents complications such as pancreatic cancer[29-31]. Cessation of alcohol has beneficial effects on clinical outcome and progression of painful CP[30,31]. An association between smoking and CP pain has yet to be determined.

We found that patients without any alcohol consumption or smoking had better prognosis than patients who had one or both addictions, regarding clinical success (P = 0.007). Cigarette smoking was associated with a poor clinical outcome in our group. Furthermore, hospital admission rates were significantly higher in patients who continued alcohol drinking and smoking. Our results add new and important data regarding the association of smoking and CP pain and emphasized the importance of smoking and alcohol cessation in those patients.

Endoscopic drainage is also recommended as a first-line treatment in uncomplicated pancreatic pseudocysts[32], with similar outcomes to surgery regarding the recurrence rate during long-term follow-up and better results in terms of mortality[33,34], medical cost and quality of life[35]. In our series, endoscopic drainage was feasible in almost all patients, and technical success was 96.43% with a short-term clinical success of 96.3%; similar results are in the literature[36-39].

The success of endotherapy in biliary drainage in CP is poor in most of the published series, with a 10%-33% rate of stricture resolution[40-47]. The choice between endoscopic and surgery drainage should be made according to the medical center's expertise, patient co-morbidities and compliance to repeat endoscopic procedures[6]. Multiple simultaneous plastic stents reportedly have better results[48-50] than single biliary plastic stenting. Recent data demonstrate good results using covered expandable metal stents[51-54]. In our series, biliary drainage was successful in 74.29% of patients during follow-up. Multiple single biliary stent sessions were performed in 12 patients (34.29%) and simultaneous biliary plastic stents in 2 patients (5.71%). Covered SEMS were used in 2 patients with multiple comorbidities and high surgical risk.

CP is a progressive inflammatory disease. Patients with long-term CP or extensive pancreatic resection can develop type 3 diabetes mellitus, characterized by a lack of insulin and other counter-regulatory islet hormones, such as glucagon[55-57]. In our series, diabetes was present initially in one-third of patients. Newly diagnosed diabetes appeared in a quarter of patients with clinical success during follow-up. We speculate that endoscopic and clinical success do not stop the progression of exocrine and endocrine pancreatic deterioration.

Our study has several weaknesses that should be considered when interpreting our results. This is a retrospective study in a single tertiary academic center and can overestimate clinical success. We did not do use any objective assessment of pain control, standardized instruments for pain quantification, quality of life and psychosocial impact evaluation in patients with CP. Also, the continuing use of concomitant medical therapy was not followed. We consider that large, multicenter randomized studies are required to evaluate ERCP efficacy *vs* surgery in patients with painful CP without response to medical therapy.

In conclusion, endoscopic therapy in painful CP is effective and safe. Technical success was achieved in 81.39% of cases. Clinical success was achieved in 82.78% of the patients, with multiple endoscopic procedures being required in the majority of cases. Positive prognostic factors of response to endoscopic treatment are age under 40 years, no smoking, no alcohol consumption, and multiple ERCP sessions. Number and size of the intraductal pancreatic stones and the location of ductal stenosis did not significantly influence the clinical and technical success. Alcohol drinking and smoking should be recognized early and aggressively advised against to improve the quality of life and to reduce hospital admission. Endoscopic treatment should be considered as the first choice of

pain treatment, especially in younger patients.

COMMENTS

Background

The endoscopic management of patients with chronic pancreatitis (CP) pain is very challenging. The intraductal stone location, complete pancreatic clearance, absence of ductal stricture, recent onset of the disease and alcohol cessation are proven factors associated with a good long-term outcome of endoscopic treatment in painful CP. Other prognostic factors, such as young age and smoking cessation, are under investigation. In this retrospective study, we evaluated new clinical or endoscopical factors which might help to achieve long-term results.

Research frontiers

Few studies have evaluated the association of smoking with CP pain. No study to date has evaluated the prognostic role of younger age in patients with painful CP who undergo endoscopic treatment.

Innovations and breakthroughs

The results of this study underline the efficacy of endoscopic treatment in patients without response to medical therapy regarding pain relief, especially in younger patients. The authors have found that younger age, no smoking, no alcohol consumption, and multiple endoscopic retrograde cholangiopancreatography (ERCP) sessions are independent factors associated with a good response to endoscopic treatment.

Applications

Most patients with painful CP undergo endoscopic treatment as first step strategy. In the present study, the endoscopic treatment in young patients with painful CP might affect long-term clinical outcome.

Terminology

ERCP is an endoscopic procedure that combines the use of endoscopy and fluoroscopy to diagnose and treat certain problems of the biliary or pancreatic ductal systems (stones, strictures, pseudocysts, tumors).

Peer-review

This is a retrospective study discussing the efficacy of endoscopic therapy in chronic pancreatitis. Overall the study is well written. Results are clear and to the point.

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ORIGINAL ARTICLE

Retrospective Study

In vivo histological diagnosis for gastric cancer using endocytoscopy

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Abstract

AIM

To examine usefulness of virtual biopsy using endocytoscopy by comparing the *in vivo* endocytoscopic and histopathological images of gastric cancers.

METHODS

Endocytoscopy was performed in 30 patients with early gastric cancer. Of these, 26 patients showed well differentiated adenocarcinomas, while 4 patients showed poorly differentiated adenocarcinomas (including one signet ring cell carcinoma). Cancerous and non-cancerous areas were observed after double staining with 0.05% crystal violet and 0.1% methylene blue. The endocytoscopic images obtained were evaluated by an expert endoscopist and an expert pathologist without knowledge of patient clinical data, and endocytoscopic and histopathological diagnoses were compared.

RESULTS

The endocytoscopic images of the cancerous area were assessed as evaluable in 25 (83.3%) and 27 (90%) patients by endoscopist A and pathologist B, respectively, and those of the non-cancerous area as evaluable in 28 (93.3%) and 23 (76.7%) patients by the endoscopist and pathologist, respectively. The sensitivity, specificity, and diagnostic accuracy of gastric cancer diagnosis using evaluable endocytoscopic images were 88.0% and 92.9%, and 90.6% by endoscopist A, and 88.9% and 91.3%, and 90.0% by pathologist B, respectively. Evaluation of the diagnostic concordance rate between the endoscopist and the pathologist by inter-observer agreement calculation revealed no significant difference between the two observers. The inter-observer agreement (κ-value) for endocytoscopic diagnosis was 0.745.

CONCLUSION

Endocytoscopy is useful for the differentiation of cancerous from non-cancerous gastric mucosa, making it a promising tool for virtual biopsy.

Key words: Endocytoscopy; Gastric cancer; Virtual biopsy; *In vivo* histopathology; Magnifying endoscopy; Double staining; Crystal violet; Methylene blue

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Core tip: Endocytoscopy, which allows for ultra-high-magnifying observation of the gastrointestinal mucosa, has recently been developed, enabling the visualization of the gastrointestinal mucosa at resolutions approaching those of histology. Although the usefulness of virtual biopsy using endocytoscopy in the diagnosis of esophageal cancer, colorectal cancer, lung cancer and ulcerative colitis has been reported, few studies are available on the diagnosis of gastric cancer. This study showed the usefulness of virtual biopsy using endocytoscopy by comparing the *in vivo* endocytoscopic and histopathological images of gastric cancers.

Tsurudome I, Miyahara R, Funasaka K, Furukawa K, Matsushita M, Yamamura T, Ishikawa T, Ohno E, Nakamura M, Kawashima H, Watanabe O, Nakaguro M, Satou A, Hirooka Y, Goto H. *In vivo* histological diagnosis for gastric cancer using endocytoscopy. *World J Gastroenterol* 2017; 23(37): 6894-6901 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6894.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6894

INTRODUCTION

The incidence of gastric cancer has been decreasing worldwide, but gastric cancer still remains a major cause of cancer-related death. The annual number of deaths from this disease is second only to that from lung cancer in Japan. A global analysis of gastric cancer-related mortality reveals higher mortality rates in East Asia (Japan, China, and South Korea) compared to those in Europeans and Caucasian populations of the United States.

Early detection, accurate staging, and appropriate treatment administration are important to improve survival outcomes and reduce mortality rates in gastric cancer. Recent advances in endoscopic technology have been remarkable, and high-resolution endoscopy has made it possible to detect endoscopically-treatable early gastric cancer more frequently. Magnifying endoscopy and image-enhanced endoscopy have been developed, and NBI-magnifying endoscopy has facilitated the differential diagnosis and histological typing of gastric cancer[1-3]. However, the gold standard for the diagnosis of malignant gastrointestinal tumors is histopathological examination, and definitive diagnosis requires endoscopic biopsy. However, diagnostic biopsy is time-consuming, increasing the risk of bleeding in patients taking antithrombotic agents, in addition to causing submucosal fibrosis, which potentially hampers endoscopic treatment.

Two endoscopic technologies (confocal endomicroscopy and endocytoscopy) have recently been developed, making it possible to observe living cells *in vivo*. To date, we have reported that confocal endomicroscopy can distinguish gastric cancer from normal gastric mucosa[4], and determine the mucin phenotype of gastric cancer by visualizing brush borders and goblet cells[5]. Fluorescent dyes used for confocal laser endomicroscopy stain cells, enabling the evaluation of structural atypia, but not that of nuclear atypia, because they do not stain nuclei. However, dye spraying during endocytoscopy results in the staining of cells and nuclei, thereby producing *in vivo* images similar to those on the histological study of biopsies (the current gold standard for pathological diagnosis).

As endocytoscopy provides real-time microscopic images *in vivo*, its application to histological diagnosis by virtual biopsy (without the need to take biopsies) is promising. Although the usefulness of virtual biopsy using endocytoscopy in the diagnosis of esophageal cancer, colorectal cancer, lung cancer, and ulcerative colitis has been reported[6-9], few studies to date have evaluated its usefulness in the *in vivo* diagnosis of gastric cancer[10-12].

In this study, we retrospectively examined the usefulness of virtual biopsy using endocytoscopy, by comparing *in vivo* endocytoscopic and histopathological images of gastric cancers.

MATERIALS AND METHODS

Subjects

This pilot study was performed at the Nagoya University Hospital between January 2011 and October 2012. Thirty patients with early gastric cancer were enrolled (Table 1). Of these, 26 patients showed well differentiated, and 4 patients showed poorly differentiated adenocarcinomas (including one signet ring cell carcinoma). This study was approved by the Ethics Committee of Nagoya University Hospital (approval no. 911), and informed consent was obtained from all patients.

Endocytoscopy system

An integrated-type endocytoscope (GIF-Y0002, Olympus Co., Tokyo, Japan) was used in the present study. As with a conventional magnification endoscope, the magnification was adjustable by pulling a lever to perform continuous observation, from conventional to ultra-high magnification. The maximum magnification was × 380, the microscopic field area 700 μm × 600 μm, and the depth of observation was 50 μm. The image quality of the endocytoscope was comparable to that of GIF-Q260 (Olympus, Japan), and was sufficient to make an accurate diagnosis.

Methods of observation

Patients were pretreated with an oral dose of 20000 units of pronase and 1 g of sodium bicarbonate, followed by an intramuscular injection of 20 mg of butylscopolamine or 1 mg of glucagon. Under intravenous conscious sedation, the patients underwent endocytoscopy. Conventional observation was performed. Then, the lesion was washed sufficiently using saline water, and sprayed with approximately 5 mL of 0.05% crystal violet and 0.1% methylene blue. One minute later, the excess dye solution was washed away, and ultra-high-magnifying observation was performed. If cells and nuclei were poorly stained, the lesion was sprayed again with the dyes. Both the lesion and the surrounding non-cancerous mucosa were observed. All examinations were performed by a single expert endoscopist (Tsurudome I), qualified as Board certified fellow of the Japan Gastroenterological Endoscopy Society.

Histopathological examination

Lesions observed by endocytoscopy underwent endoscopic biopsy or endoscopic resection. Biopsies and resected specimens were fixed, embedded in paraffin, and cut into thin sections horizontal to the mucosal surface. They were stained with hematoxylin and eosin (H&E), and examined and classified by expert pathologists according to the revised Vienna classification[13]. Lesions classified as category 4 in the Vienna classification were defined as gastric cancer, but those classified as category 3 were defined as gastric adenoma, and excluded from the category of gastric cancer.

Definitions of endocytoscopic findings compared to histopathological images

Normal mucosa (without intestinal metaplasia). In the fundic gland mucosa, the foveolar epithelium was arranged in a circle, and the glandular lumens were round (Figure 1A).

In the pyloric gland mucosa, the foveolar epithelium was arranged in ridges, and the glandular lumens were slit-like (Figure 2A). The arrangement of the epithelial cells was regular, closely resembling that seen in H&E-stained horizontal tissue sections (Figures 1B and 2B). The nuclei were faintly stained, showing no atypia.

Intestinal metaplastic mucosa

In the intestinal metaplastic mucosa, the epithelium exhibited a villous architecture (varying in size). The gland lumens were widened due to epithelial atrophy (Figure 3A). Some epithelial cells had vacuoles, suggestive of goblet cells. These findings were very similar to those in H&E-stained horizontal sections (Figure 3B). Nuclei were stained more intensely than in the normal mucosa, but demonstrated no atypia.

Gastric cancer

In well differentiated adenocarcinoma, the glands were branched irregularly and the width of the lumen varied. The epithelium was arranged in a disorderly fashion (Figure 4A). The nuclei were deeply stained and pseudostratified, and showed an increased nuclear density and nuclear atypia. In poorly differentiated adenocarcinoma, the tubular architecture was completely lost, and the nuclei were hyper-chromatic and anisokaryotic (Figure 5A). These findings closely resembled those in H&E-stained horizontal sections (Figures 4B and 5B).

Evaluation of endocytoscopic images

The endocytoscopic images obtained were analyzed separately by an endoscopist A (Ryoji M) and a pathologist B (Satou A), who were blinded to clinical information. The diagnosing physicians interpreted three photographs chosen by the examining physician,

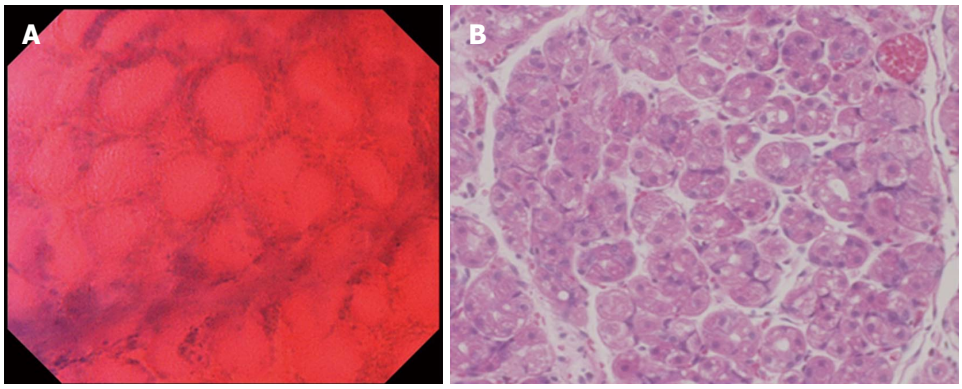


Figure 1 Normal mucosa (fundic gland mucosa). A: Endocytoscopic appearance of fundic gland mucosa. The foveolar epithelium was arranged in a circle, and the glandular lumens were round. The nuclei were weakly stained; B: Histological appearance of the corresponding normal mucosa (H&E-stained horizontal section, × 200).

Table 1 Patient clinicopathological characteristics

Variable	
Patients	30
Sex (male/female)	24/16
Mean age, yr (range)	72.2 (48-87)
Histological type of gastric cancer	
Well differentiated adenocarcinoma	26
Poorly differentiated adenocarcinoma (including signet ring cell carcinoma)	4
Histological type of non-cancerous mucosa	
Normal mucosa	10
Intestinal metaplastic mucosa	20
Mean tumor size, mm, (range)	17 (3-70)
Macroscopic type	
0-Ⅰ	1
0-Ⅱa	12
0-Ⅱc	17
Location	
Upper	6
Middle	9
Lower	15
Infiltration depth	
Mucosal	25
Submucosal	5
H. pylori infection	
Eradicated	11
Persistent infection	14
Negative	5

and classified the image quality as evaluable (nuclei and glandular structures evaluable) or unevaluable (neither nuclei nor gland structures evaluable), and determined whether the tissue was cancerous or non-cancerous if the image was of evaluable quality. The results were then compared with the actual pathology.

Outcome measures

Primary endpoint: Diagnostic accuracy: The endocytoscopic diagnosis performed independently by the endoscopist and the pathologist were compared with the pathological diagnosis, and sensitivity, specificity, and accuracy were evaluated.

Secondary endpoint: Diagnostic concordance rate between endoscopist and pathologist. To evaluate the diagnostic concordance rate between the endoscopist and the pathologist in the diagnosis of gastric cancer using endocytoscopy, inter-observer agreement was calculated.

Statistical analysis

The statistical software package SPSS 19.0 was used for data analysis. The endoscopic diagnoses by the endoscopist and the pathologist were compared using the χ^2 test, and a *P*-value of less than 0.05 was considered as significant. For evaluable images, the unweighted κ-statistic was calculated to evaluate inter-observer agreement.

RESULTS

Evaluation of endocytoscopy images

The endocytoscopy images of the cancerous areas were assessed as evaluable in 25 (83.3%) and 27 (90%) of the 30 patients by endoscopist A and pathologist B, respectively, and those of the non-cancerous areas as evaluable in 28 (93.3%) and 23 (76.7%) patients, by the endoscopist and the pathologist, respectively. The images that were assessed as unevaluable were excluded from the analysis. Endocytoscopic images of lesions in the lesser curvature of the gastric body are prone to artifacts due to cardiac pulsation, and it was difficult to place the endocytoscope in the desired position to visualize lesions in the fornix or greater curvature of the gastric body, thus complicating observation in these areas.

Table 2 shows the interpretation of endocytoscopic images by the endoscopist and the pathologist. Calculated from these findings, the sensitivity, specificity, and diagnostic accuracy of endocytoscopy in the diagnosis of gastric cancer using evaluable images were 88.0%, 92.9%, and 90.6% as performed by endoscopist A, and 88.9%, 91.3%, and 90.0% as performed by pathologist B, respectively (Table 3). Using the χ^2 test, no significant difference was found

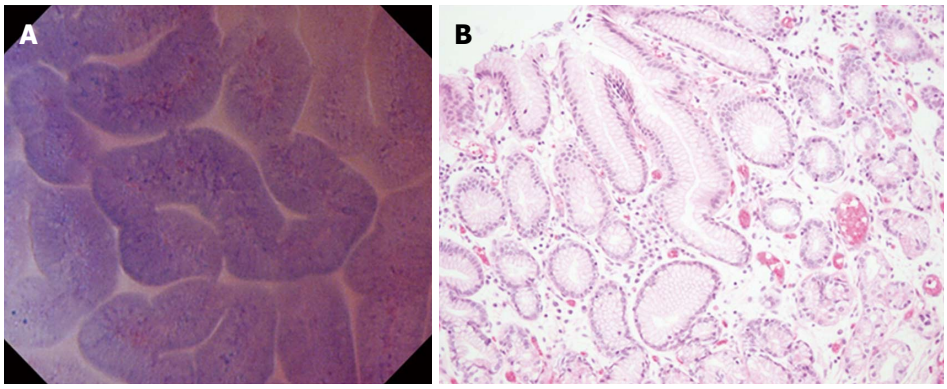


Figure 2 Normal mucosa (pyloric gland mucosa). A: Endocytoscopic appearance of pyloric gland mucosa. The foveolar epithelium was regularly arranged in ridges, and the glandular lumens were slit-like. The nuclei were weakly stained; B: Histological appearance of the corresponding pyloric gland mucosa (H&E-stained horizontal section, × 200).

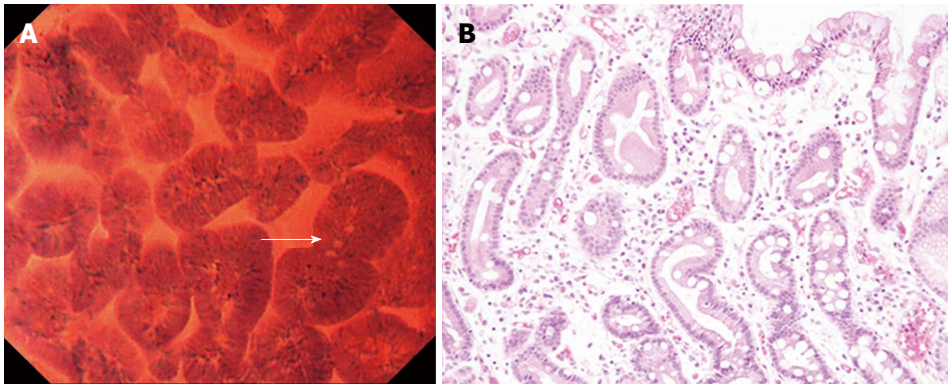


Figure 3 Intestinal metaplastic mucosa. A: Endocytoscopic appearance of intestinal metaplastic mucosa. The foveolar epithelium exhibited a villous architecture and the gland lumens were widened. Goblet cells (arrow) were observed among the epithelial cells. The nuclei were stained more intensely than in the normal mucosa; B: Histological appearance of the corresponding intestinal metaplastic mucosa (H&E-stained horizontal section, × 200).

Table 2 Comparison of endocytoscopic and histological diagnoses as performed by an endoscopist and a pathologist

	Endocytoscopic diagnosis			
	Endoscopist A		Pathologist B	
Histological diagnosis	Cancer	Non-cancer	Cancer	Non-cancer
Cancer	22	3	24	3
Non-cancer	2	26	2	21

between the two observers, and the κ-value was 0.745.

Complications

No complications were observed in this study, and none of the patients required temporary discontinuation of endocytoscopy.

DISCUSSION

At present, magnifying endoscopy facilitates observation of the mucosal surface structure as indicated by its surface patterns and the vascular structure as revealed by microvessels, thus mainly allowing evaluation of structural atypia. On the other hand, the resolution that endocytoscopy offers, allows visualization of individual cell nuclei and enables *in vivo* tissue observation, yielding results comparable to those obtained from histopathological images. Using an integrated endocytoscopy system, it is now possible to perform continuous observation of tissues with conventional to ultra-high magnification, creating the possibility of performing real-time virtual biopsies without obtaining physical lesion biopsies.

Endocytoscopy requires vital staining in order to evaluate the cellular and nuclear morphology, and methylene blue is generally used for this purpose. However, the diversity and poor staining of the gastric mucosa due to the presence of mucus[14] make observation and evaluation difficult. Therefore, to date, few studies have reported *in vivo* experiments in humans with gastric cancer[10-12].

Kodashima *et al*[15] attempted to optimize the staining conditions (staining time and dye concentration) for endocytoscopy in porcine experiments using different dyes, and reported that the ability of crystal violet to stain esophageal, gastric, and colonic tissues was inferior, and the optimal staining conditions were as follows: staining of esophageal tissue with 1% methylene blue solution for 60 s; staining of gastric

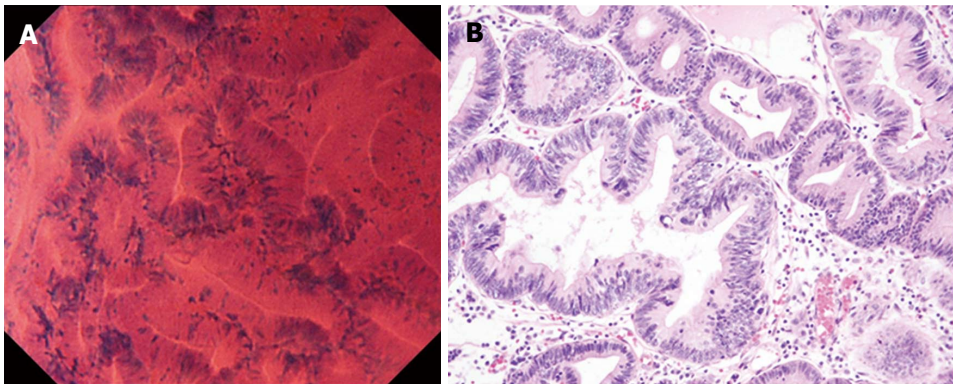


Figure 4 Well differentiated adenocarcinoma. A: Endocytoscopic appearance of a well differentiated adenocarcinoma. The glands were branched irregularly and the width of the lumen varied. The epithelium was arranged in a disorderly fashion. The nuclei were deeply stained and pseudostratified; B: Histological appearance of the corresponding well-differentiated adenocarcinoma (H&E-stained horizontal section, × 200).

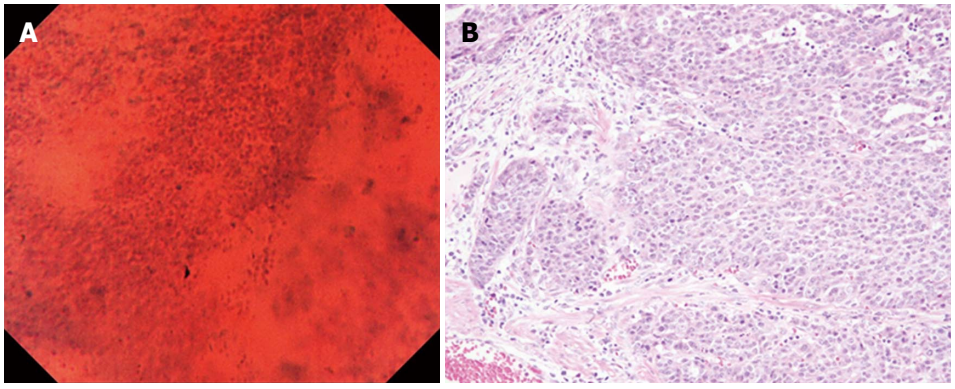


Figure 5 Poorly differentiated adenocarcinoma. A: Endocytoscopic appearance of a poorly differentiated adenocarcinoma. The tubular architecture was lost, and the nuclei were hyper-chromatic and anisokaryotic; B: Histological appearance of the corresponding poorly differentiated adenocarcinoma (H&E-stained horizontal section, × 200).

Table 3 Performance of endocytoscopy in the diagnosis of early gastric cancer, as scored by an endoscopist and a pathologist

	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Accuracy (95%CI)
Endoscopist A	88.00% (76.6-93.3)	92.90% (82.7-97.6)	91.70% (79.8-97.2)	89.70% (79.8-94.2)	90.60% (79.8-95.6)
Pathologist B	88.90% (78.4-93.8)	91.30% (79.0-97.1)	92.30% (81.4-97.4)	87.50% (75.7-93.0)	90.00% (78.7-95.3)
P-value	0.92	0.84	0.93	0.81	0.92

PPV: Positive predictive value; NPV: Negative predictive value.

and colonic tissues with 0.25% toluidine blue solution for 60 s. A double-staining method using crystal violet and methylene blue has recently been developed. In this method, methylene blue stains cellular nuclei purple, and crystal violet stains cells pink, thereby enabling the visualization of images resembling those of H&E staining. Kudo *et al*[6] and Minami *et al*[16] have demonstrated usefulness of the methylene blue and crystal violet double-staining method in the evaluation of neoplastic lesions in the colon and the esophagus, respectively. This same double-staining method has been used in the present study. Further studies are needed to compare this double-staining method with others such as the crystal violet and toluidine blue double-staining method.

The use of endocytoscopy in this study enabled us to evaluate the morphology of glandular lumens, arrangement of gastric foveolar cells, degree of nuclear stainability, and presence or absence of nuclear pseudostratification.

To determine the diagnostic performance of endocytoscopy to differentiate benign from malignant lesions, structural atypia were evaluated based on the morphology of glandular lumens and arrangement of foveolar cells; nuclear atypia were assessed based on the degree of nuclear chromasia and presence or absence of nuclear pseudostratification. Lesions with or without structural or nuclear atypia were diagnosed as cancerous or non-cancerous, respectively. Endocytoscopic images obtained from the normal gastric mucosa, gastric mucosa with intestinal metaplasia, and gastric cancers, were similar to those obtained from H&E-stained horizontal tissue sections. Thus, using endocytoscopic images, we were able to differentiate cancerous from non-cancerous lesions

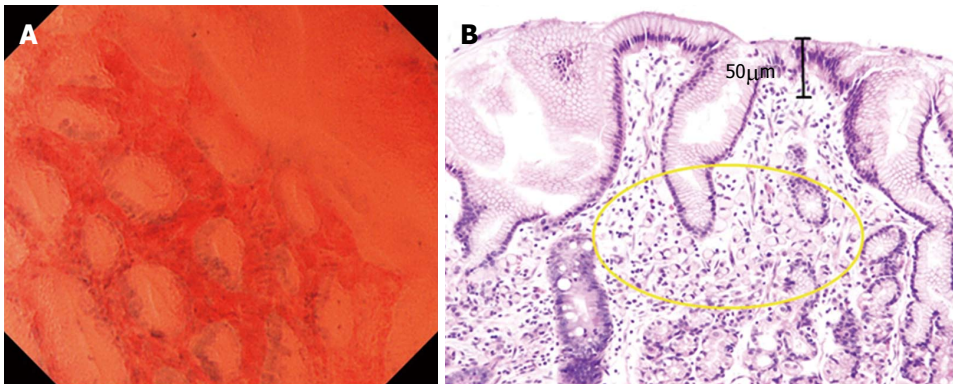


Figure 6 Signet ring cell carcinoma. A: Endocytoscopic appearance of a signet ring cell carcinoma. Slit-like glandular lumens surrounded by regularly arranged foveolar cells were observed, a finding consistent with normal mucosa in endocytoscopy; B: Histological appearance of the corresponding signet ring cell carcinoma (H&E-stained horizontal section, × 200). Signet-ring cells were observed in the lamina propria (circle), but not exposed on the mucosal surface.

with high sensitivity, specificity, and accuracy. In addition, the findings that goblet cells were observed in the intestinal metaplastic mucosa were similar to those reported by Chiu *et al*[17].

Although endocytoscopy enables *in vivo* observation of the mucosal tissue at the microscopic level, it only provides information about cells and nuclei of the superficial layer of the mucosa. In this study, both the pathologist and the endoscopist classified a signet ring cell carcinoma as a false-negative by endocytoscopy (Figures 6A and B). Although Fasoli *et al*[18]. reported that signet ring cells can be observed in resected gastric cancer specimens with endocytoscopy[18], the visualization by endocytoscopy is limited to the superficial mucosal layer (up to 50 μm in depth). In some poorly differentiated adenocarcinomas, carcinoma cells may spread in the lamina propria without any distortion to gland structure (Figure 6B). In these cases, the diagnosis of carcinoma with endocytoscopy alone is difficult.

This study has several limitations. First, only the best images that were chosen by the examining physician were evaluated. That is, the diagnostic capability of endocytoscopy was not evaluated in real-time. Second, the stomach moves actively due to peristalsis, aortic pulsation, and respiration, making focusing and fiber manipulation during endocytoscopic observation difficult for beginners. Third, the endoscopist requires knowledge of pathology when evaluating endocytoscopic findings. Fourth, this study did not include patients with adenoma. Finally, the sample size was small, implying that additional studies with a larger number of patients is indicated to confirm our findings.

In conclusion, we were able to obtain high quality endocytoscopic images, which were adequate for the differentiation of benign lesions from malignant ones. As this was a retrospective pilot study involving a small number of patients, a large-scale prospective study is indicated.

COMMENTS

Background

Endocytoscopy, which allows for ultra-high-magnifying observation of the gastrointestinal mucosa, has recently been developed, enabling the visualization of the gastrointestinal mucosa at resolutions approaching those of histology. Although the usefulness of virtual biopsy using endocytoscopy in the diagnosis of esophageal cancer, colorectal cancer, lung cancer, and ulcerative colitis has been reported, few studies are available on the diagnosis of gastric cancer.

Research frontiers

The gold standard for the diagnosis of malignant gastrointestinal tumors is histopathological examination, and definitive diagnosis requires endoscopic biopsy. As endocytoscopy provides real-time microscopic images *in vivo*, its application to histological diagnosis by virtual biopsy (without the need to take biopsies) is promising.

Innovations and breakthroughs

Endocytoscopy allows recognition of nuclei and cells, thereby producing *in vivo* images similar to those produced by histological study of biopsies. This study suggested the usefulness of virtual biopsy for gastric cancer.

Applications

Using endocytoscopy, it is expected that we will be able to diagnose gastric cancer with virtual biopsies, eliminating the need for physical biopsies. The need for tissue biopsies, and associated limitations like bleeding and fibrosis at biopsy site may be pre-empted for gastric cancer diagnosis, improving patient outcomes.

Terminology

Endocytoscopy involves an ultra-high magnification endoscope that allows observation of nuclei and cells *in vivo*. Virtual biopsy denotes a procedure to obtain a tissue image of high resolution with nuclear and cellular visualization, without performing and analyzing a physical tissue biopsy.

Peer-review

This manuscript addresses the usefulness of endocytoscopy for distinguishing gastric cancer. Although this pilot study involved a small number of patients, the content of this manuscript is interesting.

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CASE REPORT

Achalasia after bariatric Roux-en-Y gastric bypass surgery reversal

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Author contributions: All authors contributed to the manuscript; Abu Ghanimeh M, Qasrawi A and Abughanimeh O wrote the manuscript; Albadarin S edited the initial manuscript draft and provided the images; Clarkston W reviewed, edited and approved the final manuscript.

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Informed consent statement: The patient has provided permission to publish these features of his case, and the identity of the patient has been protected.

Conflict-of-interest statement: No conflict-of-interest.

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Abstract

Achalasia is a rare esophageal motility disorder that is characterized by a loss of peristalsis in the distal esophagus and failure of lower esophageal sphincter relaxation. The risk of developing esophageal motility disorders, including achalasia, following bariatric surgery is controversial and differs based on the type of surgery. Most of the reported cases occurred with laparoscopic adjustable gastric banding. To our knowledge, there are only three reported cases of achalasia after Roux-en-Y gastric bypass and no reported cases after revision of the surgery. We present a case of a 70-year-old female who had a previous history of Roux-en-Y gastric bypass with revision. She presented with persistent nausea and regurgitation for one month. Esophagogastroduodenoscopy showed a dilated esophagus without strictures or stenosis. A barium study was performed after the endoscopy and was suggestive of achalasia. Those findings were confirmed by a manometry. The patient was referred for laparoscopic Heller's myotomy.

Key words: Esophagus; Bariatric; Gastric band; Bypass surgery; Achalasia; Esophagogastroduodenoscopy; Heller's myotomy; Motility disorder

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Core tip: Achalasia is a rare esophageal motility disorder. It is uncommonly reported after bariatric surgeries. Achalasia is a very rare complication after Roux-en-Y gastric bypass. We report a case of a 70-year-old female who she presented with persistent nausea and regurgitation for one month. She had a previous history of Roux-en-Y gastric bypass with revision. As part of her inpatient evaluation, a computed tomography of the chest, a barium study and an upper endoscopy were suggestive of achalasia. Those findings were confirmed by a manometry. The patient was referred for laparoscopic Heller's myotomy.

Abu Ghanimeh M, Qasrawi A, Abughanimeh O, Albadarin S, Clarkston W. Achalasia after bariatric Roux-en-Y gastric bypass surgery reversal. *World J Gastroenterol* 2017; 23(37): 6902-6906 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6902.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6902

INTRODUCTION

Achalasia is a rare esophageal motility disorder characterized by loss of peristalsis in the distal esophagus and failure of lower esophageal sphincter (LES) relaxation[1,2]. It usually presents with dysphagia for solids and liquids as well as regurgitation[3]. Primary achalasia is an idiopathic disease with unknown etiology, whereas secondary achalasia or pseudoachalasia is associated with infections, paraneoplastic syndromes and extrinsic compression by benign or malignant processes[4-6]. Interestingly, relationships have previously been reported between abnormalities in esophageal manometry, including achalasia, and both morbid obesity and bariatric surgery[7-18]. Most of the reported cases of achalasia after bariatric surgery occurred after laparoscopic adjustable gastric banding (LAGB)[11-14]. Achalasia after Roux-en-Y gastric bypass (RYGB) is rare[15-18] and could be related to surgical trauma in the area[15]. In these cases, it is important to rule out stenosis of the gastrojejunostomy.

CASE REPORT

A 70-year-old female with a past medical history of coronary artery disease and morbid obesity (previous body mass index (BMI) of 52 kg/m^2) presented to our institution with persistent nausea and regurgitation for one month.

Her surgical history was significant for laparoscopic cholecystectomy and laparoscopic RYGB approximately two years prior to presentation. Her post-operative course was complicated by a gastro-gastric fistula and gastro-jejunal anastomosis ulcer with gastrointestinal bleeding requiring admission to the hospital, blood transfusion and endoscopic hemostasis. Her surgery was revised approximately 2 mo prior to her presentation.

Her symptoms were mostly post-prandial. She also described mild dysphasia to both solids and liquids, so she started to drink in small sips. She denied any change in her weight or appetite, abdominal pain or change in bowel habits. Her vital signs were unremarkable. Her abdomen was soft, without tenderness to palpation and with bowel sounds present. The wound from her recent surgery was clean, with no evidence of discharge or poor healing.

Initial laboratory workup showed alanine aminotransferase (ALT) 33 unit/L (normal 13-69 unit/L), aspartate aminotransferase (AST) 35 unit/L (normal 15-46 unit/L) and alkaline phosphatase (ALP) 123 unit/L (normal 42-140 unit/L). A CT scan of the abdomen and pelvis with contrast (Figure 1) showed enteric contrast within the dilated distal esophagus and was suspicious for a mild stricture at gastroesophageal sphincter (GES). There was no evidence of peri-gastric inflammatory changes. Her stool workup was negative for *Clostridium difficile* toxin, ova and parasites. She was started on IV hydration, given IV ondansetron 4 mg every 6 h as needed for nausea and admitted for further evaluation.

The next day, esophagogastroduodenoscopy (EGD) showed large amounts of thick secretions in the esophagus. The esophagus was tortuous, dilated and had a "sigmoid esophagus" appearance, but no strictures, stenosis or evidence of malignancy were noted. A Barium study (Figure 2) was performed after the EGD and showed persistent narrowing of the gastroesophageal junction with a moderately dilated, debris-filled esophagus proximally and some tertiary esophageal contractions. These findings were suggestive of achalasia.

The patient was discharged with outpatient follow-up with a manometry study. The manometry was performed 2 wk later and revealed high pressure in the LES with abnormal relaxation and high resting pressure, in addition to aperistalsis. These results were consistent with type Ⅱ achalasia (Figure 3). She was referred to surgery for evaluation and laparoscopic Heller's myotomy.

DISCUSSION

Achalasia is a rare esophageal motility disorder that is characterized by neurodegeneration, preferentially involving the inhibitory nitric oxide-producing neurons. It leads to a loss of peristalsis in the distal esophagus and failure of LES relaxation[1,2]. It is derived from the

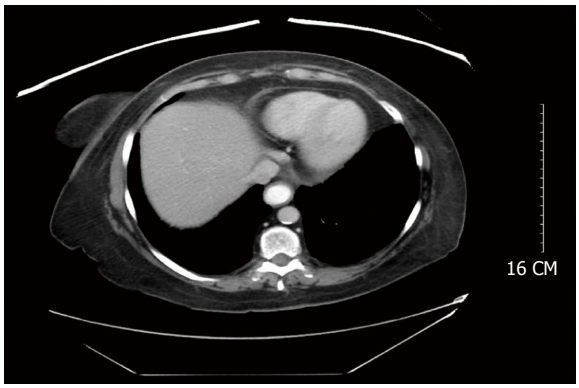


Figure 1 Computed tomography scan of the abdomen and pelvis with contrast showing enteric contrast within the dilated distal esophagus, and was suspicious for mild stricture at the gastroesophageal junction.

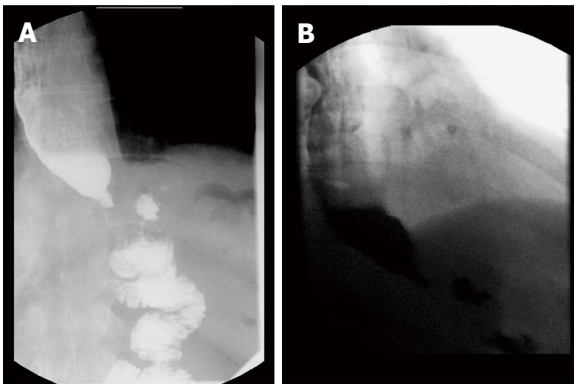


Figure 2 Barium study showing persistent narrowing of the gastroesophageal junction with a moderately dilated, debris filled esophagus proximally and some tertiary esophageal contractions.

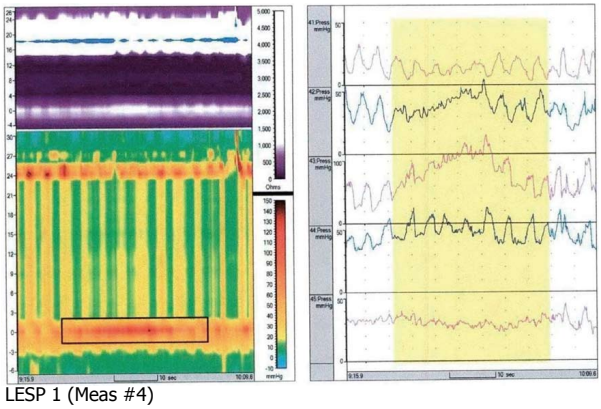


Figure 3 Esophageal manometry showing high lower esophageal sphincter pressure with abnormal relaxation and high resting pressure in addition to aperistalsis. These findings were consistent with type Ⅱ achalasia.

Greek term meaning "does not relax". Achalasia occurs equally in males and females, with an annual incidence of approximately 1.6 cases per 100000 individuals[2].

Dysphagia for solids and liquids and regurgitation of undigested food or saliva are the most common symptoms in patients with achalasia[3]. The etiology of primary achalasia is unknown, though autoimmune and viral infectious etiologies have been proposed[4,5]. In its secondary form or pseudoachalasia, there are many potential causes of esophageal motor abnormalities that are similar or identical to those of primary achalasia. Examples include infections such as Chagas disease, paraneoplastic syndromes and extrinsic compression of gastroesophageal junction by either benign or malignant processes[6].

Bariatric surgeries are among the fastest growing operative procedures worldwide[19-21]. They are recommended for adults who have a BMI of at least 40 kg/m^2 or 35 kg/m^2 with other comorbidities[21]. RYGP remains the most commonly performed bariatric procedure in the United States[19-21]. The risk of developing esophageal motility disorders, including achalasia, following bariatric surgery is still controversial and differs with the type of surgery[11-14,16-18]. Pseudoachalasia after LAGB placement has been described, though there is evidence that the pseudoachalasia may be reversible after the band is removed[11-14]. It has been postulated that malpositioning of the band near the gastroesophageal junction creates a high-pressure area, causing clinical symptoms of pseudoachalasia[11-14].

In contrast, the development esophageal motility disorders after RYGB is rare and has only been reported a few times in the literature (Table 1)[15-18]. To the best of our knowledge, there are only 3 reported cases of achalasia after RYGB[16-18], and this is the first case described after revision of RYGB. Interestingly, several case reports have been published on the simultaneous treatment of achalasia and morbid obesity with laparoscopic esophageal myotomy and gastric bypass[22-23]. The exact pathophysiology of motility disorders that develop after RYGB in general, and achalasia specifically, is unknown. Surgical trauma is one potential explanation. Shah *et al*[15] described a retrospective study of 64 patients with achalasia compared with a control group of 73 patients without achalasia evaluated by manometry and endoscopy. A significant association was found between achalasia and trauma to the upper gastrointestinal tract. Of the patients with operative trauma and achalasia, 2 had undergone gastric bypass. Finally, it is worth mentioning that another important consideration in similar cases is to rule out stenosis of the gastrojejunostomy, which we did in our patient.

Achalasia is a rare esophageal motility disorder that is characterized by neurodegeneration, of the inhibitory nitric oxide-producing neurons. It usually manifests with dysphagia to both solid and liquids and regurgitation. Obesity is a global epidemic and

Table 1 Reported cases of achalasia after Roux-en-Y gastric bypass

Case	Age and gender	Pre-operative BMI (kg/m^2)	Procedure	Presentation	Onset of symptoms postoperative	Upper GI series/ Barium swallow	EGD	Esophageal manometry	Treatment
Ramos *et al*[16] 2009	44-yr-old female	47	Laparoscopic RYGB	Dysphagia to solids, and regurgitation	4 yr	Dilated esophagus, poor esophageal emptying, and tapering of the LES	Normal gastroesophageal junction, a 4-cm gastric pouch without lesions, and a wide gastrojejunostomy	Elevated resting LES pressure, aperistalsis, and hypo contractility of the esophagus.	Laparoscopic Heller myotomy
Torghabeh *et al*[17] 2015	48-yr-old female	44.75	Laparoscopic RYGB	Dysphagia to solid, regurgitation, and chest pain	5 yr	Dilated esophagus and stricture at the LES	Tortuous esophagus with retained food products and *Candida* plaques. Stricture was balloon dilated	Elevated resting LES pressure, aperistalsis, and failure of LES relaxation	Laparoscopic Heller myotomy
Chapman *et al*[18] 2013	53-yr-old female	NA	Open PYGB	Epigastric and LUQ pain and reflux symptoms	2 yr	Dilated thoracic esophagus with reduced primary peristalsis. Contrast was slow to pass through the gastro-esophageal junction	Dilated esophagus, esophagitis and ulceration above the gastro-esophageal junction	Absence of LES relaxation and aperistalsis	Laparoscopic Heller myotomy
Our case 2016	70-yr-old female	52	Laparoscopic RYGB	Regurgitation, mild dysphagia, nausea and occasional vomiting	2 yr	Persistent narrowing of the gastroesophageal junction with a dilated, debris filled esophagus. Some tertiary contractions	Dilated, tortuous esophagus that appeared as a "sigmoid esophagus" but no strictures or stenosis was noted.	Elevated LES pressure with abnormal relaxation in addition to aperistalsis and	Scheduled for laparoscopic Heller myotomy

LES: Lower esophageal sphincter.

can be associated with esophageal motility disorders. Esophageal motility disorders, including achalasia, occur as a consequence of bariatric surgeries.

COMMENTS

Case characteristics

Persistent nausea, regurgitation and mild dysphagia to solids and liquids for one month. History of RYGB 2 yr ago with reversal 2 mo prior to presentation.

Clinical diagnosis

Hemodynamically stable with normal vital signs. Abdomen was soft, without tenderness to palpation and with bowel sounds present. The wound from her recent surgery was clean, with no evidence of discharge or poor healing.

Differential diagnosis

Distal esophageal stricture, Stenosis of the gastrojejunostomy, achalasia, tumor of the gastric cardia or distal esophagus, infectious gastroenteritis.

Laboratory diagnosis

Initial complete blood count, basic metabolic panel (kidney function and electrolytes) and liver panel were unremarkable. Stool workup was negative for infection.

Imaging diagnosis

A computed tomography scan of the chest with contrast showed enteric contrast within the dilated distal esophagus and was suspicious for a mild stricture at gastroesophageal sphincter . A Barium study was showed persistent narrowing of the gastroesophageal junction with a moderately dilated, debris-filled esophagus proximally and some tertiary esophageal contractions. Manometry was performed 2 wk after discharge and revealed high pressure in the lower esophageal sphincter (LES) with abnormal relaxation and high resting pressure, in addition to aperistalsis. These results were consistent with type Ⅱ achalasia.

Endoscopic diagnosis

Esophagogastroduodenoscopy showed a tortuous and dilated esophagus with large amounts of thick secretions in the esophagus. No strictures, stenosis or evidence of malignancy were noted.

Treatment

Supportive treatment while inpatient with intravenous fluids and anti-emetics. She was referred for evaluation and laparoscopic Heller's myotomy after her

diagnosis.

Related reports
Table 1 summarize previous reported cases of achalasia in association with RYGB.

Term explanation
Achalasia is a rare esophageal motility disorder characterized by loss of peristalsis in the distal esophagus and failure of LES.

Experiences and lessons
Achalasia and other esophageal motility disorders may occur as a consequence of bariatric surgeries including RYGB.

Peer-review
A rare case report that achalasia after bariatric Roux-en-Y gastric bypass surgery was well described by the authors. Only 3 reported cases of achalasia after RYGB had been reported in the world, and this was the first case described after revision of RYGB. It's a worth case to be reported.

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CASE REPORT

Persistent severe hypomagnesemia caused by proton pump inhibitor resolved after laparoscopic fundoplication

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Abstract

Magnesium deficiency can cause a variety of symptoms, including potentially life-threatening complications such as seizures, cardiac arrhythmias and secondary electrolyte disturbances. Hypomagnesemia can be a serious adverse effect to proton pump inhibitor (PPI) therapy, which is worrying due to the widespread use of PPIs. Current evidence suggest that the mechanism of PPI induced hypomagnesemia is impaired intestinal magnesium absorption. In this report, we present the case of a long-term PPI user with persistent hypomagnesemia with severe symptoms at presentation. He was unable to stop PPI treatment because of severe reflux symptoms, and was dependent on weekly intravenous magnesium infusions, until his magnesium levels finally normalized without the need for supplementation after a successful laparoscopic fundoplication.

Key words: Hypomagnesemia; Proton pump inhibitor; Gastroesophageal reflux disease; Drug adverse effect; Laparoscopic fundoplication

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Core tip: This case illustrates a long-term proton pump inhibitor (PPI) user with persistent hypomagnesaemia causing severe symptoms at presentation. For years, he was dependent on oral and later intravenous magnesium supplementation, until his hypomagnesemia rapidly improved upon PPI discontinuation. He was, however, not able to cope with the resulting reflux symptoms, and was not able to completely stop PPI

treatment until he underwent a successful laparoscopic fundoplication.

Semb S, Helgstrand F, Hjørne F, Bytzer P. Persistent severe hypomagnesemia caused by proton pump inhibitor resolved after laparoscopic fundoplication. *World J Gastroenterol* 2017; 23(37): 6907-6910 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6907.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6907

INTRODUCTION

Magnesium plays an essential role in several physiological and biochemical bodily processes. Consequently, magnesium deficiency can cause a variety of symptoms, ranging from nonspecific complaints of weakness, fatigue and nausea to potentially life threatening complications such as seizures, cardiac arrhythmias and secondary electrolyte disturbances. Hypomagnesemia can develop due to gastrointestinal and/or renal disease, as well as an adverse effect to several medications. Several reports of severe hypomagnesemia associated with long term proton pump inhibitor (PPI) therapy[1,2] is causing concern due to the widespread use of PPIs[3]. Current evidence suggest that PPI impair the intestinal magnesium absorption through a molecular effect on magnesium transporters in genetically predisposed individuals[4].

We present the first case of a long-term PPI user with severe and symptomatic persistent hypomagnesemia treated with surgery. Due to severe reflux, the patient was PPI dependent, and needed weekly intravenous magnesium infusions. After a successful laparoscopic fundoplication, the PPI treatment could be discontinued, and the magnesium levels normalized without the need for supplementation.

CASE REPORT

A 67-year-old male with a history of gastroesophageal reflux disease (GERD) and hypertension, first presented with severe hypomagnesemia during a hospital admission in 2012. At that time, he was taking 10 mg amlodipine and 20-40 mg omeprazole daily. Prior to admission, he had suffered from severe dizziness, double sight and vomiting for several weeks, and was initially dehydrated with electrolyte derangement (hypokalemia). A few days after admission he developed general seizures, and additional laboratory tests revealed severe hypomagnesemia [0.23 mmol/L (0.71-0.94 mmol/L)], considered a result of excessive vomiting prior to admission. His serum electrolyte levels normalized upon intravenous treatment. An extensive work-up resulted in a possible diagnosis of viral encephalitis, and after discharge, he underwent a prolonged rehabilitation course. A year later, he was re-admitted because of dizziness, balance problems and paresthesia. Laboratory results again showed severe hypomagnesemia (0.08 mmol/L). He received intravenous magnesium infusions and his symptoms disappeared. During ambulatory follow-up, he was found to have persistent hypomagnesemia, and oral magnesium was prescribed. He underwent endocrinological and gastrointestinal investigations including colonoscopy, which did not reveal any other cause of his hypomagnesemia. His urinary magnesium level was low (< 0.080 mmol/L). Despite oral magnesium supplementation, magnesium levels continued to stay low, and in 2015, he was started on weekly magnesium infusions to maintain normal magnesium levels. The only medication he was on during these years was omeprazol 20-40 mg daily. He had been advised several times during the years to stop PPI treatment, but was unable to cope with the resulting reflux symptoms. In April 2016, he did, however, agree to stop taking PPI and consequently maintained normal magnesium levels without infusions. 24-h pH testing confirmed reflux disease, and he was offered anti-reflux surgery (laparoscopic fundoplication). While awaiting the operation, he started taking daily PPI again, which resulted in a prompt drop in his serum magnesium levels demanding a resumption of weekly magnesium infusions. The patient underwent a laparoscopic fundoplication and was discharged after an uncomplicated peri-and postoperative course. After the operation, the reflux symptoms as well as the need for PPI disappeared, and his magnesium level maintained normal without supplementation at the follow-up visit. The course of the patient's magnesium levels is illustrated in Figure 1.

DISCUSSION

This rare case illustrates a long-term PPI user with persistent hypomagnesemia causing severe symptoms at presentation. For years, he was dependent on oral and later weekly intravenous magnesium supplementation, until his hypomagnesemia rapidly improved upon PPI discontinuation. Re-challenge of PPI treatment resulted in a prompt drop in serum magnesium within days, and his magnesium levels again normalized rapidly following PPI withdrawal after laparoscopic fundoplication.

PPI-induced hypomagnesemia (PPIH) was first described by Epstein *et al*[5] in 2006, and subsequently several other case reports have demonstrated the association. In 2011, the United States Food and Drug Administration issued a safety announcement regarding the association between long term PPI use and hypomagnesemia, and PPIH is now well-established as a rare, but potentially serious, adverse effect of long term PPI treatment. However, PPIH is still not widely acknowledged, and the frequency and risk

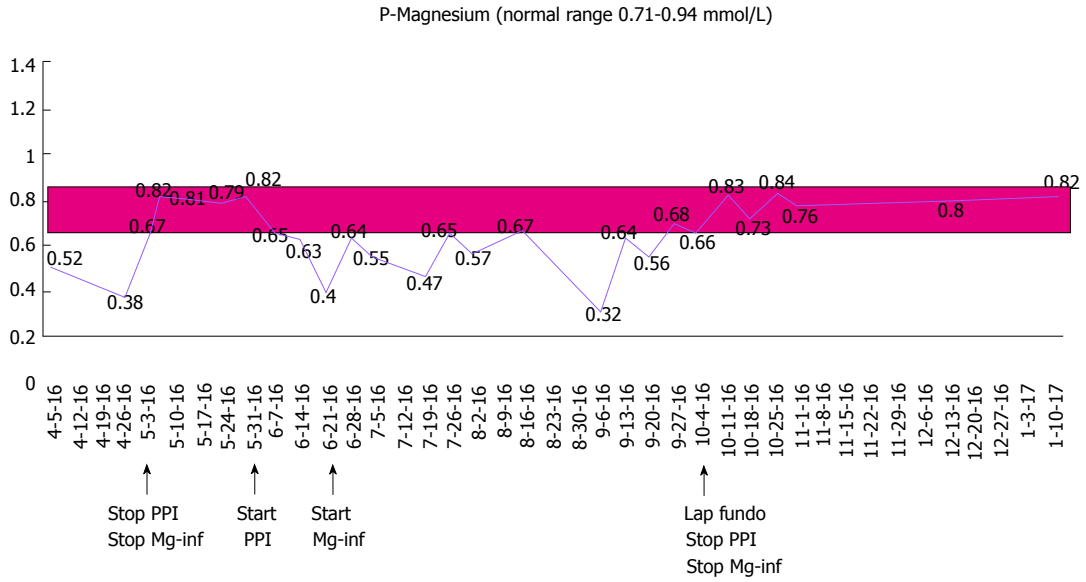


Figure 1 Course of the patient's magnesium levels. The figure illustrates how the patient´s S-Mg level stays persistently below the normal range while he is on PPI therapy despite receiving magnesium infusions. S-Mg normalizes promptly after cessation of PPI therapy. PPI: Proton pump inhibitor.

factors of PPIH are not well recognized.

PPIH has been observed with all different formulations of PPIs and is thus considered a class drug effect. Similar to our case, previous reports have documented only partial effect of oral magnesium supplements in correcting the hypomagnesemia during ongoing PPI-therapy[2], and only short term relief with intravenous magnesium infusions. In contrast, hypomagnesemia is typically improved promptly after the PPI is discontinued, and PPI re-challenge results in rapid recurrence of hypomagnesemia[1,2]. The same phenomenon has not been observed with histamine 2 receptor antagonists (H2RAs), although a recent population-based study suggested a similar but weaker association with the use of these drugs[6]. Previous reports have suggested that PPIH occurs after long term PPI use[1]; however, duration of exposure to PPI therapy is often difficult to quantify retrospectively and the duration of PPI use necessary to cause PPIH remains uncertain.

Magnesium homeostasis is mainly determined by absorption in the small intestine, and renal excretion. Intestinal magnesium absorption is complex, and occurs via both passive and active transport mechanisms. Firstly, magnesium is passively absorbed through a paracellular pathway between the enterocytes of the intestine across a concentration gradient. Secondly, a transcellular active transport mechanism occur by means of a combined action of magnesium channels in the apical membrane of enterocytes on the luminal surface, particularly transient receptor potential melastin (TRPM) 6 and 7. It has been proposed that increased luminal pH in the intestine caused by PPIs may alter the affinity of the TRPM6/7 channel for magnesium, resulting in reduced active transport of magnesium, and that individual genetic variations in the TRPM could explain why PPIH does not develop in all PPI users[4]. Several other drugs can cause hypomagnesemia, where the underlying mechanism is tubular magnesium wasting[1]. In contrast, previous case reports of PPIH have documented consistently low urinary excretion coherent with efficient tubular magnesium retention[1,2], which is thought to support the theory of impaired gastrointestinal absorption as the mechanism leading to PPIH.

Some patients might be more susceptible to PPIH due to other coexisting risk factors such as concomitant medication (diuretics) or other medical conditions associated with hypomagnesemia such as diabetes mellitus, inflammatory bowel disease, short-bowel disease, chronic pancreatitis, diarrhea and/or alcoholism. It remains controversial whether serum magnesium should be monitored in all long-term PPI users, or perhaps in specific subsets of patients with additional risk factors. Nonetheless, PPIH should be remembered as a differential diagnosis in patients with PPI use and unexplained hypomagnesemia. Patients with PPIH and persistent troublesome reflux symptoms after PPI discontinuation could be considered for anti-reflux surgery. It should be remembered, however, that a majority of patients treated with anti-reflux surgery have resumed PPI therapy 10-15 years after the operation[7], and it is mandatory to control for hypomagnesemia if patients with previous PPIH resume PPI treatment.

COMMENTS

Case characteristics

A 67-year old male with gastroesophageal reflux disease and persistent severe hypomagnesemia demanding weekly intravenous magnesium infusions.

Clinical diagnosis

Extensive work-up did not reveal any other cause of hypomagnesaemia.

Serum magnesium levels rapidly normalized upon pump inhibitor (PPI) discontinuation, and re-challenge of PPI treatment resulted in a prompt drop in serum magnesium within days. Magnesium levels again normalized following PPI withdrawal after laparoscopic fundoplication.

Differential diagnosis

Gastrointestinal or renal disease causing either impaired absorption or increased excretion of magnesium.

Laboratory diagnosis

Initially the patient had severe hypomagnesemia (0.23 mmol/L) with associated hypokalemia, which both resolved with intravenous magnesium infusions. However, the patient had continuously low S-Mg levels during the disease course despite both oral and intravenous magnesium supplementation, where other laboratory tests were normal.

Imaging diagnosis

Neither colonoscopy nor CT scans revealed any other explanation for the patient´s persistent hypomagnesaemia.

Pathological diagnosis

Biopsies taken from terminal ileum and colon during colonoscopy were normal.

Treatment

The patient underwent a successful laparoscopic fundoplication, and his serum magnesium levels again normalized following PPI withdrawal after the surgery.

Related reports

PPI-induced hypomagnesemia (PPIH) was first described in 2006, and subsequently several other case reports have demonstrated the association. In 2011, the US Food and Drug Administration issued a safety announcement regarding the association between long term PPI use and hypomagnesemia, and PPIH is now well-established as a rare, but potentially serious, adverse effect of long term PPI treatment.

Term explanation

PPIH is a rare, but potentially serious adverse effect where persistent severe hypomagnesemia is associated with of long-term PPI treatment. PPIH is probably caused by impaired intestinal magnesium absorption through a molecular effect on magnesium transporters in genetically predisposed individuals.

Experiences and lessons

PPIH should be remembered as a differential diagnosis in patients with PPI use and unexplained hypomagnesemia. Patients with PPIH and persistent troublesome reflux symptoms after PPI discontinuation could be considered for anti-reflux surgery

Peer-review

The manuscript describes surgical option in the management of hypomagnesemia induced by PPIs. The condition has been described earlier but fundoplication as an option has not been discussed in earlier reports. The manuscript is well written and may be accepted with few clarifications.

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CASE REPORT

Rupture of small cystic pancreatic neuroendocrine tumor with many microtumors

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Author contributions: Sagami R, Nishikiori H and Ikuyama S designed the study and wrote the manuscript; Sagami R and Nishikiori H collected and analyzed the patient's clinical data; and Murakami K made critical revisions to the article for important intellectual content.

Institutional review board statement: The Ethics Committee of Oita San-ai Medical Center approved this clinical study and the publication of this case report.

Informed consent statement: The patient involved in this case report authorized the disclosure of her protected health information for academic purposes.

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Abstract

Pancreatic neuroendocrine tumors (pNETs) are particularly rare. The various forms of PNETs, such as cystic degeneration, make differentiation from other similar pancreatic lesions difficult. We can detect small lesions by endoscopic ultrasound (EUS) and obtain preoperative pathological diagnosis by EUS-guided fine needle aspiration (FNA). We describe, here, an interesting case of pNET in a 42-year-old woman with no family history. Computed tomography and magnetic resonance imaging revealed an 18 mm × 17 mm cystic lesion with a nodule in the pancreatic tail. Two microtumors about 7 mm in diameter in the pancreatic body detected only by EUS, cystic rim and nodules all showed similar enhancement on contrast-harmonic EUS. Preoperative EUS-FNA of the microtumor was performed, diagnosing multiple pNETs. Macroscopic examination of the resected pancreatic body and tail showed that the cystic lesion had morphologically changed to a 13-mm main nodule, and 11 new microtumors (diameter 1-3 mm). Microscopically, all microtumors represented pNETs. From the findings of a broken peripheral rim on the main lesion with fibrosis, rupture of the cystic pNET was suspected. Postoperatively, pituitary adenoma and parathyroid adenoma were detected. The final diagnosis was

multiple grade 1 pNETs with multiple endocrine neoplasia type 1. To the best of our knowledge, no case of spontaneous rupture of a cystic pNET has previously been reported in the English literature. Therefore, this case of very rare pNET with various morphological changes is reported.

Key words: Rupture; Cystic pancreatic neuroendocrine tumor; MEN1; NET; Microtumor

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Core tip: Cystic or multiple pancreatic neuroendocrine tumors (pNETs) are very rare. This report describes a case of cystic pNET with multiple small tumors diagnosed from endoscopic ultrasonography (EUS) alone. Preoperative diagnosis was able to be obtained by EUS-guided fine needle aspiration biopsy. Postoperatively, 11 other micro-pNETs with multiple endocrine neoplasia type 1 were detected and cystic pNET morphologically changed to a small nodule because of suspected spontaneous rupture. Spontaneous rupture of cystic pNET has not been reported previously in the English literature.

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INTRODUCTION

Neuroendocrine tumors can arise in endocrine organs throughout the whole body and cause various clinical symptoms. Pancreatic neuroendocrine tumors (pNETs) are particularly rare, representing only 1%-2% of all pancreatic neoplasms[1]. PNETs sometimes show various forms, such as cystic degeneration[2] or multiple microtumors[3], but such variations appear to be rare.

Most pNETs are solid and show strong enhancement during the arterial phases on contrast-enhanced computed tomography (CT)[4-6]. Diagnosis is therefore relatively easy. However, morphologically changed pNETs, such as cystic pNETs, are difficult to differentiate from other pancreatic cystic tumors[7]. Multiple PNETs are often diagnosed in multiple endocrine neoplasia type 1 (MEN1)[8], but diagnosis of microtumors in the pancreas on CT is difficult. In such cases, endoscopic ultrasonography (EUS) is superior to CT for detecting pNETs, particularly small lesions[9]. Furthermore, contrast-enhanced harmonic EUS (CH-EUS) allows the diagnosis as pNETs with hypervascular enhanced tumors with high sensitivity and specificity, and EUS-guided fine needle aspiration (FNA) enables preoperative pathological diagnosis[10].

This paper presents a case of pNETs with microtumors detected only by EUS in a 42-year-old woman with MEN1. In addition, a rare event of spontaneous rupture of the cystic pNET was strongly suspected from the postoperative pathological findings.

CASE REPORT

A 42-year-old woman was referred to our hospital because of a pancreatic cystic lesion incidentally detected on unenhanced CT performed for inspection of painless enlargement of a cervical lymph node 1 mo earlier. No physical findings involving the abdomen were evident. No contributory family history was elicited. The patient had a history of urinary stones at 30-years-old. The only abnormality from laboratory tests was a mild elevation of liver enzymes.

Unenhanced CT revealed a 16 mm × 15 mm, somewhat obscure cystic lesion in the pancreatic tail (Figure 1A). Contrast-enhanced CT revealed an 18 mm × 17 mm cystic lesion with a well-defined, hyperenhanced thin peripheral rim and a nodule about 6 mm in diameter inside the rim (Figure 1B). The outer rim was enhanced to a greater degree than normal pancreatic parenchyma during the arterial phases of bolus contrast administration, and iso-enhanced during the portal venous phase (Figure 1B and C).

On magnetic resonance imaging (MRI), the lesion in the pancreatic tail appeared hypointense and some areas appeared relatively hyperintense compared to inside the cyst on T1-weighted images (Figure 2A), and mainly hyperintense with a hypointense peripheral rim and nodule on T2-weighted images (Figure 2B). On diffusion-weighted images, the lesion appeared relatively hyperintense (Figure 2C). The lesion was not continuous with the main pancreatic duct on magnetic resonance cholangiopancreatography (Figure 2D).

We also performed EUS, showing the cyst with a low echoic peripheral rim and nodule. Inside the cyst, no echoic fluid or solid structure was detected, and internal bleeding or necrosis was expected (Figure 3A). Apart from the main lesion, we detected two microtumors (7 mm and 6 mm in diameter) in the pancreatic body only on EUS (Figure 3B). On CH-EUS, the relatively thick rim of the cystic lesion, nodule and two microtumors showed similar enhancement from immediately after the injection of contrast medium (Figure 3C and D). We performed EUS-FNA of a 7-mm microtumor (Figure 3E).

Histopathologically, the EUS-FNA specimen comprised cells with round nuclei (hematoxylin and eosin stain, ×100; Figure 4A). Tumor cells were negative

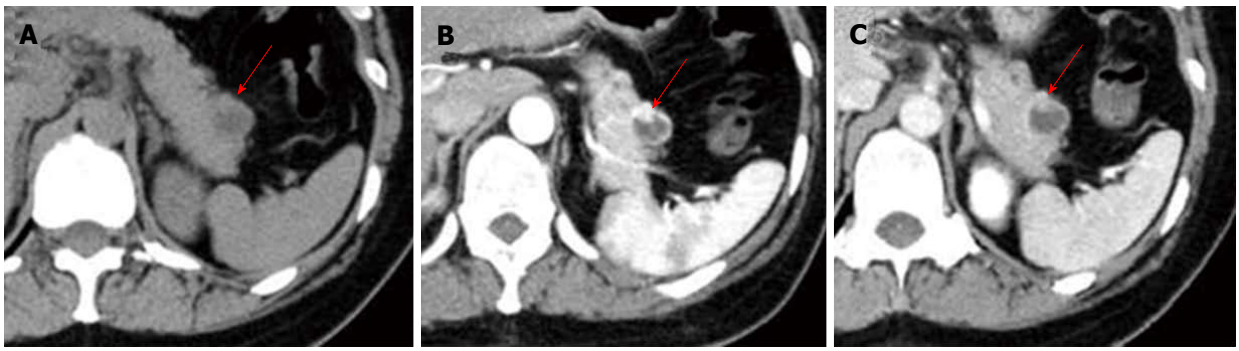


Figure 1 Findings from computed tomography. A: Unenhanced computed tomography (CT) shows a 16 mm × 15 mm, somewhat obscure cystic lesion (red arrow) in the pancreatic tail; B: Contrast-enhanced CT shows an 18 mm × 17 mm cystic lesion (red arrow) with a well-defined, hyper-enhanced, thin peripheral rim and a 6-mm nodule during the arterial phase; C: The outer rim appears iso-enhanced compared to normal pancreatic parenchyma during the portal venous phases (red arrow).

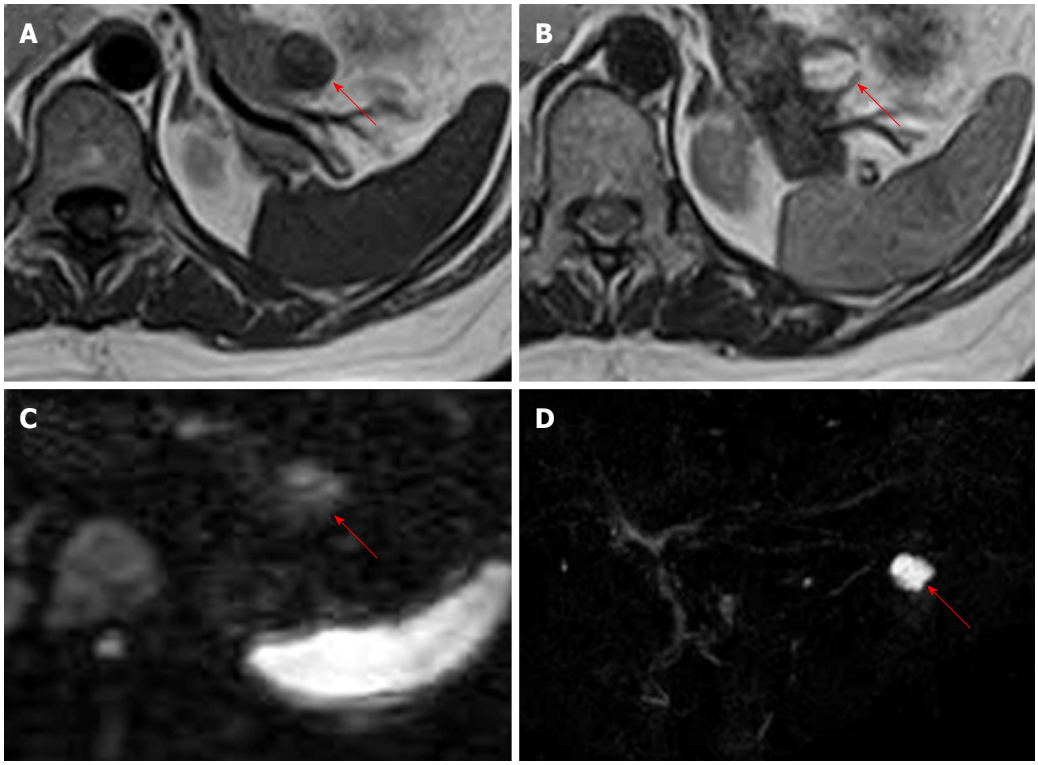


Figure 2 Findings from magnetic resonance imaging. A: The cystic lesion in the pancreatic tail (red arrow) appears hypointense and some areas appear relatively hyperintense compared to inside the cyst on T1-weighted images; B: The lesion (red arrow) appears mainly hyperintense, while the peripheral rim and nodule appear hypointense on T2-weighted images; C: The lesion (red arrow) appears relatively hyperintense on diffusion-weighted images; D: The lesion (red arrow) is not continuous with the main pancreatic duct on magnetic resonance cholangiopancreatography.

for CD10 and CD56 (Figure 4B and C), but positive for chromogranin A and synaptophysin (Figure 4D and E) by immunohistochemical staining (×100). Based on these findings, multiple grade 1 endocrine neoplasms of the pancreas were diagnosed[11].

Preoperatively, endocrine examination was performed. Abnormal laboratory results included elevated parathyroid hormone at 136 pg/mL (normal, 15-65 pg/mL) and cortisol at 25.1 μg/dL (normal, 4.5-21.1 μg/dL), while other values were within normal ranges. The tumor was non-functional pNET, because levels of pancreatic endocrine hormones such as insulin were not elevated. After sufficient preparation and obtaining consent from the patient and her family members, we performed laparoscopic spleen-preserving pancreatic body and tail resection 50 d after EUS-FNA.

Macroscopically, the main cystic lesion morphologically changed to a nodule 13 mm in diameter with internal scarring (Figure 5A and C), considered to represent traces of cystic degeneration. The microtumor in the pancreatic body on which EUS-FNA was performed was separate from the main lesion (Figure 5A and B). A scar from EUS-FNA was identified in the microtumor (Figure 5B). Besides the main lesion and

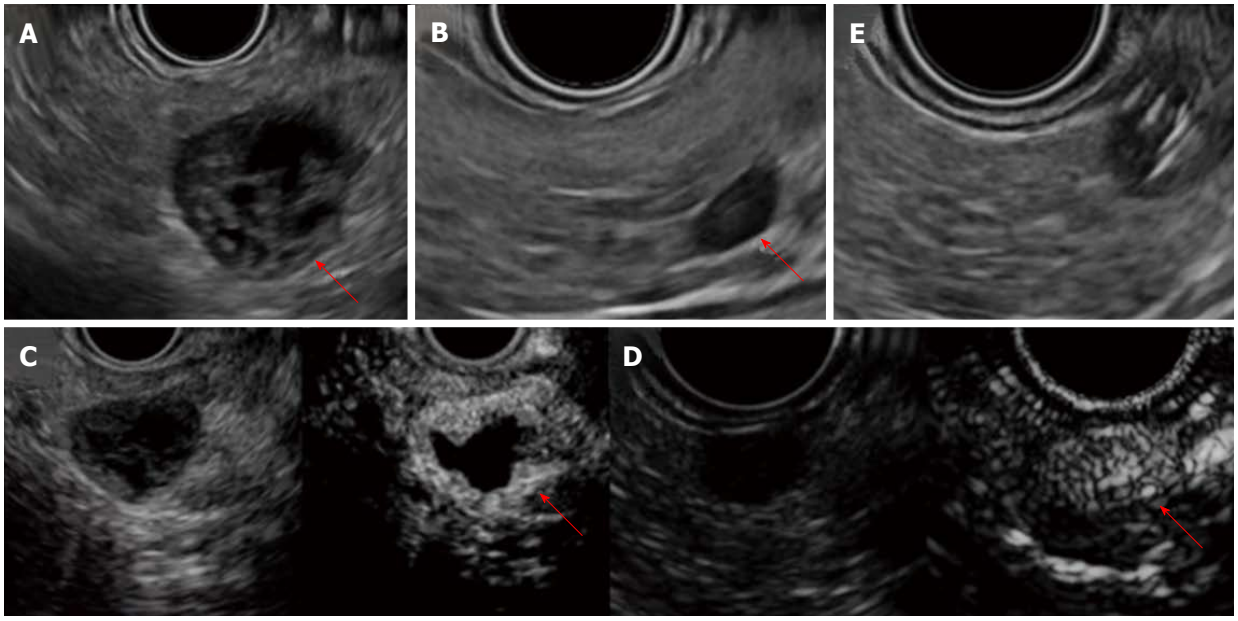


Figure 3 Findings from EUS and CH-EUS. A: The lesion in the pancreatic tail shows a hypoechoic peripheral rim and nodule. Inside the cyst, no echoic fluid or solid structure is detected; B: A new, 7-mm microtumor in the pancreatic body, apparent only on EUS; C: The rim of the cystic lesion and nodule appear enhanced immediately after injection of contrast medium on CH-EUS; D: The 7-mm microtumor is similarly enhanced on CH-EUS; E: Endoscopic ultrasound-fine needle aspiration to biopsy the 7-mm microtumor. CH-EUS: Contrast-enhanced harmonic-endoscopic ultrasound; EUS: Endoscopic ultrasound.

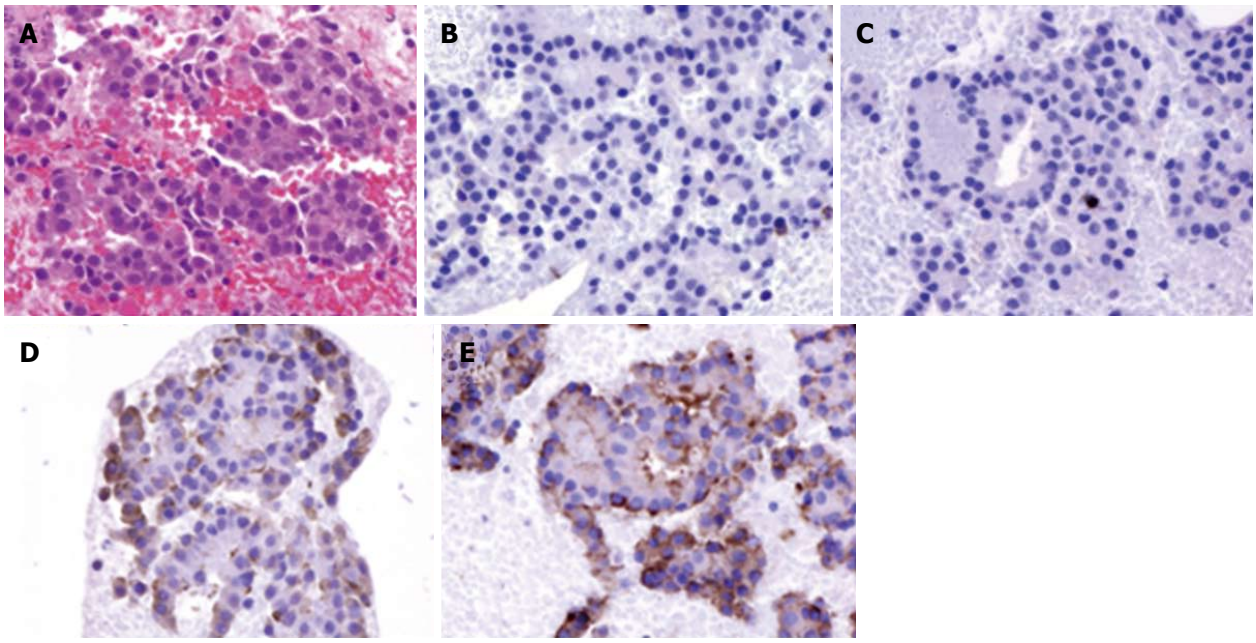


Figure 4 Histopathological findings for the specimen obtained by endoscopic ultrasound-fine needle aspiration. A: The specimen comprises cells with round nuclei arranged in sheets or rosettes (hematoxylin and eosin, ×100); B and C: Tumor cells show negative results for CD10 (B) and CD56 (C) immunohistochemical staining (×100); D and E: Tumor cells show positive results for chromogranin A (D) and synaptophysin (E) on immunohistochemical staining (×100).

two microtumors detected previously on EUS, 11 microtumors (1-3 mm diameters) were newly detected (Figure 5D).

Microscopically, the main lesion comprised cells with round nuclei arranged in sheets or rosettes (Figure 6A), with < 2 mitoses per 50 high-power fields (Figure 6B). The Ki-67 index in the main lesion was 1.01%. All other microtumors showed similar findings (Figure 6C and D). After evaluating the potential causes of morphological changes to the main cystic lesion, the peripheral rim was considered most likely to have broken in one place, followed by fibrosis after

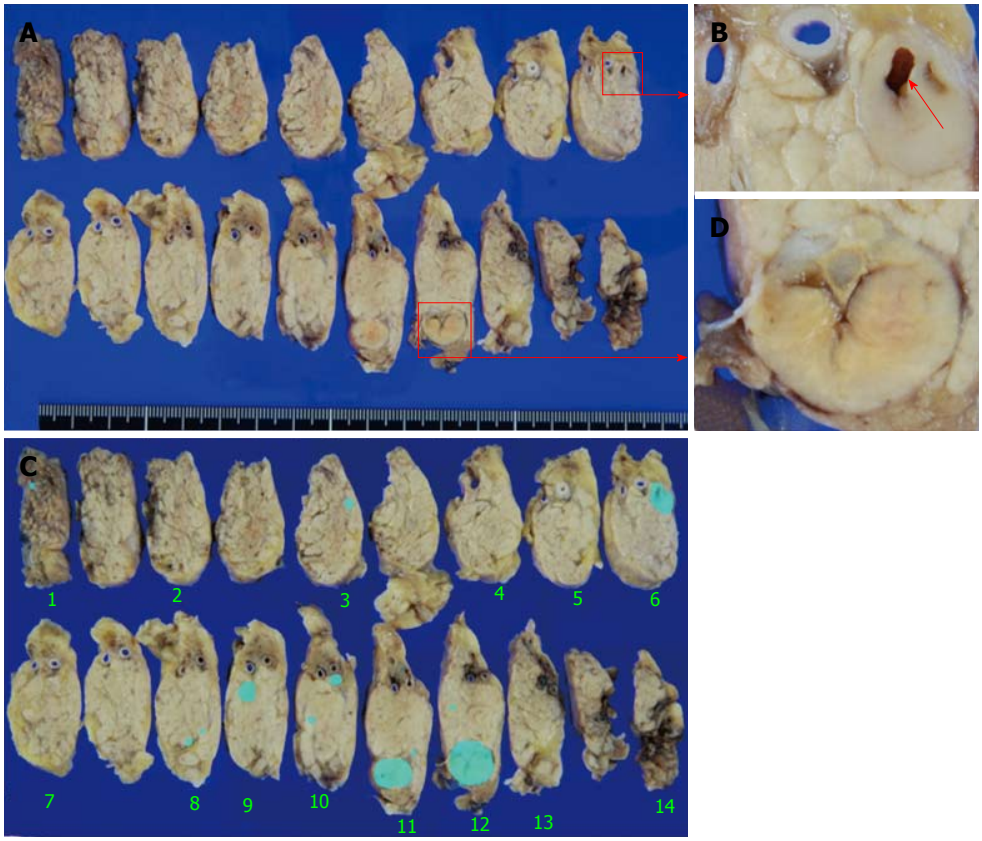


Figure 5 Macroscopic findings for the resected pancreas. A: The main cystic lesion has morphologically changed to a 13-mm nodule (red square C). The microtumor in the pancreatic body on which EUS-FNA was performed was separate from the main lesion; B: A scar from EUS-FNA is evident in the microtumor (red arrow); C: The main nodule shows internal scarring; D: The main lesion and two microtumors detected on EUS, and another 11 new microtumors (1-3 mm) (blue areas). EUS-FNA: Endoscopic ultrasound-fine needle aspiration.

inflammation (Figure 6E).

After surgery, whole-body examination detected asymptomatic pituitary adenoma and parathyroid adenoma on MRI and ultrasound. The final diagnosis was multiple pNET grade 1, stage 1 T1N0M0 with MEN1 and a strong suspicion of spontaneous rupture of the cystic pNET. As of the time of writing, the patient remains alive without recurrence for more than 2 years postoperatively. Asymptomatic pituitary adenoma and parathyroid adenoma are being followed-up by an endocrine physician, and no clinical symptoms or abnormal results from blood testing have been encountered.

DISCUSSION

Image features of pNETs and various morphological changes

PNETs are rare and represent only 1%-2% of all pancreatic neoplasms[1]. However, due to the development of increasingly sophisticated imaging modalities, more pNETs are being reported[12].

The clinical symptoms of pNET primarily depend on whether the tumor is functional or non-functional, and the type of hormone produced by a functional tumor[1]. Most pNETs are solid lesions, but some are detected as tumor with cystic degeneration[2]. Cystic pNETs were classically considered very rare, but recent studies have suggested that cystic pNETs may be more common than previously reported[13]. Some studies have reported that 9.3%-21% of pNETs are detected as cystic degeneration on imaging modalities including CT, ultrasound, MRI, and EUS or by pathological examination of a resected specimen[14-19].

Solid pNETs are typically enhanced to a greater degree than normal pancreatic parenchyma during the arterial and portal venous phases of bolus contrast administration[4-6], because of the rich vascularity[20]. On the other hand, the radiological features of cystic pNETs have been suggested to represent an enhanced outer rim with blood flow-rich tissue[21]. As another specific finding of pNETs, multiple and microtumors in the pancreas are rarely detected. Multiple PNETs are often diagnosed in cases of MEN1[8], but diagnosis of microtumors in the pancreas on contrast-enhanced CT is difficult[9].

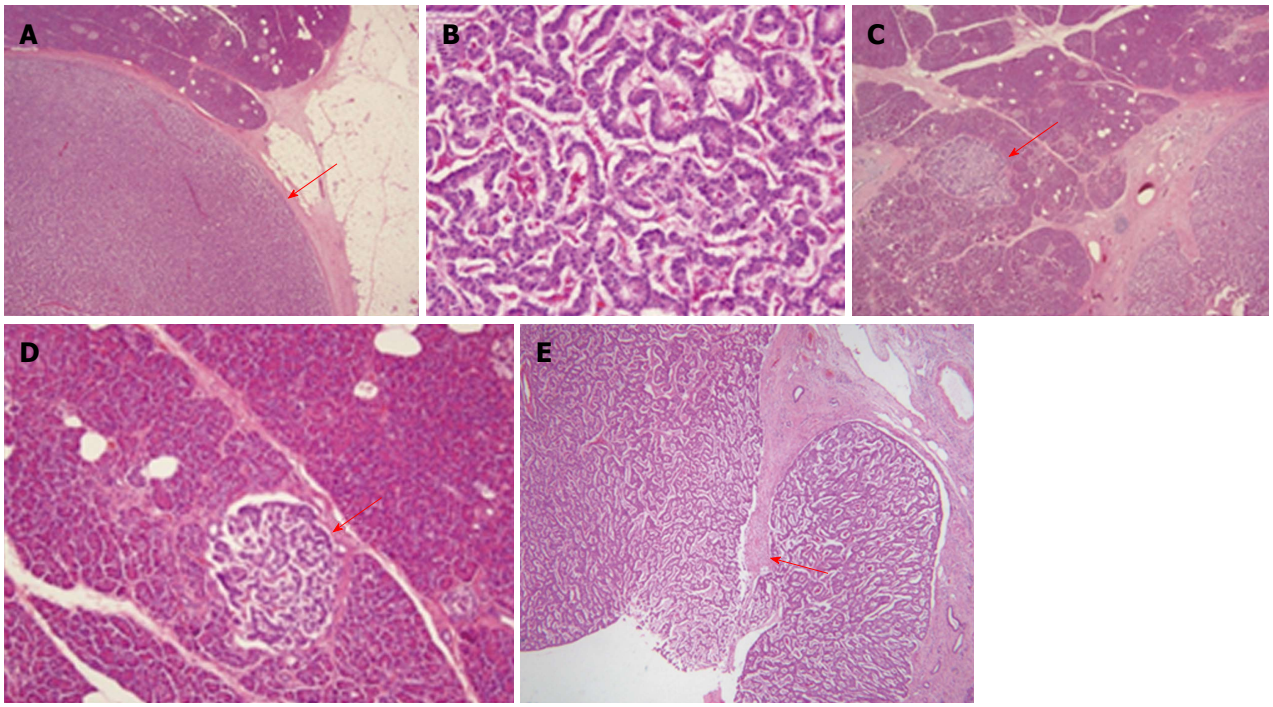


Figure 6 Microscopic findings for the resected pancreas. A: The main lesion (red arrow) comprises cells with round nuclei arranged in sheets or rosettes (hematoxylin and eosin, × 4); B: Less than 2 mitoses per 50 high-power fields are evident (hematoxylin and eosin, × 40); C and D: The other microtumors (red arrow) show similar findings (hematoxylin and eosin; C, × 4; D, × 10); E: The peripheral rim appears broken at one site, and fibrosis (red arrow) is evident with inflammation (hematoxylin and eosin, × 10). A trace of a cystic lumen is apparent below the red arrow.

EUS as one of the best imaging modalities for small pNETs

EUS is superior to CT for detecting small pNETs. In a study of 231 pNETs, CT detected 84% of tumors but was more likely to miss lesions < 2 cm in diameter (P = 0.005), while the sensitivity of EUS for detecting 56 pNETs (91.7%) was greater than that of CT (63.3%; P = 0.0002), and EUS detected 20 of 22 CT-negative tumors (91%)[10]. Furthermore, CH-EUS allowed the diagnosis of hypervascularly enhanced tumors as pNETs with 78.9% sensitivity (95%CI: 61.4%-89.7%) and 98.7% specificity (95%CI: 96.7-98.8%) respectively[11].

In this manner, EUS appears useful for the diagnosis of pNETs, but we often encounter challenges, particularly in diagnosing cystic pNET. This is because we need to differentiate the pathology from other pancreatic cystic tumors, such as intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, mucinous cystadenocarcinoma, serous cystic neoplasm, solid and cystic papillary tumors, or even non-neoplastic pseudocyst[7].

In such cases, we should consider EUS-FNA for preoperative histological diagnosis. EUS-FNA correctly diagnosed PNETs, offering 90.1% diagnostic accuracy[22]. When CH-EUS was combined with EUS-FNA, the sensitivity of EUS-FNA increased from 92.2% to 100%[11]. EUS-FNA accuracies for the malignancy of cystic and solid PNETs were 89.3% and 90% respectively[23], but EUS-FNA for cystic lesions of the pancreas is not recommended in Japan.

In our case, the cystic lesion in the pancreas tail was initially detected on CT. MRI was additionally performed, but we could not obtain a confirmed diagnosis from the imaging findings. The cystic lesion was evaluated in more detail and 2 new microtumors were detected in the pancreatic body only on EUS. In addition, the rim of the cyst and microtumors were hyperenhanced on CH-EUS, and we became confident the lesions represented pNETs. In addition, we performed EUS-FNS of a microtumor, and obtained pathological confirmation of the preoperative diagnosis.

Features of pNETs in MEN1

MEN is an autosomal-dominant inherited disease presenting with tumorous lesions, mainly in various endocrine organs[3], and 15% of MEN1 patients have no family history[24]. MEN1 is diagnosed when at least 2 of the main tumorous diseases (parathyroid tumor, pNET or pituitary tumor) are found, and hyperparathyroidism is the most frequent initial disease in MEN1 (85%)[25]. According to a Japanese nationwide survey, MEN1 was found in 10% of pNET patients. The type of pNET in MEN1 was gastrinoma in 25%, insulinoma in 14% and non-functioning in 6.1%[12]. In addition, PNETs in MEN1 often have

multiple tumors in the pancreas, and 74% of pNET with MEN1 had multiple tumors[8].

Rupture of cystic neoplasms

This was a typical case of pNETs with MEN1, but spontaneous rupture of the cystic pNET was a remarkable feature. The rupture of a pancreatic neoplasm is very rare. Ruptures of intraductal papillary mucinous neoplasm[26-28], mucinous cystic neoplasm[29], acinar cell carcinoma[30,31] and anaplastic cell carcinoma[32] have been reported, but rupture of cystic pNET does not appear to have been described. Involvement of either infarction and liquefactive necrosis of a large tumor or internal hemorrhage have been considered as causes of cystic degeneration in pNETs[21,33]. The cause of cyst formation of cystic pancreatic NET, thus, remains controversial[15,34,35].

Spontaneous rupture of cystic lesions of the pancreas has been suggested due to high pressure inside the cyst or inflammatory stimulation by others factors, such as acute pancreatitis[27], but only a small number of cases have been reported, and the exact cause remains thusly unknown. In our case, internal bleeding, rather than necrosis, might have been involved in cystic degeneration. We performed EUS-FNA of the microtumor in the pancreatic body separate from the cystic pNET in the pancreatic tail, so EUS-FNA might not have been related to rupture.

We could not rule out the possibility that rupture was associated with instrumental pressure from EUS examinations, but such high pressure on the cystic lesion was considered unlikely to arise in normal EUS examination or EUS-FNA. The scar from EUS-FNA is shown in Figure 5B. The thin part of the rim was not intact, and thinning of the tumor part and high pressure inside the cyst were considered as causes, but more detailed causes of the rupture could not be clarified.

In conclusion, pNETs are rare and show various forms of tumor, such as cystic degeneration. PNETs with MEN1 often show multiple tumors, and detecting microtumors on CT or MRI is sometimes difficult so that EUS and CH-EUS should be performed. In addition, we should search for endocrine tumors in other organs affected by MEN1. To the best of our knowledge, spontaneous rupture has not been reported previously, so this case was reported.

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COMMENTS

Case characteristics

A 42-year-old woman was referred to our hospital with a pancreatic cystic lesion found incidentally on computed tomography (CT). She had no relevant family history.

Clinical diagnosis

Cystic pancreatic neuroendocrine tumor (pNET) with many microtumors.

Differential diagnosis

Differential diagnoses included intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, solid pseudopapillary tumor and pancreatic neuroendocrine tumor.

Laboratory diagnosis

Abnormal laboratory results included mild liver enzyme, cortisol and intact parathyroid hormone elevation in blood serum

Imaging diagnosis

Contrast-enhanced CT showed an 18 mm × 17 mm cystic lesion with a well-defined hyperenhanced thin peripheral rim. On magnetic resonance imaging, the lesion appeared mainly hypointense on T1-weighted images and hyperintense on T2-weighted images. On EUS, apart from the main lesion, two microtumors were detected (7 mm and 6 mm) in the pancreatic body.

Pathological diagnosis

The final pathological diagnosis was multiple grade 1 pNETs. The main cystic lesion changed to a 13-mm nodule, and spontaneous rupture of the cystic pNET was strongly suspected.

Treatment

The patient was treated with pancreatic body tail resection.

Related reports

Recent studies suggest that cystic pNETs may be more common than previously reported, but rupture of a cystic pNET has not been reported.

Term explanation

There are no non-standard terms used in this manuscript.

Experience and lessons

The authors present this case to share important knowledge for pNET diagnosis.

Peer-review

The authors reported an interesting case, which they believed to be a spontaneous rupture of cystic pNET with many microtumors.

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LETTERS TO THE EDITOR

Resistance of *Helicobacter pylori* to furazolidone and levofloxacin: A viewpoint

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Abstract

In their review, Arslan *et al*[1] did not describe the status of *Helicobacter pylori* (*H. pylori*) treatment with furazolidone and the resistance to this antibiotic. We have presented different surveys showing the resistance of *H. pylori* to furazolidone from Asia and South America. The resistance rates varied but were mostly low (< 5%). There are not enough data on its efficacy and resistance in the United States and Europe. *H. pylori* mutations occurring in the *oorD* gene, including A041G, A122G, C349A(G), A78G, A112G, A335G, C156T and C165T, and in the *porD* gene, including G353A, A356G, C357T, C347T, C347G and C346A, have been indicated to be possibly related to the observed resistance. Additionally, to complete Arslan *et al*'s statement regarding levofloxacin resistance, it should be noted that compound mutations of N87A, A88N and V65I at codon Asn-87 were recently observed in the *gyrA* gene for the first time. However, the results on these topics are not sufficient, and more worldwide studies are suggested.

Key words: Susceptibility; Furazolidone; *Helicobacter pylori*; Resistance; Levofloxacin; Treatment

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Core tip: We have presented different surveys showing the resistance of *Helicobacter pylori* (*H. pylori*) to furazolidone from Asia and South America. The resi-

Table 1 Studies evaluating the *Helicobacter pylori* resistance to furazolidone

Continent	Country	Study year	Strains (*n*)	Method	Resistance (%)	Author
Asia	China (Shanghai)	2000-2009	293	Agar dilution	0	Sun *et al*[6]
	China (Zhejiang)	2010-2012	21	Agar dilution	0.1	Su *et al*[7]
	China (Zhejiang)	2009-2014	9687	Agar dilution	< 0.01	Ji *et al*[8]
	India (Ghaziabad and New Delhi)	NA	68	Agar dilution	22.1	Gehlot *et al*[9]
	India (Gujarat)	2008-2011	80	Disk diffusion	13.8	Pandya *et al*[10]
	Iran (Rasht)	2012-2014	169	Disk diffusion	61.9	Maleknejad *et al*[11]
	Iran (Shiraz)	2004-2005	106	Agar dilution	9.4	Kohanteb *et al*[12]
	Iran (Sari)	2009	197	Disk diffusion	61.4	Abadi *et al*[13]
	Iran (Tehran)	2001-2004	135	Disk diffusion	0	Siavoshi *et al*[14]
	Iran (Tehran)	2002-2003	24	Disk diffusion	0	Fallahi *et al*[15]
	Iran (Tehran)	2005-2008	110	Disk diffusion	4.5	Siavoshi *et al*[16]
	Iran (Tehran)	2007-2008	104	Disk diffusion	0	Sirous *et al*[17]
	Iran	2003-2005	100	Disk diffusion	9	Rafeey *et al*[18]
	South Korea	1994-1999	220	Agar dilution	1.4	Kim *et al*[19]
	Malaysia (Malacca)	2009	90	Epsilometer test	0	Goh *et al*[20]
	Pakistan (Karachi)	2008-2013	93	disk diffusion	4.3	Siddiqui *et al*[21]
South America	Brazil (Bragança Paulista)	NA	90	Agar dilution	4	Mendonça *et al*[22]
	Brazil (Bragança Paulista)	NA	138	Agar dilution	13	Godoy *et al*[23]
	Brazil (Sao Paulo)	NA	39	Agar dilution	0	Eisig *et al*[24]
	Brazil (Sao Paulo)	2008-2009	77	Agar dilution and disk diffusion	0	Ogata *et al*[25]
	Brazil (Sao Paulo)	2008-2009	77	Agar dilution	0	Ogata *et al*[26]

stance rates varied but were mostly low (< 5%). *H. pylori* mutations occurring in the *oorD* gene, including A041G, A122G, C349A(G), A78G, A112G, A335G, C156T and C165T, and in the *porD* gene, including G353A, A356G, C357T, C347T, C347G and C346A, have been indicated to be possibly related to the observed resistance. Regarding levofloxacin resistance, compound mutations of N87A, A88N and V65I at codon Asn-87 were recently observed in the *gyrA* gene for the first time.

Zamani M, Rahbar A, Shokri-Shirvani J. Resistance of *Helicobacter pylori* to furazolidone and levofloxacin: A viewpoint. *World J Gastroenterol* 2017; 23(37): 6920-6922 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i37/6920.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i37.6920

TO THE EDITOR

We have read with great interest the valuable article by Arslan *et al*[1], titled "Importance of antimicrobial susceptibility testing for the management of eradication in *Helicobacter pylori* infection". One of the main subjects of the review was the description of the resistance rates of different antibiotics and the potential mechanisms leading to decreased in *Helicobacter pylori* (*H. pylori*) antimicrobial susceptibility. However, the authors should consider clarifying two important issues.

The authors did not allude to the status of *H. pylori* treatment with furazolidone and the resistance to this antibiotic. We have provided existing surveys reporting the resistance of *H. pylori* to furazolidone in Table 1. The resistance rates have been mostly reported to be lower than 5%; however, these rates can vary geographically. Furazolidone is not used widely in the United States and Europe; therefore, there are not enough data on its efficacy and resistance in these regions.

One of the main reasons for the emergence of resistance is related to the extensive use of furazolidone. In addition, regarding the molecular mechanisms, some genetic mutations have been identified. Mutations occurring in the *2-oxoglutarate:acceptor oxidoreductase* (*oorD*) gene, including *A041G*, *A122G*, *C349A(G)*, *A78G*, *A112G*, *A335G*, *C156T* and *C165T*, and in the *pyruvate oxidoreductase* (*porD*) gene, including *G353A*, *A356G*, *C357T*, *C347T*, *C347G* and *C346A*, are possibly related to the resistance[2,3]. *The oor and por* genes are involved in the generation of acetyl coenzyme A (acetyl-CoA) and succinyl-CoA[4]. Despite these findings, additional molecular methods are proposed to reach a better understanding of the mechanisms that were mentioned.

Arslan *et al*[1] accurately documented the mechanism of levofloxacin resistance; *i.e.*, point mutations in the *gyrA* (DNA *gyrase*) gene were stated to be potentially linked to the resistance. However, to complete their statement, it should be noted that compound mutations of N87A, A88N and V65I at codon Asn-87 were recently observed in the *gyrA* gene for the first time. L45F, A55S, A97V, D91N, R130K and G60S are other possible mutations that need to be assessed in studies with broader sample bases[5].

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EDITORIAL

Evolving role of FDG-PET/CT in prognostic evaluation of resectable gastric cancer

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Abstract

Gastric cancer (GC) remains a leading cause of cancer death worldwide. Radical gastrectomy is the only potentially curative treatment, and perioperative adjuvant therapies may improve the prognosis after curative resection. Prognosis largely depends on the tumour stage and histology, but the host systemic inflammatory response (SIR) to GC may contribute as well, as has been determined for other malignancies. In GC patients, the potential utility of positron emission tomography/computed tomography (PET/CT) with the imaging radiopharmaceutical ^{18}F-fluorodeoxyglucose (FDG) is still debated, due to its lower sensitivity in diagnosing and staging GC compared to other imaging modalities. There is, however, growing evidence that FDG uptake in the primary tumour and regional lymph nodes may be efficient for predicting prognosis of resected patients and for monitoring tumour response to perioperative treatments, having prognostic value in that it can change therapeutic strategies. Moreover, FDG uptake in bone marrow seems to be significantly associated with SIR to GC and to represent an efficient prognostic factor after curative surgery. In conclusion, PET/CT technology is efficient in GC patients, since it is useful to integrate other imaging modalities in staging tumours and may have prognostic value that can change therapeutic strategies. With ongoing improvements, PET/CT imaging may gain further importance in the management of GC patients.

Key words: Gastric cancer; Prognosis; ^{18}F-fluorodeoxyglucose; Positron emission tomography-computed tomography; Bone marrow

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Core tip: Gastric cancer (GC) is still a leading cause of cancer death worldwide. Prognosis depends on surgical curability, response to adjuvant therapies, tumour stage and histology, but also on the systemic inflammatory response to malignancy. While the diagnostic role of positron emission tomography with ^{18}F-fluorodeoxyglucose (FDG) in GC is still debated, due to unsatisfactory sensitivity, there is growing evidence that FDG uptake, either at the tumour sites or in the bone marrow, may represent an efficient tool for predicting prognosis of resected patients and for monitoring tumour response to adjuvant treatments, and may have prognostic value in directing therapeutic strategies.

De Raffele E, Mirarchi M, Cuicchi D, Lecce F, Cola B. Evolving role of FDG-PET/CT in prognostic evaluation of resectable gastric cancer. *World J Gastroenterol* 2017; 23(38): 6923-6926 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/6923.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.6923

INTRODUCTION

Gastric cancer (GC) remains a leading cause of cancer death worldwide, with poor prognosis despite significant advances in diagnosis and treatment. Survival rates are progressively increasing in western countries[1-3], and are highest in Japan, due to focused management of preventive and prognosis-related factors (*i.e.* infection and smoking, respectively)[2]. Prognostic factors related to GC are quite well established, such as local extension, lymph node involvement and presence of distant metastases, and can be adequately defined by the conventional imaging modalities, including endoscopic ultrasound (EUS), computed tomography (CT) and magnetic resonance imaging (MRI). However, some emerging prognostic factors related to the metabolism of tumour cells, such as the glucose avidity, or to the systemic inflammatory response (SIR) to the tumour can be better evaluated through the metabolic information that are provided by positron emission tomography (PET) integrated with CT, even though the role of PET/CT imaging in the evaluation of GC is still controversial.

CLASSIFICATION, THERAPEUTIC STRATEGIES AND PROGNOSIS

GC can be categorized according to anatomical location, as either true GC (non-cardia) or gastro-oesophageal-junction (cardia) cancer (GEJ)[1,2]. In general, GC are predominantly adenocarcinomas, classified according to the World Health Organization (WHO) classification into tubular, papillary, mucinous (MAC), poorly cohesive and rare variants[1-3]. The Lauren classification distinguishes GC according to intestinal type, diffuse type (including signet ring cell carcinoma (SRC)), mixed type and indeterminate type[1-3]. Classification of GC based on molecular subtyping has been proposed recently[1] and is promising for helping to improve the accuracy of prediction of individual prognosis and for providing individually-tailored therapies.

Radical surgical resection is the only potentially curative therapeutic option for resectable GC presently. Adequate surgery includes complete resection of the primary tumour and appropriate lymphadenectomy. Tumours of the lower two-thirds of the stomach can be selectively treated with distal subtotal gastrectomy; otherwise, total gastrectomy is recommended[2-4]. This approach has contributed in part to the amelioration of cure rates from 30% to over 50% in selected series over the past decade[1]. Early GC (EGC) is defined as limited to the mucosa or submucosa (T1 stage or lower), regardless of nodal status. Endoscopic resection is considered appropriate for small ($\leqslant$ 20 mm), non-ulcerated, superficial GC that are well differentiated and limited to the mucosa (T1a), because the incidence of regional lymph node metastases is very low[3]. If, however, the tumour has invaded the submucosa (T1b), radical gastrectomy with lymphadenectomy is required, since lymph node involvement is observed in up to 20% of cases[1,2].

Locally advanced GC (AGC; invading the muscularis propria and beyond (T2 stage or higher)) presents in most cases with metastases to lymph nodes, distant organs, or both. Patients without distant metastases are candidates for potentially radical surgery, either conventional or minimally invasive by laparoscopy[1-4]. Perioperative therapies for resectable GC include chemotherapy (CHT), radiotherapy and chemoradiotherapy, performed before and/or after surgery. Even though adjuvant and neoadjuvant therapies have been demonstrated to improve prognosis after potentially curative resection of locally AGC, the optimal strategy is still debated[1-3].

Despite substantial advances in the staging procedures, imaging techniques and treatment options, prognosis of GC remains poor, with postoperative 5-year survival rates of 25%-30% in western countries, because of the high incidence of advanced tumours[3]. Cardia GC and diffuse-type non-cardia GC have the worst prognosis. For resectable locally AGC, outcome depends on the surgical disease stage. Resection of EGC provides excellent 5-year survival rates, up to 90%. However, at the time of diagnosis GC is usually advanced, with reported involvement of the regional lymph nodes in 70% to 80% of cases. If the tumour invades the subserosa (T3 stage), 5-year survival decreases to less than 50%. Moreover, the presence of nodal involvement in T3 lesions further decreases 5-year overall survival to less than 30%[2].

Besides tumour-related factors, the survival of GC patients, as for other malignancies, is also dependent on the host's reaction to the cancer. SIR plays a critical role in carcinogenesis and tumour diffusion[5]. Several host SIR markers (SIRMs) have been identified as prognostic factors. Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), albumin and C-reactive protein (CRP) have been indicated, alone or in combination, as significant factors for predicting postoperative prognosis of GC patients[6,7].

ROLE OF PET/CT IN DIAGNOSIS AND PROGNOSTIC EVALUATION

Clinical evaluation of GC has greatly improved with the availability of EUS, CT, MRI, PET/CT and laparoscopic staging. PET/CT using ^{18}F-fluorodeoxyglucose (FDG) has proven useful for staging, detecting recurrence, evaluating treatment response and predicting prognosis[1-4,8,9]. However, the overall sensitivity of FDG-PET/CT for detecting GC is lower than for most other malignancies, so that its effective role in GC patients is still controversial[8,9]. FDG-PET may have different sensitivities for different histotypes, with better sensitivity for GEJ tumours, but significantly lower sensitivities for diffuse type adenocarcinoma, including SRC, or for MAC[8,9]. Since tumour size and depth of invasion are significant factors influencing FDG-PET detection of GC, sensitivity is low for EGC and far higher for AGC. Altogether, the role of PET/CT is limited in T staging due to its low spatial resolution[9]. For N staging in GC, the sensitivity and specificity of FDG-PET/CT range between 33.3%-64.6% and 85.7%-97.0%, respectively[8]. The low sensitivity in detecting lymph node metastases may be related to the histotype of the primary tumour, or even to the size of the metastatic lymph nodes; some small lymph nodes may be difficult to visualize because of the radioactive volume effect generated by the nearby primary cancer[8,9]. Nonetheless, FDG-PET/CT is considered to have higher specificity than CT and MRI in the N staging of GC, especially for the N2 and N3 groups[9]. FDG-PET/CT has lower sensitivity than CT for the diagnosis of peritoneal seeding, while being more efficient in the detection of solid organ metastases, including those involving the lung, liver, bone or adrenal gland, with near 100% sensitivity and specificity[8,9].

Despite these limitations, FDG-PET/CT is emerging as an effective tool for therapeutic and prognostic evaluation of AGC. Preoperative FDG uptake has been demonstrated as an independent, significant prognostic factor following curative gastrectomy[8,9]; although, the collective data are not in full agreement. Patients with lower preoperative FDG uptake in the GC have shown significantly lower incidence of recurrence and better recurrence-free survival after surgery[8,9]. Lower preoperative FDG uptake has been reported as a predictor of tumour curability at the time of surgery, since higher FDG uptake in the primary tumour and positive FDG uptake in local lymph nodes have been significantly associated with non-curative resection, suggesting that these patients should be candidates for neoadjuvant CHT[9].

Neoadjuvant treatments have been increasingly used for AGC to reduce tumour stage, plan the optimal surgical timing and strategies, and improve the overall prognosis[9]. About 30% to 60% of histologically partial or even total responders have been reported with different therapeutic regimens[8]. Since patients with clinical and pathological response to neoadjuvant therapies are considered to gain significant survival benefit, the prompt identification of responders seems to be essential. FDG uptake in PET/CT scans is actually considered an early and sensitive indicator of response to treatment[2,3,8,9], concordant with histopathological analysis for tumour response. Changes in FDG uptake soon after the initiation of treatment have been related to final outcome also. In some studies, metabolic responders have shown better prognosis than non-responders, while FDG non-avid tumours seem to have poor response rates to CHT and unfavourable prognosis, indicating that neoadjuvant therapies may be ineffective in metabolic non-responders and in patients with low FDG uptake at baseline PET imaging[8].

In neoplastic patients, FDG uptake in bone marrow (BM) on PET/CT has been shown to be significantly associated with SIRMs, suggesting that this imaging finding has a significant relationship with SIR to malignancy[7]. In non-small cell lung cancer patients with curative surgical resection, Lee *et al*[7] have recently shown that the FDG uptake in BM and the BM to liver uptake ratio (BLR) were significantly correlated with albumin and CRP levels, white blood cell count, NLR and PLR; moreover, the BLR was identified as an independent prognostic factor of recurrence-free survival. The authors concluded that the FDG uptake in BM for non-small cell lung cancer patients reflects the degree of SIR and can be used as a prognostic factor after curative surgery[7].

In a recent retrospective series of 309 GC patients undergoing curative surgical resection, Lee *et al*[10] demonstrated that the preoperative BM FDG uptake, and BLR especially, are correlated with SIRMs of GC. In addition, patients with AGC, recurrence and positive FDG uptake of primary cancer were shown to have higher BM FDG uptake than those with EGC, no recurrence and negative FDG uptake, respectively; thus, GC patients with advanced stage and aggressive features might have higher degrees of SIR. BLR was identified as an independent prognostic factor for predicting survival, along with T4 stage, lymph node metastasis and positive resection margin. The authors conclude that for GC, both tumour factors and SIR could play important roles in long-term prognosis of resectable patients, and that BM FDG uptake could

reflect the degree of SIR to cancer and provide information on prognosis after curative surgery.

CONCLUSION

In conclusion, PET/CT technology represents an efficient tool for use in GC patients, since it is useful to integrate other imaging modalities in staging tumours. Moreover, it can be effective in monitoring tumour response to treatments and may have prognostic value with the potential to change therapeutic strategies. Although some problems still persist, PET/CT imaging remains promising, and with ongoing improvements may gain further importance in the evaluation and treatment of GC patients.

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EDITORIAL

Staging chronic pancreatitis with exocrine function tests: Are we better?

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Abstract

Chronic pancreatitis (CP) is an inflammatory disease of the pancreas evolving in progressive fibrotic disruption of the gland with exocrine and endocrine pancreatic insufficiency. Although imaging features of CP are well known, their correlation with exocrine pancreatic function tests are not obvious, particularly in the early stage of the disease. There are many clinical classification of CP, all suggested for better distinguish and manage different forms based on etiological and clinical factors, and severity of the disease. Recently, a new classification of CP has been suggested: the M-ANNHEIM multiple risk factor classification that includes etiology, stage classification and degree of clinical severity. However, more accurate determination of clinical severity of CP requires a correct determination of exocrine function of the pancreas and fecal fat excretion. Recently, Kamath *et al* demonstrated that the evaluation of exocrine pancreatic function by acid steatocrit and fecal elastase-1 (EF-1) was helpful, but EF-1 was able to detect exocrine pancreatic insufficiency in more patients, upgrading some patients in higher stage of disease according to M-ANNHEIM classification. So, EF-1 is a more accurate test to determine exocrine pancreatic insufficiency and to stage chronic pancreatitis in the M-ANNHEIM classification. On the contrary, EF-1 determination shows low sensitivity in detecting exocrine pancreatic insufficiency in early stage of the disease.

Key words: Chronic pancreatitis; Exocrine pancreatic insufficiency; Fecal elastase-1; Pancreatic function tests; Steathorrea

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Core tip: Classification of chronic pancreatitis is useful for planning adequate diagnosis and management of the disease, particularly in the early detection and prevention of related-complications. Recognition of pancreatic exocrine insufficiency is useful for graduating severity of chronic pancreatitis in modern classification systems, and fecal elastase determination appears the better method in term of simplicity and sensitivity to stage exocrine function of the pancreas. However, sensitivity of elastase-1 is low in early stage of chronic pancreatitis, and new diagnostic tools or combination of different procedures are needed to better stage pancreatic function.

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INTRODUCTION

Chronic pancreatitis (CP) is the most commonly known cause of pancreatic exocrine insufficiency (PEI)[1]. Every patient with a new diagnosis of CP should be screened for PEI and in order to detect maldigestion prior to the occurrence of overt clinical symptoms, the presence of PEI should be evaluated annually in patients with CP. PEI can lead to poor quality of life, steathorrea, abdominal pain and malabsorption, and early diagnosis of PEI is important to prevent malnutrition-related complications. Many tests are nowadays available for the diagnosis of PEI, but each one has some diagnostic limitations. As a consequence, PEI is still underdiagnosed and undertreated. In addition to its clinical relevance, PEI represents an helpful method for staging CP in different classification systems. The M-ANNHEIM classification is a new system for staging and grading the severity of CP[2]. The M-ANNHEIM classification system is based on the categorization determined by etiological factors, clinical stage and severity of CP. This system constitutes a simple, objective, accurate and non-invasive method for clinicians which combines the influence and interaction of several risk factos on the course of the disease. These multiple (M) risk factors included the subsets of alcohol consumption (A), nicotin consumption (N), nutritional factors (N), hereditary factors (H), efferent pancreatic duct factors (E), immunological factors (I), and various rare miscellaneous and metabolic (M) factors[2]. The M-ANNHEIM staging of CP is divided into an asymptomatic phase (stage 0) and a symptomatic phase (stages I, II, III, IV) of the disease[2]. The latter phase represents the period of clinically evident chronic inflammation of he pancreas. The evaluation of the symptomatic phases of CP is based on pain and on the presence and degree of PEI[2]. An accurate evaluation of PEI is therefore necessary to validate the diagnosis and allow the proper treatment of the disease. In fact, a correct diagnosis of PEI suggests the pancreatic enzyme replacement therapy (PERT) and it is essential to monitor the efficacy of treatment in order to avoid complications of CP. Only few studies in the literature have compared the different diagnostic power of the tests available to evaluate PEI, and only few data are available for determining their role in the present staging system of CP.

STUDY ANALYSIS

In the recent issue of the *World Journal of Gastroenterology*, Kamath *et al*[3] reported a prospective analysis comparing two tests for PEI to use in M-ANNHEIM staging for pancreatitis. In this study, 116 patients with CP were included. PEI was analyzed by faecal elastase-1 (FE-1) value and fecal fat excretion by the acid steatocrit method. Based on the results of the two tests, the patients were separately categorized as per M-ANNHEIM stages. Among the 116 patients with CP, the presence of PEI was evident in 61 (52.5%) and 79 (68.1%) by the acid steatocrit method and FE-1, respectively. A statistically significant difference was seen between the M-ANNHEIM stages as classified separately by acid steatocrit and the FE-1. The Authors concluded that FE-1 estimation permits better staging of pancreatitis by the M-ANNHEIM classification, since it diagnosed a higher number of patients with exocrine pancreatic insufficiency. They recommend the use of FE-1 test for staging CP by the M-ANNHEIM classification. The study of Kamath *et al*[3] is interesting because it deals with the assessment of PEI, which still remains a diagnostic challenge in patients affected by CP.

The ideal test for the diagnosis of PEI should be accurate, non-invasive, widely available and easy to perform. In our Center, in twenty years of experience, we observed 325 patients with a diagnosis of CP, and among them 253 received surgery[4,5]. In this period, different tests were used to diagnose PEI (fecal fat excretion, p-aminobenzoic acid test, coefficient of fat absorption, fecal chymotrypsin)[4-6], and in more recent years they have been replaced by FE-1 test. Nowadays, two different groups of tests (direct and indirect tests) are available for the diagnosis of PEI[1] (Table 1). Among direct tests, the most sensitive method is derived from the aspiration of the pancreatic secretions during secretin-cholecystokinin/cerulein administration[7]. However, this test is invasive and it is available only in few specialized centers[7]. Among indirect test, the coefficient of fat absorption (CFA), the fecal-elastase-1 determination and the acid steatocrit test are the most frequently used[7]. FDA have approved treatments for PEI based on randomized

Table 1 Advantages and pitfalls of function tests for pancreatic exocrine insufficiency

Test	Advantages	Pitfalls
Aspiration of pancreatic contents (during secretin-cholecystokinin/cerulein administration)	High sensitivity	Invasive; only available in specialized centers
CFA	Gold standard; useful in monitoring PERT	Need of a strict diet; unpleasant and long stool collection; no simultaneous PERT
FE-1	Easy test; widely available; no need to stop PERT	Low sensitivity in mild PEI; not clear cut-off
Acid steatocrit	Good correlation with CFA	Lack of standardization; Influenced by dietary fat intake
^{13}C-mixed Triglyceride Breath Test	Good sensitivity in detecting mild to moderate PEI; useful in monitoring PERT	Only available in specialized center; false positive results in non pancreatic fat malabsorpion

CFA: Coefficient off at absorption; PERT: Pancreatic enzyme supplementation therapy; FE-1: Fecal elastase-1; PEI: Pancreatic exocrine insufficiency.

controlled trials that used CFA to define PEI[8]. The CFA is also useful to monitor pancreatic enzyme replacement therapy (PERT). The CFA requires patients to maintain a strict diet containing 100 g of fat per day over five days, and to collect the total amount of stools excreted over the last three days[9]. A CFA < 93% is considered pathological[9]. However, this test is not easy to perform, it is difficult to control the amount of fat consumed, especially in alcoholic patients, and collection of faeces is unpleasant and cumbersome for patients. Fecal Elastase-1 (FE-1) is an indirect assessment of the pancreatic secretion. This test is easy to perform, widely available and only requires a small stool sample for analysis[10]. However, FE-1 test is not able to exclude mild to moderate PEI, and there is no consensus concerning the ideal cut-off for PEI in patients with CP: figures of < 15, 50, 100 and 200 mg/g have been proposed, and a threshold of 200 mg/g has been used most frequently in accordance with the intended use label of the test[11]. The acid steatocrit method is a quantitative measurement of fat expressed as a proportion of an entire centrifugated homogenized stool sample; it correlates well with the 72 h quantitative faecal fat estimation[12]. However, the acid steatocrit method has some disadvantages which include a lack of standardization of the test and the possible effect of dietary fat intake during the sample collection[12].

Other tests are currently available. The 13 C-Mixed Triglyceride Breath test (TGBT) is a valid alternative to the CFA, both for the diagnosis of PEI and for evaluating the efficacy of PERT in clinical practice[13]. Modifications of the test may allow the detection of mild to moderate PEI[13]. However, the test also has limitations in terms of specificity (false positive results in non-pancreatic fat malabsorption), it is not easily available since it is commercialized only in few European countries. Pancreatic secretion volume can be evaluated semiquantitatively by secretin enhanced-MRCP (s-MRCP)[14]. Pancreatic secretion evaluated by this technique correlates with FE-1 test results; however, its sensitivity for PEI is as low as 69%[14]. In addition, there is very limited evidence supporting this technique for the diagnosis of PEI in clinical practice.

In conclusion, many tests are available for the diagnosis and evaluation of PEI in CP, but every test has some limits and pitfalls. In particular, a diagnostic, non-invasive method to differentiate mild and moderate PEI is still required. Further studies are needed to identify the ideal method in the diagnostic setting of PEI.

PERSPECTIVE

Accurate staging of pancreatitis is crucial to study both the natural history of the disease and the effect of treatment. In the study by Kalmath *et al*[3], acid steatocrit and FE1 were compared, while other standard tests like CFA or the 13 C-Mixed Triglyceride Breath test were not considered. It would be interesting in future studies to investigate the comparative usefulness of these tests comprehensively. Moreover, it is reasonable to believe that the additional use of biomarkers could improve the staging systems and this aspect should be explored in future studies. Recently, it has been reported that serum monocyte chemoattractant protein-1 levels were lower in patients with CP and PEI as compared to patients with CP without PEI[15]. A panel of 6 serum miRNA has been recently suggested as potential useful investigation for diagnosis of CP, especially for the early diagnosis of CP[16]. Future studies concerning the association of tests, such as pancreatic function tests, biomarkers or radiological-endoscopic imaging, could be helpful for the early detection of CP.

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MINIREVIEWS

How to perform gastrointestinal ultrasound: Anatomy and normal findings

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Abstract

Gastrointestinal ultrasound is a practical, safe, cheap and reproducible diagnostic tool in inflammatory

bowel disease gaining global prominence amongst clinicians. Understanding the embryological processes of the intestinal tract assists in the interpretation of abnormal sonographic findings. In general terms, the examination principally comprises interrogation of the colon, mesentery and small intestine using both low-frequency and high-frequency probes. Interpretation of findings on GIUS includes assessment of bowel wall thickness, symmetry of this thickness, evidence of transmural changes, assessment of vascularity using Doppler imaging and assessment of other specific features including lymph nodes, mesentery and luminal motility. In addition to B-mode imaging, transperineal ultrasonography, elastography and contrast-enhanced ultrasonography are useful adjuncts. This supplement expands upon these features in more depth.

Key words: Ultrasound; Intestinal; Inflammatory bowel disease; Guidelines; Teaching

Core tip: In general terms, gastrointestinal ultrasound examination principally comprises interrogation of the colon, mesentery and small intestine using both low-frequency and high-frequency probes. In addition to B-mode imaging, transperineal ultrasonography, elastography and contrast-enhanced ultrasonography are useful adjuncts.

Atkinson NSS, Bryant RV, Dong Y, Maaser C, Kucharzik T, Maconi G, Asthana AK, Blaivas M, Goudie A, Gilja OH, Nuernberg D, Schreiber-Dietrich D, Dietrich CF. How to perform gastrointestinal ultrasound: Anatomy and normal findings. *World J Gastroenterol* 2017; 23(38): 6931-6941 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/6931.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.6931

INTRODUCTION

Gastrointestinal ultrasound (GIUS) is an accurate diagnostic imaging tool for inflammatory bowel disease[1-3]. Utilisation has steadily increased in different global regions including Asia-Pacific[4]. To correctly interpret GIUS findings, it is necessary to have a firm grounding in intestinal anatomy, the fundamentals of ultrasonography, as well as the examination techniques and approach. The indications for intestinal ultrasound are wide-ranging including inflammatory bowel disease, assessment of functional aspects and general gastroenterological conditions such as diverticular disease. Various educational theories are relevant in the process of learning intestinal ultrasonography including the learning process itself, using formative assessments such as DOPS (direct observation of procedural skills), adopting the apprenticeship or core competencies model and formulation of a GIUS curriculum. These principles and a process of learning GIUS have been recently proposed[1].

The purpose of this paper is to review our knowledge of intestinal embryology relevant to GIUS. Understanding the origins and evolution of abdominal structures during the embryological process can assist in highlighting the reasoning behind abnormalities found on GIUS. We then expand upon examination techniques relevant to different segments of and structures around the intestine, including an overview of transperineal ultrasonography. We describe specific intestinal luminal parameters to be assessed in GIUS including special techniques such as contrast-enhanced ultrasound.

REVIEW OF INTESTINAL EMBRYOLOGY

In the 6th and 8th week of intrauterine life, the primitive mid-gut intestinal tube elongates on the mesentery around the superior mesenteric artery (SMA), herniating into the umbilical cord. As the gut grows and returns into the peritoneal cavity, it eventually rotates 270 degrees counter-clockwise, such that the duodenum rests behind the SMA[1]. The caecum, initially in the upper abdomen, descends to the right lower quadrant. Thus the mesentery attachment of the small bowel takes an oblique course from the duodeno-jejunal junction at the level of the left L2 process, over the 3rd part of the duodenum, down to the level of the right sacroiliac joint[5]. Though the mesentery attachment is only 15-20 cm long, it supplies a length of small intestine approximately 40 times its length, a feat achieved through progressive fan-like ruffles. Seen with traditional barium enterography, each curve of the intestine has a concave and convex aspect, the concave generally pointing towards the mesentery whilst the convex aspect representing the anti-mesenteric border[6]. These can be viewed in real time with GIUS.

Towards the end of the first trimester, the peritoneum of the newly forming ascending colon and the hind-gut derived left colonic segments, begin to fuse with the posterior abdominal wall. Although traditionally described as retroperitoneal structures, modern post-mortem studies have found that two thirds of the ascending and a third of the descending colonic segments have mobile portions of elongated mesentery[7]. Nonetheless, peritoneal attachments have significance for the flow of free fluid within the abdomen as fluid tends to flow caudally, medial to the ascending colon towards the ileocaecal junction and thus metastatic deposits may become lodged in the mesenteric ruffles en route.

Relative thickenings of the mesocolon provide ligamental support to the colonic flexures; the nephrocolic ligament runs from the inferior aspect of the right kidney to the hepatic flexure which then becomes intimately related to the descending duodenum before the transverse mesocolon begins; the splenic flexure is suspended by (1) the phrenicocolic ligament which runs from the diaphragm and also supports the spleen; (2) the splenocolic; and (3) the pancreaticocolic ligaments which are essentially extensions of the transverse

mesocolon[5,7]. These attachments provide fixed points for ultrasound evaluation of the colon, which can at times, be highly mobile within the abdomen.

The taenia coli, thickenings of the longitudinal muscle layer grow from diffuse sheets at the caudal end of the bowel, become more defined in the proximal colon until they encase the caecum[6]. The taeniae are one sixth shorter than the colon[5], forming the haustrae. The muscle fibres in the longitudinal layer end by turning at right angles to merge with the circular fibres and thereby acting as fixed linkage points for contraction. Contractions can occur asymmetrically obliterating some haustrae, giving the false impression of small-bowel-like semi-circular folds and bowel wall thickening.

Vascular supply of the colon flows from the mesenteric border, vessels spreading around the colon. The vasa recta penetrate through oblique connective tissue clefts in the bowel wall, the site of diverticular protrusion, but importantly enter these clefts on the anti-mesenteric aspect. In practice, diverticula are rare on the anti-mesenteric border between the taenia omonetalis and taenia libera. A vasa recta vessel runs over the long aspect of each diverticulum before entering the submucosa at the antimesenteric border[6].

EXAMINATION TECHNIQUE

General considerations and examination technique

An optimal environment for United States is within a dedicated space or consulting room, offering indirect low light sources and facilitating patient comfort. Prior cross-sectional imaging and endoscopy reports should be available to inform of post-surgical and anatomical variants.

In most scenarios patient preparation is not required but specific measures can be used. Fasting for 4-6 h decreases bowel motility whilst two cups of water can be used to improve visualisation of the duodenum[8]. Negative oral contrast may improve detection of jejunal and proximal ileal stenosis, particularly where examination findings are negative; 250-800 mL of polyethylene glycol solution generally reaches the terminal ileum after an average duration of 30 min[9]. Once contrast is seen to flow into the caecum, retrograde examination of the small bowel can be performed.

Examination of the intestinal tract begins with a comfortable patient, relaxed in a supine position so as not to tense the abdominal wall. The transducer is held maintaining contact with the patient's skin to gauge pressure, whilst the left hand is free to optimize image characteristics on the machine. A systematic approach in examining the whole intestine is encouraged (Figure 1). Firstly, the low frequency 3-8 MHz (multifrequency) curvilinear probe initially allows orientation to the anatomy and detection of gross changes, whilst high frequency linear probes (7-17 MHz) are preferable for interrogating specific regions of interest in depth.

Colonic examination

Beginning at the right anterior superior iliac crest and moving medially to the edge of the rectus muscles in a sagittal plane, the common iliac (iliacal) vessels are identified. Rotating anticlockwise to a transverse plane and moving cranially, the first bowel loop crossing from medial to lateral is identified as the terminal ileum. This is followed to the ileocaecal (Bauhin's) valve and caecum. The base of the appendix can be identified at the deep margin of the caecum where the colonic taenia meet before the ascending colon is followed up towards the hepatic flexure. The rest of the colon can be followed *via* the transverse segment distally towards the rectum. Alternatively, the same technique can be used on the left side identifying the sigmoid colon as the first loop of bowel crossing the left iliac vessels, which can be followed to the descending colon and towards the spleen as far as the rib margin allows. The iliopsoas muscle can be used as an alternative landmark for identification of the terminal ileum and sigmoid colonic segments in the right and left iliac fossae respectively. Intercostal imaging may be required to visualise the left or splenic flexure where it has attachment to the spleen; elevating the left arm and rotating to a partial right decubitus position with a straight left leg can spread the ribs and improve image acquisition (Figure 1C). Placing the probe in the epigastric region in sagittal orientation demonstrates the liver and stomach; one can then follow the gastrocolic ligament to the transverse colon. Although the transverse colon can be followed on ultrasound, it may not be reliably viewed in its entirety. Be aware that the rectum and distal parts of the colon cannot always be displayed satisfactorily by transabdominal United States. Transperineal imaging, in such cases, can be useful to evaluate the distal rectum and perianal tissues.

Mesentery and small intestine examination

Mesenteric fat is evident sonographically and is considered to be abnormal if it extends over more than half the circumference of the bowel loop, if it is thickened beyond 5-6 mm or consistently greater than the normal bowel wall thickness[10].

Examination of the mesentery begins in the epigastrium at the duodenojejunal flexure which then runs obliquely towards the right iliac fossa. To aid visualization, the patient is asked to breath in deeply and as they exhale, pressure is applied to the transducer following which excellent views of the small bowel mesentery sheets and abnormalities can be achieved. A systematic scanning of the small bowel may start in the right iliac fossa by defining the terminal ileum and following its course in a proximal direction as far as possible. Finally, a systematic overlapping "Lawn Mowing" strategy is used, sweeping up and down the abdomen to provide an overview of the small bowel. This is performed with the probe in horizontal,

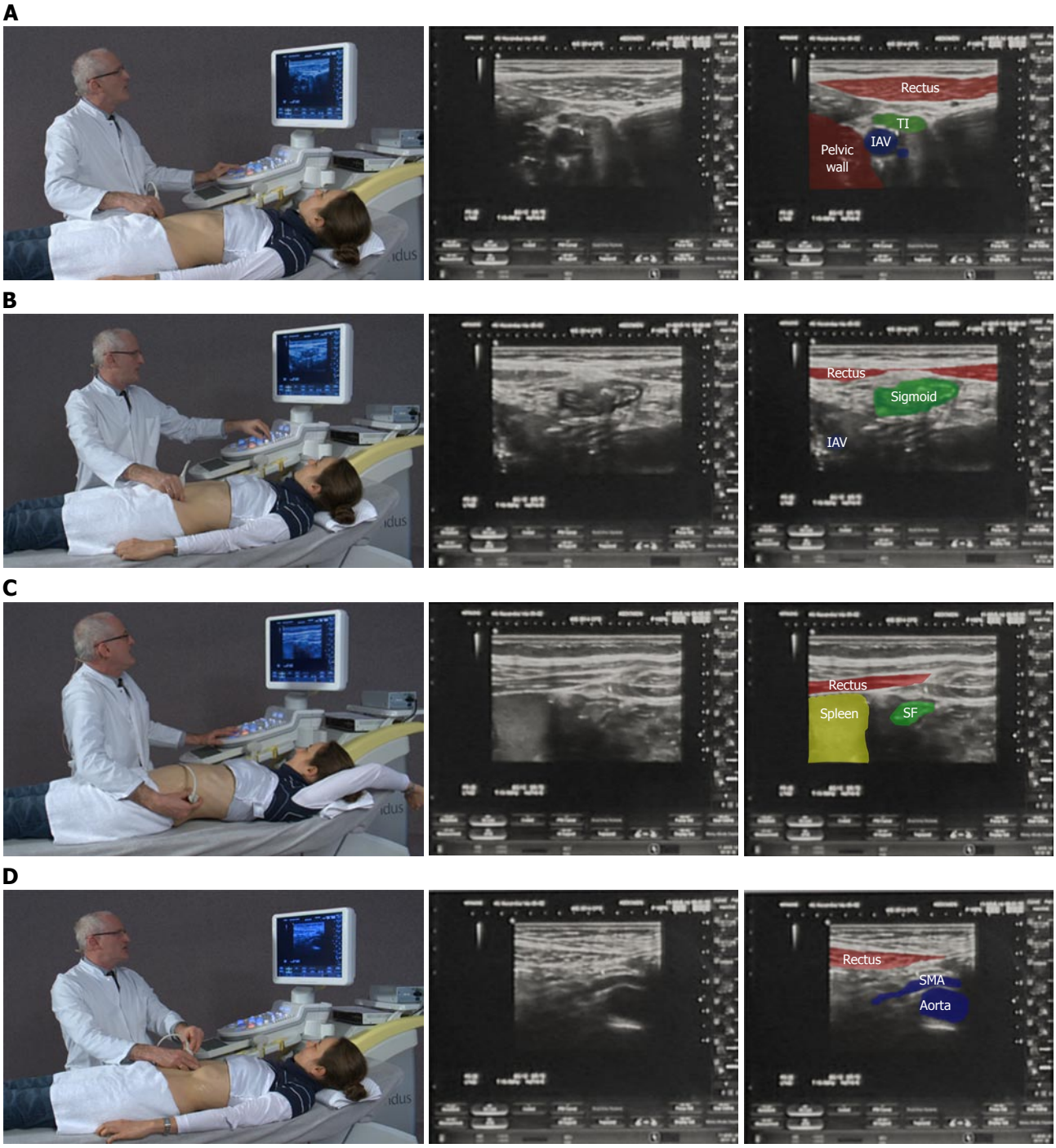


Figure 1 A systematic approach in examining the whole intestine. A: Examination begins in a relaxed ventral position; B: Beginning medial to the right anterior superior iliac spine, the iliacal vessels (IAV) are identified and the first bowel loop crossing medial-to-lateral is the terminal ileum (TI). The same technique on the right identifies the sigmoid colon; C: Elevating the arm spreads the rib spaces to improve visualisation of the splenic flexure (SF); D: Gentle pressure as the patient breaths out improves visualization of the mesentery and superior mesenteric artery (SMA) to exclude lymphadenopathy. The videos can be accessed *via* the efsumb website [www.efsumb.org/education/cfd-videos001.asp].

sagittal and oblique (parallel to the mesenteric attachment) orientations in order to allow one's eye to follow structures and detect abnormalities. A full video explanation of abdominal and intestinal ultrasound examination technique is freely available on the EFSUMB website[11] (www.efsumb.org/education/cfd-videos001.asp).

Perineal ultrasound

TPUS allows visualisation of the perianal tissues, anal canal, sphincters, the distal 5-7 cm section of the rectum, vagina and a part of urinary bladder. A point for orientation is the symphysis. Knowledge of the pelvic anatomy is essential[12,13] (EFSUMB Case of the Month). No specific patient preparation is required.

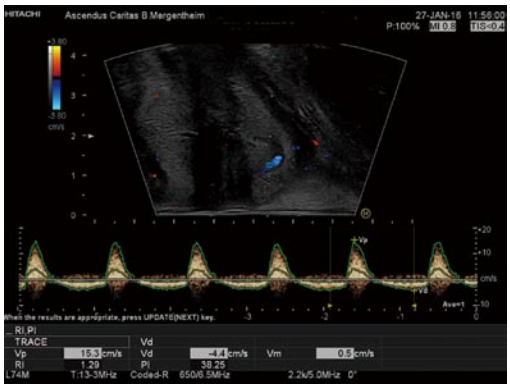


Figure 2 Example on the use of color doppler imaging and continous duplex scanning. Perineal ultrasound showing the hemorrhoidal pleaxus using color doppler imaging and continous duplex scanning with the typical spectrum of the hemorrhoids.

The patient is placed in the left lateral position as for a digital rectal examination. The probe is covered in either a sterile cover or an examination glove with ultrasound gel between the layers.

Examination begins in the midline just above the anus with the probe in a sagittal plane. The ultrasound probe can be moved laterally, however angulated views have reduced sensitivity for identification of pathology. Fistulous tracts can be followed by first placing the probe over the external opening. If necessary, the probe can be placed in a coronal angle, although this is usually less comfortable for the patient. It is useful to start with an abdominal convex probe (lower frequency) for the deeper structures and then continue with a higher frequency probe (7-15 MHz). Examination is also possible after rectal amputation (*e.g.*, Quénu-Operation).

The anal canal, sphincter complexes, hemorrhoidal plexus (Figure 2), recto-vaginal plus ano-vaginal septums, the walls of the vagina and distal rectum can be defined. Fistulae should be classified as per Parks' classification[14] although TPUS has reduced sensitivities for sphincteric relationships and therefore the American Gastroenterological Association (AGA)[15] distinction of "simple" from "complex" is of more clinical utility; the former including low fistulae (superficial, intersphincteric or intrasphincteric) below the dentate line, with a single external opening and without perianal complications or active proctitis[16]. Fistula and abscesses visibility can be improved with ultrasound contrast agents (UCA) using contrast enhanced ultrasound techniques[17,18]. Colour Doppler improves the differentiation of inflammatory reactions.

INTESTINAL LUMINAL FEATURES

Bowel wall thickness

Bowel wall thickness (BWT) is the measure most consistently reported in diagnostic and activity trials. Wall thickness of the alimentary tract differs by region and depends on the degree of distension and contraction[19,20]. The overall thickness should be measured under mild compression from just above an air-mucosal interface to the outside of the outer muscularis propria layer border, including the whole bowel wall[21]. Under these standardised conditions, the stomach wall thickness measures 3-6 mm; terminal ileum 1-3 mm; and colon 0.5-2 mm. In fact, the normal range is likely to be even lower than this[22]. Values in children can be reliably obtained without the need for sedation[22] but bear in mind that values do increase over childhood[23] whilst still remaining < 2 mm. The optimal threshold for abnormal thickness is debatable, as specificity improves with increasing wall thickness at the cost of sensitivity (Figures 3 and 4).

Bowel wall layers

The GI wall has five layers that usually can be visualized with ultrasound. The sonographic layers are a combination of interface echoes and the echo characteristics of the histological layers[24-26]. When imaged in the anterior wall of a bowel loop starting from the lumen the hyperechoic layer 1 corresponds to the interface between the mucosa and the lumen and is not a part of the actual GI wall. The hypoechoic layer 2 corresponds to the mucosa without the interface between the submucosa and mucosa, the hyperechoic layer 3 to the submucosa including this interface echo, the hypoechoic layer 4 to most of the proper muscle and layer 5 to the hyperechoic interface echo between the proper muscle and the serosa.

Interface echoes are always hyperechoic and located distally to the actual tissue interface. Therefore, the correspondence between histology and sonographic layers differ slightly in the dorsal wall. Specifically, the interface between lumen and mucosa (layer 1) is a part of the actual mucosa and layer 2 represents the rest of the mucosa without muscularis mucosae, which normally is covered by an interface echo and add thickness to layer 3. Moreover, the interface between submucosa and the proper muscle adds thickness to layer 3 and reduces the thickness of layer 4. Finally, the interface between the proper muscle and serosa (layer 5) extends beyond the actual serosa[27,28].

The interface from the serosa is hard to delineate. Accordingly, the measurement should be made from the start of the hypoechoic layer of the proper muscle to the end of the hypoechoic layer of the mucosa. Transducer-compression of the bowel wall will reduce thickness and can make it challenging to distinguish wall layers[29,30]. However, some operators practice mild compression suggesting that this improves reproducibility of measurements[21,22,31-33]. The examiner should also be aware of interpretation difficulties due to mucosal folds and haustrations and to keep the probe angle perpendicular to the bowel wall to avoid tangential measurements. In conclusion, dosed compression is a prerequisite for a reproducible examination for some authors whereas others use it with caution.

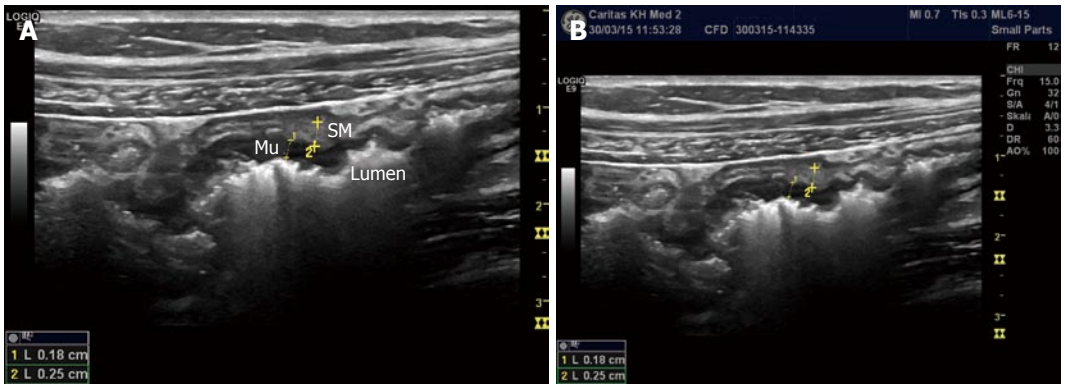


Figure 3 Measurement of the bowel wall. The measurements are best taken ventrally since posterior artefacts occur (A) and the measurements (B) are not reliable. Mu: Mucosa; SM: Submucosa.

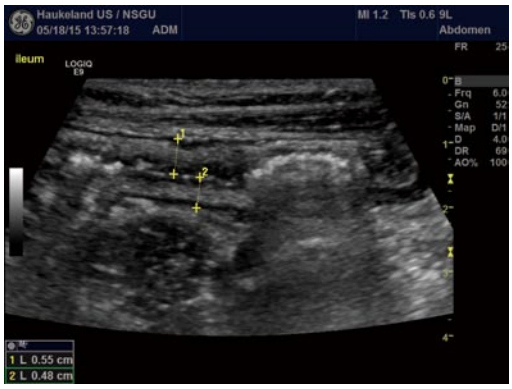


Figure 4 Measurement of the bowel wall. In a patient with Crohn's disease of the small intestine, ultrasound was applied to evaluate disease extension and wall thickness. B-mode image shows moderate wall thickening in the ileum with well-preserved layer structure. Be aware the marked thickening of the submucosal layer in white, often seen in IBD. The crosses mark the wall thickness in the anterior and posterior wall denoting a slight difference in thickening.

Table 1 Differential diagnosis of asymmetrical terminal ileal thickening with chronic symptoms

Crohn's disease
Actinomycoses
Mycobacteria tuberculosis
Lymphoma
Neoplasia
NSAID enteropathy

Table 2 Differential diagnosis of chronic inflammatory diseases of the bowel

Inflammatory bowel disease
Mycobacterium tuberculosis
Sarcoidosis
Diverticulitis
Neoplasia
Lymphoma
Ischemia

The layered wall structure changes with disease[34]. In severe disease the stratification may disappear due to deep mural ulcers, increasing inflammatory infiltrate and neovascularisation. In chronic inactive disease, accentuated wall layers are more common. The distinctions are less apparent with milder disease phenotypes.

Symmetry

The symmetrical nature of changes is relevant. Asymmetry has been assumed to correspond to endoscopic signs of focal ulceration or polypoid mucosal changes, whilst diffuse thickening is evident with ulcerative colitis (UC) or infectious colitis. The differential diagnoses for chronic inflammatory bowel findings, with or without asymmetry are listed in Tables 1 and 2.

Luminal diameter and motility

The small and large bowel can usually be distinguished by scanning the haustrae of the colon and/or the circular folds of Kerckring in the small intestine. In unclear cases, scanning of the intestine during various stages of filling may be helpful. Changes in Kerckring's folds and luminal fluid quantity can be associated with disease[35]. The small bowel diameter varies widely depending on recent meals and activity, but dilatation beyond 25 mm should be regarded as abnormal, particularly when motility is reduced[36]. Assessment of peristaltic activity and lumen compressibility are two advantages of ultrasound over other imaging modalities. Strictures may be identified by the co-existence of thickened and stiffened bowel wall with narrowing of the intestinal lumen, particularly if less than 10 mm[9]. The presence of proximal loop dilatation with fluid or echogenic content is not required for the diagnosis[36] but may carry clinical significance.

Dilatation of the proximal small bowel loops with hypo- or hyperperistalsis can be caused not only by chronic fibrotic strictures but also by acute inflammatory stenosis or passenger invagination. Functional ultrasonography is helpful in differentiation[37-40].

Extent of disease

Evaluating the length and extent of involved bowel segments is performed by estimated longitudinal

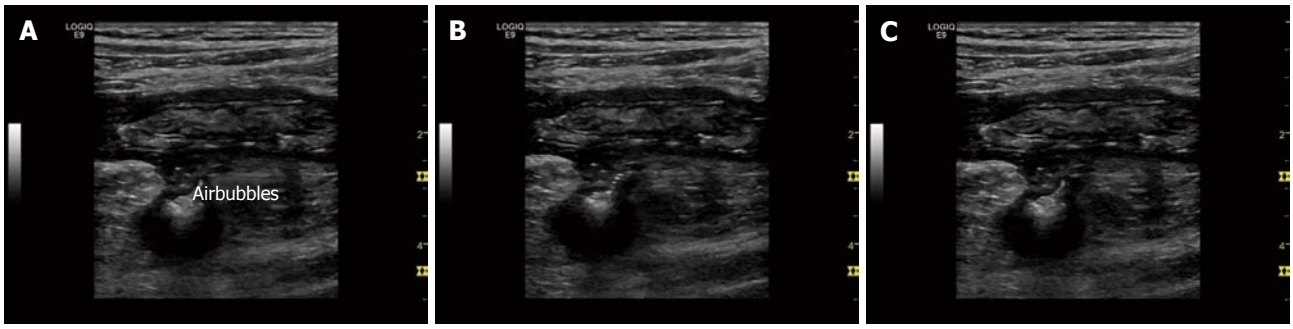


Figure 5 Typical complications in Crohn's disease, fistula. Typical ultrasound findings in Crohn's disease include transmural inflammation, fistula and abscess formation. A-C: The typical sign of fistula is hypoechoic transmural inflammation with (moving) air bubbles outside of the bowel lumen. The air bubbles are best visualised using a real-time examination or video. Here we demonstrate single images of a video to demonstrate the changes within one second.

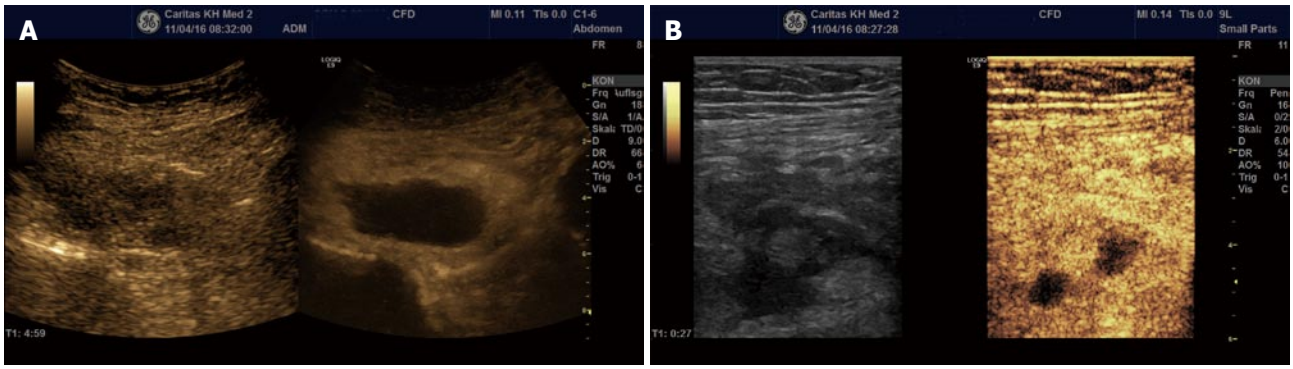


Figure 6 Typical complications in Crohn's disease, abscess. Typical ultrasound findings in Crohn's disease include transmural inflammation, fistula and abscess formation. Contrast enhanced ultrasound allows to better delineate larger (A) and smaller abscess formation (B) not clearly suspected using B mode ultrasound.

measurement and taking note of skip lesions. Bowel wall thickening, luminal diameter for stenosis or dilatation > 25 mm, and motility should be noted in each region. Chronic inflammation tends to produce an isolated, fixed segment without peristalsis and abnormal angulation due to the fibrofatty proliferation of the mesentery[41,42].

Transmural reactions

Transmural inflammation and fibrosis may result in an asymmetrically thickened, stratified bowel wall, which are the typical findings of Crohn's disease (CD). A hypoechoic extension through the normal bowel wall stratification correlates with cellular and oedematous tissue infiltration. Even though the extent of the fibro-fatty proliferate correlates with the degree of intestinal inflammation in CD, there is no standardized method to date to quantify the mesenteric fat by using GIUS. Note should be made if the reaction extends beyond the muscularis propria layer, and whether it does so into the mesenteric or anti-mesenteric border. Fistulae are identified as hypoechoic tracts extending through the bowel wall, often with reverberations (circumscribed bright air echoes) within them (Figure 5). Rounded hypoechoic areas (non-contrast imaging) within the mesentry are suspicious for abscesses or inflammatory phlegmons often with an irregular wall or internal echoes (Figure 6). The occurrence of free peritoneal fluid is important to note, though clinical data corroborating its significance are scant[21].

Fistulae and abscesses

Two prospective studies suggest a sensitivity and specificity for the GIUS detection of fistulae of 72%-87% and 90%-96%, respectively[43]. This performance is equivalent to CT/MR studies in meta-analysis[44], whilst small intestine contrast ultrasound (SICUS) may have a sensitivity as high as 96%. Estimates of the sensitivity and specificity for detecting abscesses have been reported in a somewhat higher range; 71%-100% and 77%-94% respectively[21,45-49]. The direct application of contrast agents into the orifice of the fistula may be helpful in determining the route and connection(s)[12,13,50].

SECONDARY MESENTERIC FEATURES

Mesenteric lymph nodes

Lymph node enlargement is a frequent sonographic finding in CD[10], however their interpretation and clinical implications remain to be further clarified in the literature. It has been suggested that they may represent a very early manifestation of CD in children for example[22]. They are correlated with duration of disease and the presence of fistulae but more importantly, for the ultrasound learner, they provide a

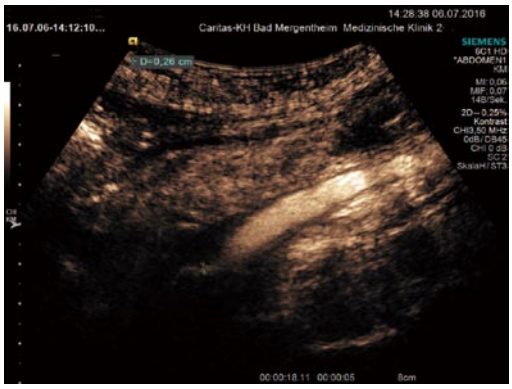


Figure 7 Complications of inflammatory bowel disease. Thrombosis of the superior mesenteric vein. Partial recanalisation is shown by the markers.

marker of procedural competence and interpretation. B mode characteristics of lymph nodes to consider include their length and particularly for those < 15 mm; their short axis dimension should be less than half their longitudinal diameter. Furthermore, the normal lymph node architecture and hilum should be preserved in normal or inflammatory nodes[51].

Mesenteric inflammation, oedema and vascularity

The supporting structures of the intestine run within the sheets of mesentery, seen as layers of mixed echogenicity with hyperechoic serosal layers on either side, which does not have peristaltic movement and appears similar in both transverse and sagittal planes. Fat wrapping has long been recognised by surgeons as a common and specific feature of CD. So-called (creeping) fat, extending from the mesenteric attachment to partially cover the small or large intestine resulting in loss of bowel mesentery angle, is seen as an early event in the disease course and plausibly plays a role in the inflammatory milieu[52]. In practical terms the serosal planes on either side of the mesentery may be detected and should cover less than half of the bowel circumference. It is also the most common cause of bowel loop separation[41]. A subjective impression of increased thickening and echogenicity has been applied in the literature[10], correlating with clinical severity and primary luminal findings; although in long standing disease it can become more heterogeneous and hypoechoic[41].

TECHNIQUES FOR INTERROGATION OF FEATURES OF INTEREST

Doppler imaging of tissue and SMA parameters

Colour Doppler imaging of the bowel wall is part of standard assessment of the intestine and mesenterial vessels (Figure 7). Hyperaemia is associated with inflammation, usually seen in the submucosal layer and the penetrating vessels of the muscularis propria. Use of Doppler evaluation increases the sensitivity of US for evaluating disease activity[33,39,53-59]. The degree of vascularity can be graded by the Limberg scale, a semi-quantitative assessment[60] that lacks routine practical relevance. Other more complex quantitative measurements of Doppler parameters have been proposed, however a standardised protocol to compensate for confounders has not yet become widely used. Power Doppler assessment of the arterial inflow, in particular the inferior mesenteric artery (IMA) for left sided colonic disease and SMA for proximal colon and small bowel activity, can be assessed in the majority of patients and correlates with other ultrasound markers of disease activity. It should therefore be interpreted in the overall context of ultrasound findings[61]. A prognostic role for Doppler parameters was previously proposed[62] but awaits further study and validation.

Contrast-enhanced US

Second generation contrast agents such as SonoVue®, produce harmonic frequencies from micro-bubbles approximately the size of a single red blood cell, and are stable within the circulation[17,63]. Imaging systems thereafter allow visualisation of individual blood vessels through a tissue and thereby improve the accuracy of Doppler US in evaluating bowel wall vascularity. This technique has been shown to be useful in the assessment of disease activity in CD, in particular differentiating inflammatory masses from abscesses and may help to distinguish inflammatory from fibrotic strictures in certain situations[64-66]. Use of Contrast-Enhanced US (CEUS) during GIUS has been standardized, does not requires specific expertise, and its use in IBD presently is increasing[67].

Elastography

Similar to palpation, the elastic properties of a tissue can be evaluated by assessing the speed of a sheer wave through tissue or the amount of deformity created by the sheer stress (strain imaging). Various sonographic approaches to generating and measuring these parameters are available[68], which may compliment standard B-mode assessment of a lesion. Fibrotic lesions may appear stiff and inflammatory lesions soft using elastography, which can help to characterise intestinal lesions and has been correlated with endoscopic findings (Figure 8)[69,70].

CONCLUSION

Understanding the anatomy and embryology of the intestinal tract is highly relevant in identifying sonographic abnormalities relevant to GIUS. The general principles of examination involve specific interrogation of the colon, small intestine and mesentery. Further work is required to validate and understand the significance of certain sonographic parameters where understanding is limited; this includes further evaluation of abnormal wall thicknesses and quantifying this to a higher degree of accuracy, understanding the significance of peritoneal fluid present as a reflection of transmural reactions,

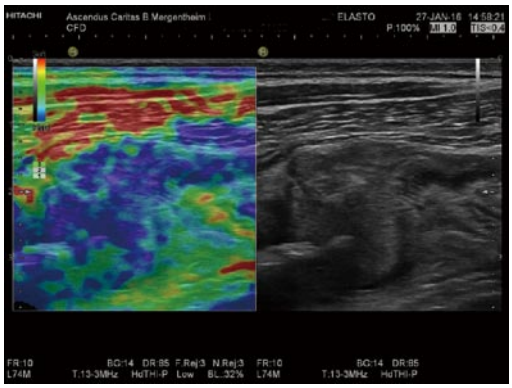


Figure 8 The evaluation of bowel wall stiffness. Elastography is helpful to determine stiff tissue as shown in this patient with colorectal carcinoma and infiltration of the abdominal wall.

and the accurate interpretation and implications of lymph nodes. Despite the presence of semi-quantitative measures, such as the Limberg score, they lack practical relevance and so there is a need for further multi-centre prospective studies.

Various sonographic abnormalities can be detected and interpreted currently but a standardized scoring system for GIUS in inflammatory bowel disease, akin to validated endoscopic scores (such as the Ulcerative Colitis Endoscopic Index of Severity) is lacking. Ultimately, formulating a reproducible and validated scoring system integrating different sonographic parameters to reflect severity will be highly relevant; this will require agreement amongst GIUS experts and validation in multi-centre prospective studies. Finally, a standardized method of documentation, including how to capture images, needs to be developed.

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MINIREVIEWS

Dysphagia: Thinking outside the box

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Abstract

Dysphagia is a common symptom that is important to recognise and appropriately manage, given that causes include life threatening oesophageal neoplasia, oropharyngeal dysfunction, the risk of aspiration, as well as chronic disabling gastroesophageal reflux (GORD). The predominant causes of dysphagia varies between cohorts depending on the interplay between genetic predisposition and environmental risk factors, and is changing with time. Currently in white Caucasian societies adopting a western lifestyle, obesity is common and thus associated gastroesophageal reflux disease is increasingly diagnosed. Similarly, food allergies are increasing in the west, and eosinophilic oesophagitis is increasingly found as a cause. Other regions where cigarette smoking is still prevalent, or where access to medical care and antisecretory agents such as proton pump inhibitors are less available, benign oesophageal peptic strictures, Barrett's oesophagus, adeno- as well as squamous cell carcinoma are endemic. The evaluation should consider the severity of symptoms, as well as the pre-test probability of a given condition. In young white Caucasian males who are atopic or describe heartburn, eosinophilic esophagitis and gastroesophageal reflux disease will predominate and a proton pump inhibitor could be commenced prior to further investigation. Upper gastrointestinal endoscopy remains a valid first line investigation for patients with suspected oesophageal dysphagia. Barium swallow is particularly useful for oropharyngeal dysphagia, and oesophageal manometry mandatory to diagnose motility disorders.

Key words: Dysphagia; Oesophagus; Eosinophilic; Manometry; Gastroesophageal reflux; Aspiration; Food bolus impaction

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Core tip: Dysphagia may represent serious and life-threatening pathology such as oesophageal neoplasia or oropharyngeal dysfunction capable of causing aspiration. In young white Caucasian males, eosinophilic esophagitis and gastroesophageal reflux

disease are predominant causes, and a trial of proton pump inhibitor is suggested whilst awaiting endoscopy.

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INTRODUCTION

Dysphagia is a common symptom in patients presenting to both generalist and specialist medical practitioners[1,2]. The key initial consideration for clinicians is to rule out life-threatening pathology such as neoplasia, and to identify patients who are at risk of aspiration such that immediate intervention can occur[3]. Dysphagia is most commonly due to chronic benign disorders, although it can be associated with appreciable morbidity and impaired quality of life (QOL)[4]. Gastroesophageal reflux disease (GORD) is the major cause in younger individuals, whilst oropharyngeal dysphagia (OD) secondary to cerebrovascular disease is more frequent in the elderly[1,4]. Dysphagia as a symptom attributable to GORD in younger adults does not automatically mandate investigation, rather a trial of a proton pump inhibitor (PPI) and strict follow-up to ensure symptomatic resolution might be sufficient[5]. OD and oesophageal dysphagia can often be reliably differentiated symptomatically and might help reduce the risk of aspiration in OD[6]. A comprehensive awareness of the multitude of potential causes of dysphagia, including common medications and rheumatological conditions, is necessary to allow for efficient and thorough evaluation. Judicious investigation with the appropriate choice of tests in order to consider both common and unusual aetiologies is required; high quality endoscopy with care to take sufficient oesophageal biopsies even if mucosa looks normal [*e.g.*, eosinophilic oesophagitis (EoE)], high resolution oesophageal manometry (HRM) in suspected motility disturbance, barium swallow and where appropriate, cross-sectional imaging [*e.g.*, computed tomography (CT) scan]. The escalating frequency of GORD and EoE, and the increase in mean age (and associated conditions such as CVA) will ensure that dysphagia remains a common presentation in the future[7,8]. This review attempts to present a contemporary understanding and approach to patients with dysphagia.

EPIDEMIOLOGY

Dysphagia is the subjective awareness of an impairment in swallowing of saliva, liquid or solid food. The prevalence varies by age of cohort, method of data collection, and if the dysphagia is classified as acute or chronic[1,4,9]. A community survey of 1000 individuals in Sydney Australia, demonstrated a prevalence of dysphagia of 16% (defined as ever having the symptom), although few (1%) reported dysphagia as chronic or severe[4]. GORD, hypertension and psychological symptoms (anxiety and depression) were independently associated with dysphagia[4]. Subjectivity in symptom reporting and the contribution of GORD have emerged as important themes in contemporary studies of dysphagia[1]. A study of patients with GORD reported resolution of dysphagia with PPI[10]. General practice cohorts have found a frequency of dysphagia of up to 23%, thus reflecting the significant health care burden taken up by this patient group[2]. Self-reported dysphagia is often variably defined[11]; an isolated globus sensation can erroneously be considered a form of dysphagia.

In a large survey of an adult United States population (n = 7640, 3669 respondents), 17% of respondents reported "infrequent" (< 1 episode per week) and 3% "frequent" (> 1 episode per week) dysphagia[1]. On interrogation of these respondents' medical files, symptoms of heartburn and endoscopic diagnosis of GORD were significantly associated with both the "frequent" and "infrequent" cohorts[1]. Perhaps due to the limited sample size and old age of the respondents (mean 62 years), other typical conditions were not statistically associated with dysphagia. Primary oesophageal conditions (EoE, achalasia, cancer) and systemic conditions (scleroderma, rheumatoid arthritis) were only reported amongst the "frequent" dysphagia group[1]. Therefore, clinically significant (or frequent) dysphagia should alert the clinician to serious underlying pathology, whilst infrequent dysphagia is most likely to represent GORD and a trial of acid suppression may be worthwhile in the first instance provided there are no alarm symptoms. This view is supported by several experts in the field (see below).

AETIOLOGY AND PATHOGENESIS

Dysphagia is the subjective awareness of impairment in the passage of food from the oropharynx to the stomach, and therefore may signify an actual delay in bolus transit, or merely the sensation thereof[12]. It is useful to consider the anatomical structures implicated in swallowing (including innervation), and the location of pathology within (Figure 1)[10]. The process of swallowing, whereby food or liquid travels from the mouth to the stomach, involves a complex sequence of muscular contraction and relaxation, involving striated (oropharynx and upper 1/3rd of the oesophagus) and smooth muscle (lower 2/3rd of the oesophagus) that is controlled by motor neurons of the brainstem, and autonomic innervation (of the myenteric plexus) respectively[10,11]. The oropharyngeal component is

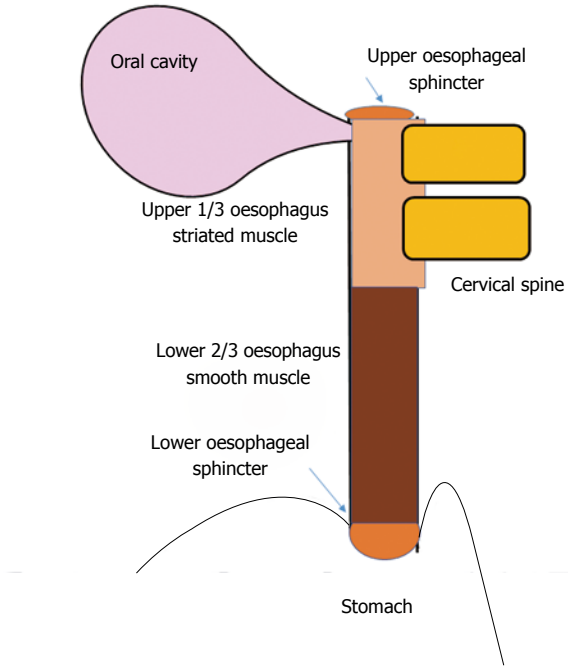


Figure 1 Anatomy of the oesophagus. Disease of the upper 1/3 of the oesophagus causing dysphagia may include extrinsic compression (*e.g.*, cervical osteophytes), or dysfunction secondary to rheumatological conditions (*e.g.*, Sjogrens's syndrome) or in eosinophilic oesophagitis (along with the lower oesophagus). The lower oesophagus can be afflicted in scleroderma, gastroesophageal reflux disease and in eosinophilic oesophagitis.

initially voluntary (including chewing). This is followed by an involuntary phase initiated by food entering the pharynx, whereby the swallowing reflex causes simultaneous relaxation and contraction of the soft palate, upper oesophageal sphincter, oesophagus and lower oesophageal sphincter[6].

Causes of dysphagia vary across the world, and there is evidence of a change in the relative proportion of the various disease states in the last few decades[8]. In the United States, benign oesophageal stricture has become less common, whilst EoE is increasing, along with oesophageal adenocarcinoma[8]. This pattern is mirrored in other Western nations[18]. Oesophageal squamous cell cancer remains a common condition in Asia, whilst Chagas disease still is prevalent is some regions of South America[18].

The oesophageal lumen can become narrowed by inflammation (*e.g.*, erosive reflux disease or EoE), neoplasia or fibrous stricture, or can be compressed externally by lymph nodes or degenerative disease of the cervical spine[8,12-15].

Luminal stenosis

Narrowing of the oesophageal lumen by inflammation, stricture, web or tumour can result in dysphagia[16]. Distal oesophageal inflammation from GORD can result in dysphagia due to transient decrement in peristaltic vigour, and if sustained can lead to fibrous stricture formation[8,15]. Barrett's oesophagus and/or sliding hiatus hernia (HH) are present in > 80% of those with chronic severe GORD and there is some evidence that both may independently influence oesophageal peristalsis[17,18]. Treatment of Barrett's oesophagus with endoscopic mucosal resection (EMR) and radiofrequency ablation can lead to fibrosis[19]. The Schatzki ring (a mucosal ring of the distal oesophagus), occurs just proximal to the gastroesophageal sphincter, often within a hiatus hernia, and is relatively common[20,21]. Barium swallow reveals Schatzki ring in 5%-15% of patients with HH[8]. The association with dysphagia and and its cause (congenital, secondary to GERD or even EoE) are debated[22]. Proximal oesophageal webs have been described in individuals with dysphagia. An association with iron deficiency anaemia (the Plummer-Vinson syndrome) was apparently common in the first half of last century, although is rarely reported today[23,24]. EoE is perhaps the most important, benign cause of dysphagia behind GORD. It is increasingly common and may be responsible for some cases previously diagnosed as fibrous strictures or webs[7]. This is an important consideration amongst Caucasian patients in western countries, and is readily treatable (see below)[8,25]. The prevalence of EoE is estimated at between 1 in 500 and 1 in 1000 adult Caucasian males[26]. The importance of EoE is redoubled by the frequent occurrence of food bolus obstruction, a complication that is associated with significant morbidity including the need for hospitalisation, upper gastrointestinal endoscopy (UGE) and (rarely but catastrophically) oesophageal perforation[27].

Non-obstructive gastro-oesophageal reflux disease

Many patients that describe dysphagia will have normal investigations including UGE and high-resolution manometry (HRM), suggesting that a dysfunction of the somatosensory as opposed to neuromuscular apparatus might be present[28,29]. A unifying hypothesis to explain this apparent dichotomy is that a minor disturbance in peristalsis (caused for example by GORD, or non-erosive reflux disease - NERD) may be below the threshold of detection even by sensitive HRM, yet be experienced by the patient as dysphagia. Indeed, many people will describe a resolution of symptoms with proton pump inhibition (PPI). Interventions aimed at modulating the somatosensory system have hence been advocated[28,29].

Primary motility disturbance

Achalasia is the prototypical condition classified as a primary (isolated to the oesophagus) disorder of oesophageal motility, yet is quite rare, with an estimated prevalence of 0.17% in individuals undergoing endoscopy[30]. Patients typically present with a chronic history of dysphagia for liquids and/or solids, and often with retrosternal discomfort, heartburn and weight loss[31]. The underlying pathophysiology is of an imbalance between excitatory

and inhibitory neurons of the myenteric plexus at the distal oesophageal sphincter, with a decrement in inhibitory innervation (nitrous oxide, or vasoactive intestinal peptide) leading to aperistalsis and failure of relaxation of the lower oesophageal sphincter during primary swallowing in classical (Type I) achalasia, pan-oesophageal pressurisation in early (Type II) achalasia or spasm of the oesophageal body (Type III achalasia), but manometry is required to accurately define subtype[32]. Current literature suggests that achalasia is an autoimmune condition associated with antibodies directed at the myenteric plexus and linked to HLA class II DR and DQ alleles[33]. Distal oesophageal spasm and "jackhammer" oesophagus (formerly, nutcracker oesophagus) are disorders of distal oesophageal hypercontractility which can present as dysphagia and/or chest pain and are defined with high resolution manometry as per the recent Chicago classification update (see below)[34-37]. These primary, major disorders of oesophageal motility, may be part of a disease spectrum with shared pathogenesis[38-40]. It is important to always consider secondary achalasia (pseudo-achalasia) that can result from neoplastic infiltration of the distal oesophagus, or be secondary to infection (Chagas disease, primarily occurring in South America)[41,42].

The advent of HRM and subsequent diagnostic algorithms has led to a reconsideration of minor disorders of peristalsis as a cause of dysphagia (defined by the Chicago Classification)[35]. Many patients presenting with dysphagia will have ineffective oesophageal motility in the distal oesophagus or fragmented peristalsis (defined by HRM and the Chicago Classification)[35]. Previously, it had been demonstrated that these abnormalities are frequently found in patients with GORD[43]. The symptom of dysphagia did not correlate with minor disorders or peristalsis on HRM[28,44]. An evolving consensus is arguably emerging that these "abnormalities" may lack clinical application and relevance[44].

Rheumatological conditions

To ensure a systematic evaluation occurs and that relevant pathology is not overlooked, an awareness of rheumatological and neurological conditions, medications that may cause oesophageal dysmotility, and causes of extrinsic anatomical compression is required[3,45-48]. Dysphagia is frequently reported by patients with Sjogren's syndrome, systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD) and rheumatoid arthritis, not to mention the more commonly recognised association with systemic sclerosis (as part of the CREST syndrome)[45] (Table 1). More than 1/3 of patients with Sjogren's syndrome report dysphagia, caused both by xerostomia and thus deficient lubrication of the food bolus, and by abnormal peristalsis demonstrable by oesophageal manometry (particularly in the upper 1/3 of the oesophagus)[45,49].

Table 1 Medications implicated in causing dysphagia

Medication	Putative mechanism
Antipsychotic, *e.g.*, olanzapine[63], clozapine[62]	Block dopamine receptors
Tricyclic antidepressant, *e.g.*, amitriptyline[56]	Anticholinergic, decreased saliva and impairment secondary peristalsis
Opioids[90]	Increase lower oesophageal contractility, oesophageal spasm
Iron supplements	Localised oesophagitis[58,59]
Potassium supplements	
NSAIDs	
Tetracyclines	
Macrolides	
bisphosphonates	
Calcium channel blockers	Smooth muscle relaxation (including lower oesophageal sphincter)[56]
Nitrates	
Alcohol	
Theophylline	

Similarly, patients with SLE and MCTD frequently describe dysphagia, and proximal oesophageal peristalsis is often weak or fragmented[45]. The cause of the abnormal motility in these conditions is unknown, although an inflammatory myopathy or neuropathy has been postulated[45]. Manometry was unable to correlate these symptoms with dysmotility[45]. In patients with rheumatoid arthritis, distal oesophageal peristalsis can be weak or fragmented; although life-threatening subluxation of the cervical spine needs to be considered[50]. Oesophageal dysmotility as a feature of the CREST syndrome is the best known and most studied of the rheumatological conditions, although dysphagia is often overshadowed by heartburn[51,52]. The pathophysiological process of fibrous replacement of the smooth muscle of the mid and lower oesophagus leads to incompetence of the lower oesophageal sphincter (LOS) and the clinical sequalae of GORD and dysphagia[53].

Extrinsic compression of the oesophagus can be a feature of osteoarthritis of the cervical spine, with (particularly) anterior osteophyte formation[13]. This is also occurs in a more florid fashion with diffuse idiopathic skeletal hyperostosis (DISH)[54]. Ironically, neurosurgical interventions for degenerative cervical spine disease (such as anterior discectomy) can result in dysphagia both in the short term (with post-operative swelling, or rarely because of damage to the cranial nerves) or long term (compression secondary to a cage implant, if placed)[48]. Other causes of extrinsic compression include mediastinal or hilar lymphadenopathy (*e.g.* due to haematological or disseminated solid organ neoplasia) and goitre[14,55].

Medications

Medications cause or contribute to dysphagia by influencing oesophageal peristalsis, xerostomia, as a secondary consequence of esophagitis ("pill oesophagitis"), or by causing GORD (Table

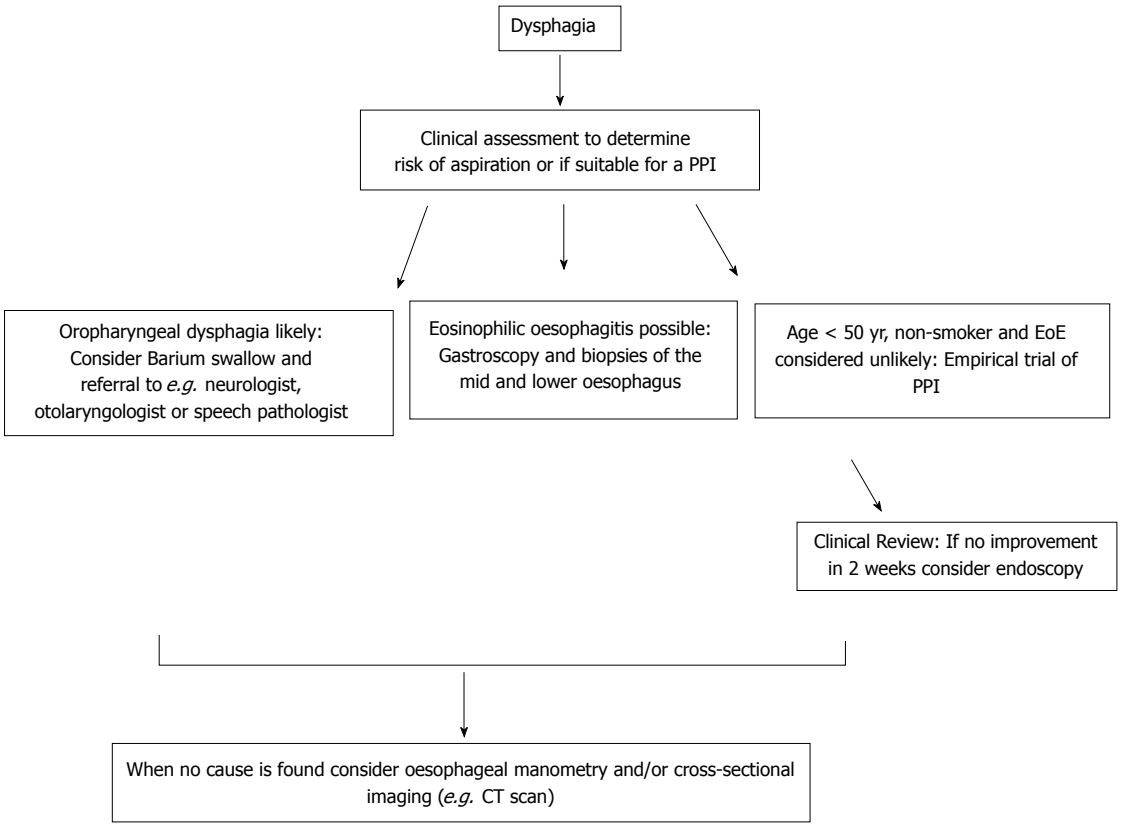


Figure 2 Proposed management algorithm for patients presenting with dysphagia.

Table 2 Neurogenic dysphagia - Common causes

Condition	Description
Parkinson's disease[68]	Oropharyngeal and oesophageal dysphagia is possible
Multiple sclerosis[64]	Oropharyngeal and oesophageal dysphagia is possible
Cerebrovascular disease[3,67]	Oropharyngeal dysphagia typical
Motor neuron disease[11,64]	Features of bulbar and pseudobulbar palsy possible
Myasthenia gravis[64,91]	
Myopathy (various, including inflammatory)[45,64]	Oropharyngeal and proximal oesophageal
Cerebellar pathology (various)[64]	

2)[47,56-59]. Sedative agents can lead to compromise of airway maintenance and thus aspiration is a risk. Finally, medications that have a local or systemic immunosuppressant effect can predispose to infective oesophagitis[60]. Among individuals with a psychiatric diagnosis, dysphagia may relate, at least in part, to the use of psychotropic medication[61]. The anticholinergic and antidopaminergic effects of antipsychotics and tricyclic antidepressants have been implicated, not only in causing a dry mouth (anticholinergic) thus limiting lubrication of the food bolus, but also in decreasing the coordination and strength of peristalsis and the timing of lower oesophageal sphincter relaxation[62,63]. It is also possible that serotonergic pathways may be responsible for the dysphagia caused by clozapine, although this hypothesis is perhaps refuted by the absence of case reports relating SSRIs to dysphagia[63]. Individuals with psychiatric diagnoses may also have an increased rate of neurological disorders (such as Parkinson's disease), particularly among older individuals in residential care, that can cause dysphagia[3,61]. The use of opiates, even if low dose and over the counter, can lead to hypercontractile or hypertensive sequelae of the oesophagus and/or LOS, sometimes even mimicking Type III achalasia.

Neurological disorders

Neurogenic dysphagia, a term loosely applied to cases where the pathology involves the central, autonomic or peripheral nervous system (and not isolated to the oesophagus such as achalasia) is more often characterised by an oropharyngeal component, with neurologists and speech pathologists primarily involved in management[64,65]. Dysphagia is common after cerebrovascular accidents (CVA) involving the cortex often causing hemiplegia[66]. Rapid improvement in swallowing function occurs in the majority[67]. Brainstem infarcts with cranial nerve involvement is another (albeit less common) lesion, but can be severe and sustained[3]. The basal ganglia are the major site of pathology in Parkinson's disease, and oropharyngeal involvement (with difficulty initiating swallowing and impairment in bolus transit between the pharynx and proximal oesophagus) demonstrable at video fluoroscopy[68].

Table 3 Rheumatological causes of dysphagia

Condition	Description
Scleroderma[52]	Distal oesophageal dysmotility, part of the CREST syndrome
Sjogren's syndrome[49]	Xerostomia limits bolus lubrication and food passage, proximal oesophageal dysmotility
Rheumatoid arthritis[45]	Proximal oesophageal dysmotility. Always consider and rule out associated cervical spine disease
Systemic lupus erythematosus (SLE)[45]	Proximal oesophageal dysmotility
Degenerative cervical spine disease, surgery on the cervical spine and diffuse idiopathic skeletal hyperostosis (DISH)[48,54]	Anterior cervical osteophytes cause extrinsic compression of the oropharynx and proximal oesophagus

Awareness of Parkinson's disease as a cause of dysphagia is of importance to gastroenterologists given the recent finding of neurological pathology beyond the brainstem. Furthermore other gastrointestinal symptoms such as constipation may precede the characteristic clinical syndrome of tremor, rigidity and bradykinesia[68-70]. Other neurogenic causes of dysphagia are outlined in Table 3.

CLINICAL ASSESSMENT

The aetiology of dysphagia can be variably classified, but traditionally oropharyngeal dysphagia (OD) and oesophageal dysphagia are defined as distinct groups because the immediate treatment approach to the former is to consider aspiration risk and dietary modification (Figure 2)[6]. It has been estimated that the presence of one or more of a 4-symptom cluster can predict OD accurately: delay in oropharyngeal initiation of swallowing, deglutitive cough, deglutitive nasal aspiration and the need to repetitively swallow to clear nasal secretions[71]. OD is principally managed by neurologists, ENT surgeons and speech pathologists, and is therefore beyond the focus of this review. Further useful sub-classifications of oesophageal disease include dysphagia to solids only (implying an anatomical distortion) or to liquids and solids (implying a physiological or motility disturbance), although the precision of any such clinical "rule of thumb" is not absolute. Longstanding symptoms of mixed dysphagia would normally suggest a motility disturbance[31].

INVESTIGATIONS

UGE remains a central tool in the investigational algorithm, being essential for the diagnosis of oesophageal malignancy, refractory GORD and EoE[72]. At least middle and lower biopsies of the oesophagus should be taken in patients with dysphagia, even when the macroscopic appearance is normal, primarily with the intention of excluding EoE. The role of UGE as a mandatory primary investigation is debated. Whilst dysphagia can signify life-threatening pathology such as oesophageal or gastric neoplasia, in the majority UGE will be normal or demonstrates only mild erosive disease , leading several experts to advocate a trial of PPI first in otherwise healthy subjects[5]. This is a departure from orthodox teaching where dysphagia is considered an alarm symptom mandating gastroscopy[11]. Thus, a trial of PPI can be considered both diagnostic and therapeutic. The significance of dysphagia however should not be minimised, as the condition is common, and the morbidity in terms of utilisation of healthcare and impairment in quality of life considerable[1,4].

Barium oesophogram (barium swallow) is the next investigation to consider after UGE, particularly when upper oesophageal anatomical or structural pathology is suspected (*e.g*, a pharyngeal pouch), or to exclude a major motility disorder (*e.g*, achalasia, spasm) when HRM availability is limited[73,74]. Indeed, experts disagree when it comes to the sequence of the investigations, with some advocating barium swallow as the initial test in all, citing the ability to pre-plan endoscopic inventions such as dilatation, to avoid unnecessary repeated testing and to avoid potential risks including pharyngeal pouch perforation and aspiration in patients with suspected oropharyngeal dysphagia[75]. Barium esophagography can also be considered when extrinsic compression is being considered, or when there is a strong suspicion of para-oesophageal (previously known is "rolling") HH. Cross-sectional imaging may assist in some cases, with communication between reporting radiologists being imperative in this setting[76,77].

HRM is most useful when a motility disorder is suspected, or if a HH is thought to be contributing to refractory GORD and dysphagia. HRM is more accurate in the diagnosis of sliding HH, and is the gold standard for major disorders of peristalsis (achalasia, distal oesophageal spasm, jackhammer oesophagus and aperistalsis), demonstrating superiority over conventional manometry and barium studies[78-80].

The last decade has seen not only the advent of HRM, but also of intraluminal oesophageal impedance, and intraluminal oesophageal impedance planimetry (commercially known as Endoflip)[81,82]. As well as being a measure of gastric reflux (alkaline and acid), IOI can detect bolus transit abnormalities[83]. Many would argue that whilst this technology has proved of value to researchers, the clinical value is limited or yet unrealised[84]. This assertion is based on data showing that impairment in bolus clearance defined by IOI correlates poorly with both the symptom of dysphagia

and with manometric findings[28]. Endoflip has only recently become available and can measure the hitherto unknown parameter of resistance to distension (distensability)[85] .This technology has shown promise in assessing reduced distensiblity in EoE and in post-operative measurement of achalasia response. On the other hand, inclusion of Impedance sensors with HRM has helped provide a visual impression of bolus transport or interruption, which is akin to radiology.

TREATMENT

Once a malignant process is excluded, the aims of treatment are to avoid aspiration or food bolus impaction, and minimise the morbidity associated with ongoing symptoms. Effective management of dysphagia obviously starts with early identification of the cause with judicious use of investigations. Requests for endoscopy for patients with oropharyngeal dysphagia where the risk of periprocedural aspiration is considerable and the utility questionable should ideally be preceded by an assessment of crico-pharyngeal function and speech pathologist evaluation of oral intake.

Proton pump inhibitors should be used when GORD is suspected as well as in cases of EoE to determine if this might be proton pump Inhibitor responsive oesophageal eosinophilia (PPI-REE)[25]. Patients with persistent eosinophilia will require swallowed topical corticosteroids (*e.g.*, budesonide 1mg orally twice daily administered as an oral viscous solution) or an elimination diet[25]. Specific treatment for oesophageal neoplasia is beyond the scope of this review and will depend on the extent of local invasion or occurrence of metastasis. The treatment of hypercontractile disorders of peristalsis may entail botulinum toxin injection of the distal oesophagus, and of achalasia might include pneumatic dilatation, or myotomy (surgical or per-oral endoscopic myotomy (POEM)][32,38]. Botulinum toxin injection will last only for a few months, and commonly requires repeated therapy[86,87]. Surgical myotomy can result in durable improvement, whilst POEM may offer a less invasive approach, although long-term data is currently unavailable[88,89]. Medications such as oral nitrates or calcium channel blockers lack efficacy but can be used as a holding measure while definitive therapy is being considered[87].

CONCLUSION

Dysphagia is a common symptom with a diverse range of aetiologies. In young healthy patients that do not describe additional red flag symptoms, a trial of PPI is reasonable and will result in symptomatic resolution in many cases. GORD can cause not only a decrement in peristaltic vigour, but may contribute to hyperalgesia, such that whilst food passes freely into the stomach, the patient experiences dysphagia indicating a somatosensory dysfunction. When further investigation is required, reconsideration of the site and the nature of symptoms (*e.g.*, oropharyngeal *vs* oesophageal), and early recourse to upper gastrointestinal endoscopy is suggested, particularly given the increasing frequency of EoE and thus the need for oesophageal biopsies. HRM is required to exclude major disorders of peristalsis (achalasia, jackhammer oesophagus, distal oesophageal spasm and aperistalsis), although the finding of minor disorders of peristalsis is of unclear significance. Finally, gastroenterologists require a comprehensive knowledge of conditions that may cause or worsen dysphagia so that timely and efficient management occurs.

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MINIREVIEWS

Role of endoscopic ultrasound in idiopathic pancreatitis

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Abstract

Recurrent acute pancreatitis (RAP) is defined based on the occurrence of two or more episodes of acute pancreatitis. The initial evaluation fails to detect the cause of RAP in 10%-30% of patients, whose condition is classified as idiopathic RAP (IRAP). Idiopathic acute pancreatitis (IAP) is a diagnostic challenge for gastroenterologists. In view of associated morbidity and mortality, it is important to determine the aetiology of pancreatitis to provide early treatment and prevent recurrence. Endoscopic ultrasound (EUS) is an investigation of choice for imaging of pancreas and biliary tract. In view of high diagnostic accuracy and safety of EUS, a EUS based management strategy appears to be a reasonable approach for evaluation of patients with a single/recurrent idiopathic pancreatitis. The most common diagnoses by EUS in IAP is biliary tract disease. The present review aims to discuss the role of EUS in the clinical management and diagnosis of patients with IAP. It elaborates the diagnostic approach to IAP in relation to EUS and other different modalities. Controversial issues in IAP like when to perform EUS, whether to perform after first episode or recurrent episodes, comparison among different investigations and the latest evidence significance are detailed.

Key words: Endoscopic ultrasound; Pancreatitis; ERCP; MRCP; USG; CT; Idiopathic recurrent pancreatitis; Ascariasis; Pancreatic cancer; Biliary sludge; Gallstones; Microlithiasis; Common bile duct stones

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Core tip: The initial evaluation fails to detect the cause of recurrent acute pancreatitis (RAP) in 10%-30% of patients, whose condition is classified as idiopathic RAP. Idiopathic acute pancreatitis (IAP) is a diagnostic challenge for gastroenterologists. In view of high diagnostic accuracy and safety of endoscopic ultrasound

(EUS), a EUS based management strategy appears to be a reasonable approach for evaluation of patients with a single/recurrent idiopathic pancreatitis. The most common diagnoses by EUS in IAP is biliary tract disease. This review aims to discuss the role of EUS in the clinical management and diagnosis of patients with IAP.

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory process of the pancreas affecting peripancreatic tissues and distant sites. In most patients after an attack of AP, aetiology can be found with gallstones and alcohol use being most often implicated. Approximately 20% of patients will develop subsequent attacks, defined as recurrent acute pancreatitis (RAP). Most aetiologies of AP can lead to recurrent attacks, if the underlying etiology persists[1,2,3]. RAP is a challenging condition, because it leads to significant patient morbidity, can progress to chronic pancreatitis (CP), and has limited treatment options[3]. In up to 10% of patients with a single episode of AP and in up to 30% of patients with RAP, the aetiology is not identified after the initial evaluation. This condition is known as idiopathic pancreatitis[4]. Evaluation and treatment is important as 50% of untreated patients with IRAP experience current episodes that may lead to CP[2]. Endoscopic ultrasound (EUS) is minimally invasive, highly accurate imaging modality for studying the pancreas and the biliary tree[5]. The goal of this review is to discuss the role of EUS in idiopathic pancreatitis, focusing on the methodology, findings, and limitations of the available literature.

WHAT IS IDIOPATHIC PANCREATITIS?

RAP has various definitions. RAP is defined as the occurrence of two or more episodes of AP without concurrent clinical or imaging evidence suggestive of CP[3]. Another definition is two or more episodes of AP with or without complete or near complete resolution of symptoms between episodes[6]. AP or RAP can be from an identifiable etiology or idiopathic (unidentifiable etiology). Initial evaluation of a patient with AP/RAP can identify the aetiology in 70%-90% of cases. When the etiology could not be identified on initial evaluation as it happens in 10%-30% of the patients, AP/RAP can be defined as IAP (idiopathic acute pancreatitis)/IRAP[2,3,7]. The initial evaluation includes a thorough history (including medication review and family history of pancreatitis), physical examination, laboratory studies (serum liver tests, calcium and triglyceride levels), transabdominal right upper quadrant ultrasound, contrast-enhanced abdominal computerized tomography (CT), or magnetic resonance cholangiopancreatography (MRCP). Various explanations for non-detection of aetiology of AP includes microlithiasis as the cause being difficult to diagnose using standard imaging analysis; pancreatic inflammation/necrosis, preventing visualization of pancreatic solid or cystic tumours; and biological abnormalities occurring during the initial days of AP, making difficult to diagnose lipid- or calcium metabolism abnormalities[8]. There are limited studies regarding the natural history following a single attack of IAP. These studies suggest relapse rates varying from 14% to 24%[9-11]. According to the latest prospective study by Wilcox *et al*[12], the relapse rate is 24. Various diagnostic studies to investigate the remaining 10%-30% cases includes microscopic bile examination (MBE), pancreatic function testing, genetic testing for mutations associated with pancreatitis, secretin-enhanced MRCP (MRCP-S), EUS, EUS elastography, and endoscopic retrograde cholangiopancreatography (ERCP) with sphincter of oddi manometry (SOM). This additional workup usually leads to the diagnosis of microlithiasis or biliary sludge, sphincter of oddi dysfunction (SOD), pancreas divisum (PD), hereditary pancreatitis, cystic fibrosis, a choledochocele, annular pancreas, an anomalous pancreatobiliary junction, pancreatobiliary tumours, duodenal duplication cyst, periampullary diverticulum or pancreatic-biliary ascariasis (Figures 1 and 2)[2,4,13-16]. After a complete additional advanced work-up, the aetiology remains unknown in no more than 10% of RAP, which can then be defined as true IRAP[2,3]. However, which test(s) to perform is not standardized. EUS is emerging as a diagnostic tool of choice for the patients with idiopathic pancreatitis[12].

Diagnostic yield of endoscopic ultrasound in idiopathic acute pancreatitis and its advantages

Since the early 1980s, EUS has been in use and is a safe, minimally invasive diagnostic procedure in patients with RAP. Various advantages includes close proximity of EUS probe to the pancreas and availability of high frequency EUS probes leading to high resolution images, and non-interference of the intestinal gases with image acquisition. EUS has few complications[17,18]. The diagnostic yield of EUS in various studies varies from 29%-80%. In a recent systemic review of 13 studies evaluating the role of EUS in IAP, the most frequent aetiology was

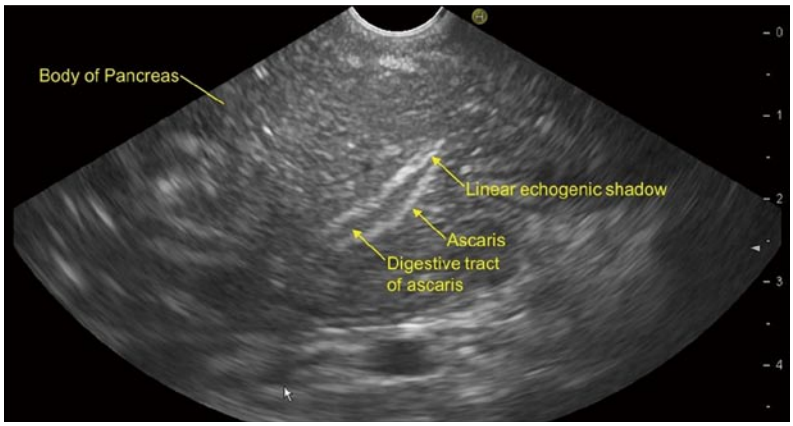


Figure 1 Linear endoscopic ultrasound from the stomach showing *Ascaris lumbricoides* in the body of the pancreas.

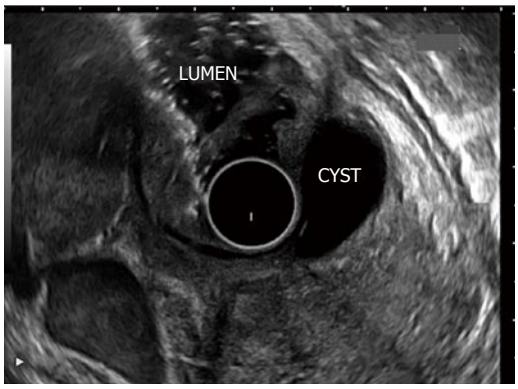


Figure 2 Radial endoscopic ultrasound from the duodenum shows duodenal duplication cyst as etiology of idiopathic pancreatitis.

biliary tract disease (biliary stones, microlithiasis and sludge). In the above mentioned 13 studies, different diagnostic modalities (1 or combination of 2 or more) were mentioned for diagnostic accuracy. They were ultrasonography, CT, MRCP, ERCP, SOM. Reference standard varied depending on the study. EUS identified pancreatic disease like CP, PD, periampullary tumours or pancreatic parenchymal change and/or pancreatic ductal change in 22.1% ± 26.6% of patients with IAP. Overall, EUS identified additional diagnostic information in 61% of patients with IAP, with 41% having biliary tract disease[19]. Various studies on the sensitivity of EUS to detect biliary tract disease suggests that EUS has superior sensitivity to other commonly used tests like ultrasonography (USG), CT, MRCP or MBE[12,17,18-22]. EUS is a reliable diagnostic method to detect PD[23,24]. EUS is quite useful to diagnose biliary and pancreatic tumours with a diagnostic accuracy higher than CT particularly in tumours smaller than 2.5 cm in diameter[25]. EUS is highly specific in the diagnosis of pancreatic cancer with a negative predictive value of 100%[26]. EUS can detect pancreatic solid tumours and cystic tumours at an early stage before they are identified by USG or CT. Detecting them earlier can lead to early operative management and better prognosis[27]. EUS is a useful diagnostic technique to detect the presence of CP in patients initially diagnosed with IRAP[28]. EUS is the least expensive initial investigation for the diagnostic evaluation of patients with IAP with gallbladder *in situ*[29].

Endoscopic ultrasound vs endoscopic retrograde cholangiopancreatography in idiopathic pancreatitis

Before the advent of EUS, ERCP was a primary diagnostic and therapeutic tool with a diagnostic yield of about 80%; however the contribution of EUS and MRCP has changed the diagnostic algorithm of IAP[3]. Established (microlithiasis, neoplasms) and controversial (PD, SOD) aetiologies of IAP may now be identified with EUS, limiting the role of ERCP to its therapeutic arm[3]. EUS has a diagnostic yield of about 80% which is about the same as the diagnostic yield of ERCP but with much less complication rates compared to ERCP which is 10%-15%[4].

Along with the lesser complication rates compared to ERCP, the added advantage of EUS is, EUS can diagnose biliary and pancreatic tumors especially tumors communicating with the pancreatic duct which can cause IAP[4]. In the past, ERCP was advised only after the second episode of IAP or after the first in severe IAP[30,31]. ERCP and EUS are considered the "gold standards" in clinical practice; however, MRCP has been proposed as a non-invasive alternative imaging technique to ERCP[32].

Endoscopic ultrasound vs magnetic resonance cholangiopancreatography in idiopathic pancreatitis

MRCP is a non-invasive investigation revealing detailed images of the hepatobiliary and pancreatic systems[32]. MRCP is indicated to diagnose PD, choledochocele, anomalous pancreatobiliary junction, or annular pancreas in patients with IARP[2,33]. Advantages includes no administration of intravenous contrast or ionizing radiation, can be used in all patients

including infants or those with allergies to iodine-based contrast materials[33,34]. It is less operator-dependent than USG or ERCP[35]. Disadvantages includes no therapeutic manoeuvres possible as it is primarily diagnostic investigation. MRCP is less sensitive than EUS for microlithiasis, small ampullary lesions, and ductal strictures[36-38].There are various studies showing better diagnostic yield of MRCP after secretin stimulation(MRCP-S) in CP,PD, SOD and pancreaticobiliary malformation as compared to simple MRCP[32,39-43]. In view of the available literature, MRCP-S may be more beneficial in IAP instead of MRCP if available.

There are three studies which has directly compared EUS and MRCP in IAP[18,43,44]. All have found higher diagnostic yield of EUS particularly to exclude biliary causes. In a prospective study of 49 patients with IAP, diagnoses were compared between EUS and MRCP. The diagnostic yield of EUS was more than that of MRCP (51% *vs* 20%, $P = 0.001$). It was concluded that EUS should be performed for establishing a possible biliary aetiology in patients with intact gall bladder (GB)[44]**.** In a study of 40 patients with MRCP negative IAP by Rana *et al*[18], the diagnostic yield of EUS was 55%. The most common aetiology was occult biliary tract disease in 50 percent[18].

Endoscopic ultrasound in pancreas divisum

PD is controversial aetiology of IAP[3]. Various studies have assessed the role of EUS as a reliable substitute for ERCP in patients of PD. Some believe that the sensitivity and specificity of EUS need further evaluation[5,45]. However, various studies considers EUS as an accurate, minimally invasive diagnostic method for PD[23,24,46-48]. In a study by Rana *et al*[46], the sensitivity, specificity, positive predictive value, negative predictive value of EUS for diagnosis of PD was 100%, 96%, 80%, 100% and 96%, respectively. In a recent retrospective cohort study of 45 consecutive patients diagnosed with PD on ERCP, diagnostic accuracy of EUS was compared to CT and MRCP. The sensitivity of EUS was 86.7%, significantly higher than CT (15.5%) or MRCP (60%).It was concluded that EUS is a sensitive test for diagnosing PD and is superior to MDCT and MRCP[48]. As per recent systemic review comparing diagnostic accuracy of MRCP with MRCP-S, MRCP-S has significant higher diagnostic accuracy and should be preferred for diagnosis of PD[42]. However, MRCP-S is not easily available. We believe that EUS is excellent diagnostic investigation for PD instead of MRCP[24].

Endoscopic ultrasound in biliary tract disease (biliary stones, microlithiasis and sludge)

USG (abdomen) has high accuracy in detecting gallstones, with reported sensitivities ranging from 92% to 96%[49]. However, when stones are less than 3 mm in diameter or are located in the GB infundibulum, the sensitivity is only 65%[50]. EUS is superior than USG for GB imaging due to its high-image resolution and close proximity to the biliary system. In a prospective study of 100 consecutive patients with AP, EUS was more sensitive than USG in detecting gallstones (100% *vs* 84%). The sensitivities of ERCP and EUS for choledocholithiasis were both 97%. It was concluded that EUS can be useful in selecting patients with AP who require therapeutic ERCP, thereby avoiding complications associated with diagnostic ERCP[49].

In a recent systemic review of 13 studies evaluating the role of EUS in IAP, the most frequent aetiology was biliary tract disease (41%)[19]. Various studies have found biliary microlithiasis/sludge (Figures 3, 4 and 5) as a cause of IAP in up to 75% of these patients[7,51,52]. However, there are few studies where biliary aetiology is not the most common cause of IAP[27,53].

In patients with intact GB, the most common cause of IAP is biliary microlithiasis/sludge which is detected in up to 80% of patients[7,51]. In the past, MBE was considered the gold standard for microlithiasis[54]. However, the disadvantages include high false negative rate, time consuming and high failure rate[4]. EUS has now replaced ERCP and bile sampling to diagnose microlithiasis in IRAP[8]. There are various reasons due to which EUS is highly accurate in detecting biliary microlithiasis. These includes GB being closely related to the stomach/duodenal wall; the median distance between the EUS transducer and the gallbladder is small (0.5 mm); the whole gallbladder is examined, even under acute pancreatitis; and use of high frequency probe[18].

The prevalence of the various aetiologies of RAP depends on whether the gallbladder is present or absent. If GB is present insitu, then the prevalence of microlithiasis would be about 50%, SOD-20%, CP-15%, PD-10% and choledocholithiasis 5%[29]. In patients who underwent cholecystectomy the prevalence of microlithiasis falls to 10%-15% whereas the prevalence of SOD, PD and CP rises[45].

Endoscopic ultrasound in chronic pancreatitis

RAP is a diagnostic and therapeutic challenge in clinical practice. It is sometimes impossible to differentiate clearly between recurrent attacks of AP and the early stages of CP[55]. Recurrent attacks of AP may complicate the course of chronic subclinical pancreatitis[13] (Figure 6). Although one of the limitations of EUS is being operator dependent, EUS has high diagnostic accuracy for the pancreatic parenchyma. EUS can detect early signs of CP, although the diagnostic significance of some changes is still debated[43]. In a study by Yusoff *et al*[27], EUS findings consistent with CP were reported

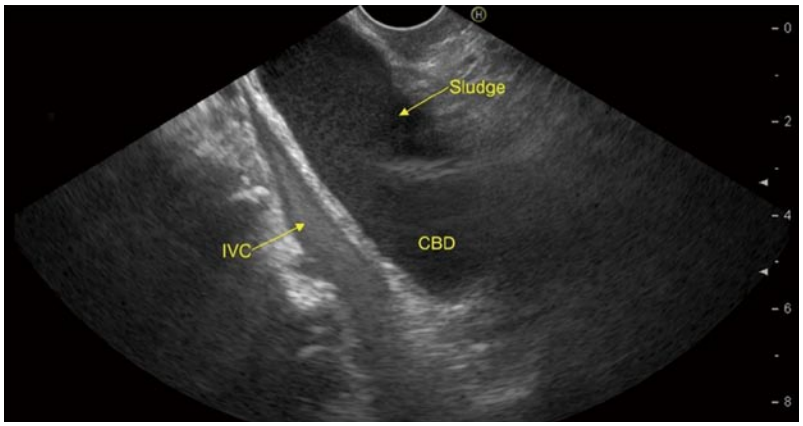


Figure 3 Linear endoscopic ultrasound from the duodenal bulb shows echogenic biliary sludge in the common bile duct.

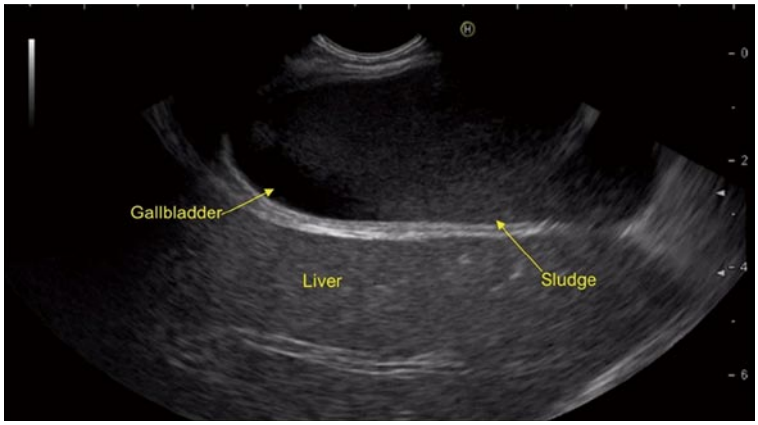


Figure 4 Linear endoscopic ultrasound from the duodenal bulb shows echogenic sludge in the gallbladder.

as the most common finding in patients with IAP.

When to perform endoscopic ultrasound, after first episode or recurrent episodes of idiopathic acute pancreatitis?

There is controversy in the literature about the timing of EUS in IAP whether to perform after first episode or recurrent episodes[21,27,56]. In a study by Yusoff *et al*[27], the diagnostic yield of EUS was not significantly different after a single attack or recurrent attacks of IAP. Therefore, the authors concluded that it is reasonable to perform EUS after the first episode, especially in older patients. Many authors have supported this opinion[12,18,21,57]. On the other hand there are few studies where the diagnostic yield is more in recurrent episodes than a single episode[51,58].

IAP is a common condition but the natural history is not well studied and the best diagnostic approach to both single and multiple attacks remains undefined. The suggested diagnostic approach in various studies and guidelines to IAP is highly variable[12]. As per the American Gastroenterological Association, EUS is not recommended after first episode of IAP in patients less than 40 years of age. It recommends EUS as investigation of choice in IRAP and after first episode in patients with more than 40 years of age[59]. As per American College of Gastroenterology, extensive evaluation using EUS or MRCP is recommended following recurrent attacks of IAP[60]. As per the International Association of pancreatology, patients with IAP should undergo EUS as the investigation of choice irrespective of first or recurrent episodes and, if negative, then, a MRCP-S as the next step[61].

In a recent landmark prospective study of patients with IAP over a 10-year period, 201 patients were enrolled. 80 were with single attack and 121 with multiple attacks of IAP. After EUS, 54% of patients with a single attack were categorized as idiopathic, while for multiple attacks 14 % were idiopathic. Long-term follow-up documented recurrence of pancreatitis in 24% of the patients with a single attack and in 49% of the patients with multiple attacks. On multivariate analysis, number of previous attacks was found to significant predictor of recurrence. It was concluded that following a single idiopathic attack of pancreatitis and a negative EUS examination, relapse was infr-

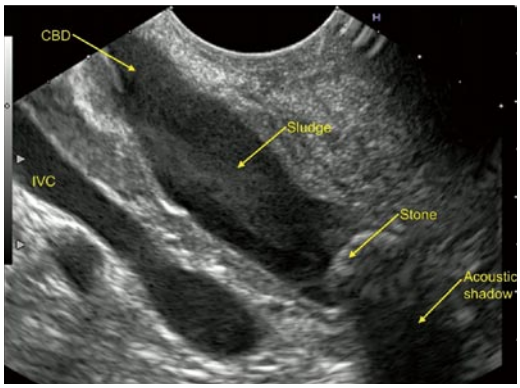


Figure 5 Common bile duct stone with sludge seen on linear endoscopic ultrasound from the duodenal bulb in a 36-year-old male presenting with 3 episodes of idiopathic acute pancreatitis in last 7 mo.

equent. A negative EUS after a single attack had significant prognostic value as it identified low rate of recurrence. This study suggest that EUS may be an useful test following a single attack given its yield and potential prognostic value. This study had prospective EUS based approach to idiopathic pancreatitis[12]. In a recent systemic review of 13 studies by Smith *et al*[19], the diagnostic yield of EUS was not influenced by recurrent disease. Considering the available literature, we recommend EUS after first episode of idiopathic pancreatitis.

Endoscopic ultrasound vs secretin enhanced endoscopic ultrasonography

Intravenous secretin when used with MRCP (MRCP-S) or EUS (EUS-S) may help the visualization of the pancreatic ductal system and can recognise some undetected ductal abnormalities that are of diagnostic importance in IRAP patients. In a study by Mariani *et al*[43], the diagnostic yield of EUS-S in IRAP was 13.6% and 16.7% higher than MRCP-S and ERCP respectively. ERCP alone did not find a diagnosis in any case missed by the other two investigations. It was concluded that both MRCP-S and EUS-S should be used as complementary, first-line, imaging techniques, rather than ERCP in the diagnostic work-up of IAP[43]. However, there are limited studies on the EUS-S and further data are required regarding its efficacy in IAP as compared to EUS without IV secretin.

When to perform endoscopic ultrasound after idiopathic pancreatitis?

The timing of performing EUS examination after an episode of IAP remains controversial and unclear. Different studies have used different timings[4]. In a recent prospective study of patients with IAP, EUS was performed 1 mo or more after hospital discharge[12]. Rana *et al*[18] performed EUS at least 1 mo after the episode of AP when the patients were asymptomatic for abdominal pain. In a study by Norton *et al*[62], EUS

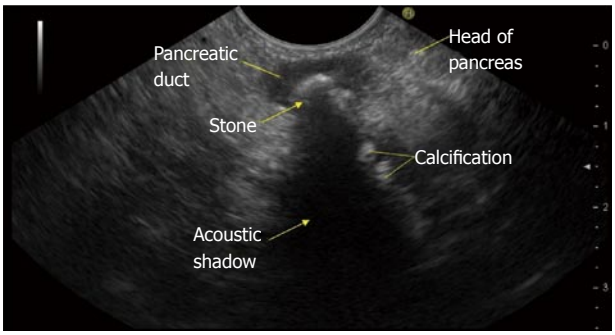


Figure 6 Features of chronic pancreatitis seen on linear endoscopic ultrasound from descending duodenum.

was performed when patients resume food intake while Liu *et al*[20] perform EUS after resolution of AP during admission. Yusoff *et al*[27] performed EUS atleast 4 wk after episode of AP to make sure that acute pancreatic parenchymal changes have resolved when EUS is performed.

Thevenot *et al*[8] suggested to perform MRCP/EUS at a longer interval after the initial AP as inflammation and/or necrosis can prevent visualization of pancreatic lesions during the acute phase. In two recent studies investigating the role of EUS/MRCP in IAP, these examinations were performed1 mo after initial AP when patients were eating normally. The explanation for delayed examination is that fasting is usually advised as initial treatment of AP which can induce GB sludge and, consequently, can result in a false-positive diagnosis of biliary AP if the examination is made early although this is not proven by studies[43,44].

Disadvantages of late EUS after 4 wk of AP includes papillary/ampullary stones (Figure 7) not being diagnosed early with the risk of relapse of AP, early/small periampullary tumours (Figure 8) might be missed as patient may lost to follow up after hospital discharge[4] and possible low diagnostic accuracy as microlithiasis may be missed[21]. Further studies exploring the role of early EUS in IAP are required to come to final conclusion regarding the ideal timing.

Which should be the first investigation after idiopathic acute pancreatitis: Magnetic resonance cholangiopancreatography vs endoscopic ultrasound

There is slight controversy regarding the first investigation after an episode of IAP. Different studies/authors/guidelines have variable opinions on this topic. Although MRCP is less invasive, cheaper and widely available than EUS, and has recently benefited using IV secretin, performance of EUS remains higher than that of MRCP[8]. However, few studies[43,44] have demonstrated that MRCP can detect few aetiologies undiagnosed by EUS. Further prospective studies are required regarding the cost benefit analysis for EUS/MRCP but at present they

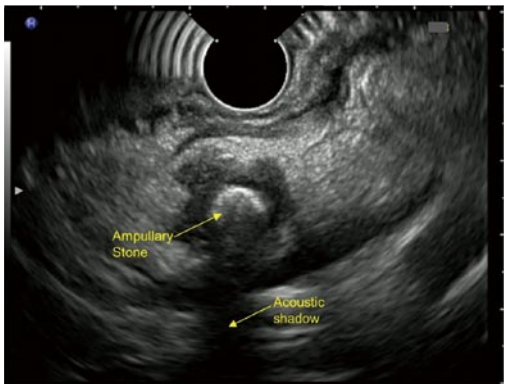


Figure 7 Ampullary stone with acoustic shadow seen on radial endoscopic ultrasound from descending duodenum.

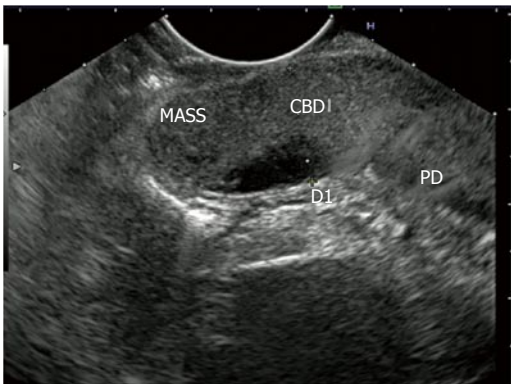


Figure 8 Hypoechoic ampullary mass extending into common bile duct.

should be considered complimentary rather than competitive.

After reviewing the extensive literature on IAP, our opinion which is shared by many authors/guidelines [12,19,21,22,52,57,59,61] and not agreed by few authors/guidelines[32,59], is performing EUS as the first investigation after IAP with intact GB. There is some debate regarding the choice of investigation in post cholecystectomy patients where CP, SOD and PD are the most common diagnoses in which MRCP has demonstrated good diagnostic accuracy[21,52]. As per the latest systemic review in 2015 comparing the diagnostic accuracy of EUS and MRCP in diagnosis of choledocholithiasis, both tests were found to be similar and the choice of test will be decided by availability and contra-indications to each test[63]. However, EUS is superior to MRCP in detecting choledocholithiasis smaller than 5 mm[64]. Various authors and studies believe that the decision to perform EUS or MRCP as the first diagnostic investigation in post cholecystectomy patients must take into account various factors like local expertise, availability and patient details such as claustrophobia, gastric surgery etc. ERCP should always be considered a therapeutic intervention when required[61,63-65].

The role of PD and SOD as causes of IAP remains highly controversial. In a study by Coté *et al*[66] and Wilcox *et al*[12], inspite of endoscopic therapy, RAP occurred in almost 50% patients with SOD. In addition, despite repeat endoscopic interventions, further relapse was also very common. These studies questions the role of endoscopic therapy in PD and SOD. These studies shows that diagnosing SOD and PD may not alter the natural history/prognosis of these patients presenting with IAP. Hence, MRCP and ERCP with SOM may not be that useful in diagnostic workup of IRAP. This also supports EUS as first investigation of choice after IAP irrespective of gallbladder status and is agreed by many studies[12,19, 21,22,54,59].

CONCLUSION

As per the recent prospective study done by Wilcox *et al*[12] on EUS based approach to the evaluation of Idiopathic Pancreatitis and after reviewing the literature, EUS as a first strategy towards the etiological evaluation of IAP appears to be useful not only as a diagnostic but as an important prognostic yield irrespective of gallbladder status. In view of high incidence of biliary tract disease as a cause of IAP in most of the studies and given the high diagnostic accuracy of EUS in identifying them, EUS should be considered as an initial diagnostic step for IAP after conventional radiography fails to identify the aetiology.

Considering all the prior studies, the diagnostic yield of EUS is not influenced by whether the episode is first or recurrent. Hence, EUS should be performed after the first episode of IAP if possible. MRCP preferably MRCP-S can be performed if EUS expertise is not available. MRCP can be complimentary to EUS in identifying controversial aetiologies like PD and SOD. ERCP can be then be performed to treat biliary stones and PD. However, in view of high risk of post ERCP pancreatitis, ERCP with SOM should be reserved in those patients in whom MRCP and EUS has found to be negative for diagnostic and therapeutic evaluation especially with post cholecystectomy cases. We recommend an EUS as the first-line examination in the evaluation of patients with idiopathic pancreatitis, because it is minimally invasive, low risk and accurately identifies most occult causes of pancreatitis.

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ORIGINAL ARTICLE

Basic Study

Delayed and short course of rapamycin prevents organ rejection after allogeneic liver transplantation in rats

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Abstract

AIM

To test whether a delayed and short course of rapamycin would induce immunosuppressive effects following allogeneic orthotopic liver transplantation (OLT) in rats.

METHODS

Allogeneic OLTs were performed using Dark Agouti

livers transplanted into Lewis recipients, and syngeneic OLTs were performed using the Lewis rat strain. Rapamycin (1 mg/kg per day) was administered by gavage from day 4 to day 11 post-transplantation. Lymphocyte cellular compartments were analyzed by flow cytometry in draining lymph nodes, non-draining lymph nodes and the spleen at days 11 and 42 in rapamycin-treated rats, untreated control rats and syngeneic grafted rats. Skin grafts from Dark agouti or from F344 RT were performed at day 30 on liver grafted rats treated with rapamycin.

RESULTS

An 8-d course of rapamycin treatment initiated 4 d following transplantation resulted in the survival of grafted rats for more than 100 d. In contrast, untreated rats died of liver failure within 13 to 21 d. The analysis of the cellular compartment revealed an increase in two cellular subpopulations, specifically myeloid-derived suppressor cells (MDSCs) and $CD8^{+}CD45RC^{low}$ T cells, without major modifications in the regulatory T cell (Treg) compartment in treated rats in the early stages after grafting. We evaluated the ability of treated rats to reject third-party allogeneic skin grafts to confirm their immune competence. In contrast, when skin was collected from rats syngeneic to the grafted liver, it was not rejected.

CONCLUSION

Our results demonstrate that short and delayed rapamycin treatment allows for tolerance in allogeneic OLT. The results also allowed for the identification of the mechanisms of tolerance induced by rapamycin by identifying MDSCs and $CD8^{+}CD45RC^{low}$ T cells as associated with the state of tolerance.

Key words: Myeloid-derived suppressor cells; Liver transplantation; Rapamycin; Regulatory T cells

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Core tip: Rapamycin is an immunosuppressive drug that is rarely used for liver transplantation treatment due to its side effects. Here, we show that a delayed and short course of rapamycin initiated on day 4 following allogeneic orthotopic liver transplantation in rats resulted in the survival of grafted rats for more than 100 d. The analysis of the cellular compartment revealed an increase in two cellular subpopulations, specifically myeloid-derived suppressor cells and $CD8+CD45RC^{low}$ T cells. The treated liver grafted-rats rejected third-party allogeneic skin grafts, but tolerated skin from syngeneic liver donor rats. Our results identify one of the mechanisms by which the state of tolerance is established.

Hamdani S, Thiolat A, Naserian S, Grondin C, Moutereau S, Hulin A, Calderaro J, Grimbert P, Cohen JL, Azoulay D, Pilon C. Delayed and short course of rapamycin prevents organ rejection after allogeneic liver transplantation in rats. *World J Gastroenterol* 2017; 23(38): 6962-6972 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/6962.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.6962

INTRODUCTION

Current immunosuppression treatments following solid organ transplantation are based on calcineurin inhibitors (CNIs). However, prolonged CNI exposure leads to nephrotoxicity[1], neurotoxicity[2], an increased risk of cancer[3], and hypertension[4], all of which are associated with long-term morbidity and mortality. Reducing the use of CNIs by combining or replacing them with immunosuppressive drugs that exert different mechanisms of action remains the primary strategy to lower the incidence of these adverse events. The drug rapamycin, a mammalian target of the rapamycin (mTOR) inhibitor, is a potential alternative due to its strong immunosuppressive properties in humans.

Rapamycin mediates immunosuppressive effects by preventing T cell cycle progression from the G1 to the S phase, thereby blocking T cell proliferation[5]. Rapamycin also acts on dendritic cells (DCs) by inhibiting their capacity to differentiate from immature into mature DCs, thus reducing their ability to activate T cells. In turn, immature DCs promote the expansion of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs) while concomitantly inducing T cell anergy and apoptosis and promoting graft tolerance[6]. In a model of experimental graft-*vs*-host disease facilitating the detection of donor-derived luciferase-labeled conventional T cells *in vivo*, the combination of Tregs and CNIs, but not rapamycin, suppresses Treg function[7]. In an allogeneic transplantation model, myeloid-derived suppressor cells (MDSCs) can also be recruited during mTOR inhibitor treatment[8]. Furthermore, following the activation of nitric oxide synthase (iNOS) and arginase (Arg1), MDSCs inhibit T cell proliferation and induce their apoptosis[9].

Currently, rapamycin is widely used to prevent rejection following renal[10], liver[11], and heart[12] transplantation. However, its use is primarily restricted to renal transplantation and is not recommended as a first-line treatment for liver transplantation due to deleterious side effects such as delayed wound-healing processes[13], thrombosis of the hepatic artery inducing graft loss, and increased mortality[14,15]. In liver transplantation, previous clinical trials have successfully demonstrated the possibility to introduce mTor inhibitors at day 30 in combination with reduced CNI treatments[16]. Here, we evaluated the possibility of using the mTor inhibitor alone in order to avoid CNI side effects in patients. Therefore, we tested a novel delayed and shortened rapamycin administration protocol in a model of orthotopic liver transplantation rejection in rats.

MATERIALS AND METHODS

Animals

Inbred Lewis RT1^{l} male (LEW) and Dark Agouti RT1^{av1} male (DA) rats weighing 220 to 280 g were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and F344 *RT1^{1v}* male rats weighing 220 to 280 g were purchased from Charles River Labs (L'Arbresle, France). The rats were maintained in animal facilities for at least one week prior to surgery under standard conditions. All experimental protocols were performed according to European Union guidelines and with the approval of the Regional Ethics Committee in Animal Experimentation no. 16, Ile-de-France, France (authorization no.11/12/12-11B).

Orthotopic liver transplantation

Non-arterialized OLT was performed according to Kamada's technique[17]. The allografts were transplanted orthotopically. Vena porta clamping ranged from 19-23 min. Allogeneic OLTs were performed by grafting a DA liver (donor) into a LEW rat (recipient). Syngeneic OLTs were performed with LEW rats (donor and recipient).

Treatment

Rapamycin[18] was administered by oral gavage (1 mg/kg body weight per day) every day starting on day 4 after transplantation until day 11. Non-draining lymph node (non-dLN; axillary), dLN (periportal), spleen, and liver samples were collected for analysis on day 11 and day 42 after sacrifice. The residual concentration of rapamycin was analyzed in naive and liver-grafted rats after the administration of rapamycin for 8 d. Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes for chromatographic analysis on days 0, 4, 9 and 11.

Flow cytometry

Cell suspensions obtained by mechanical shredding (draining lymph nodes, not draining lymph nodes and the spleen) were used for phenotypic analysis. Anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CTLA-4, anti-CD62L, anti-CD278 (ICOS), anti-CD11b/c, anti CD161, and anti-MHC-Ⅱ, anti-Foxp3, anti-IFNγ, anti-TNFα labelled with phycoerythrin (PE), phycoerythrin cyanine 7 (PE-Cy7) fluorescein isothiocyanate (FITC), peridinin chlorophyll protein complex eFluor 710 (perCP eFluor 710), allophycocyanin (APC), and eFluor 450 were purchased from ebioscience (Paris, France). Anti-CD45RC-PE was purchased from BD Biosciences and Anti-CD3-Vioblue and Helios-APC were purchased from Miltenyi Biotec (Paris, France). Events were acquired on a FACS Canto Ⅱ flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR, United States) software.

Skin grafting

Skin grafting was performed according to the method of Billingham and Medawar[19]. Full-thickness tail skin (1 cm^2) from two donors (DA and F344) was grafted onto the right and left dorsal thorax, respectively, of liver-grafted recipients after 30 d of tolerance (DA skin and F344 skin onto LEW rats). Rejection was defined as more than 80% graft necrosis upon daily inspection.

Histologic analysis

Grafted livers were excised, fixed in 10% formaldehyde buffer, paraffin-embedded, sectioned (5 μm) and stained with hematoxylin and eosin (H and E) according to a standard protocol. The histological grading of rejection was performed based on the BANFF classification[20].

Serum biochemical testing

Serum samples were stored at -20 ℃ until the analysis was performed. Transaminase enzyme and total bilirubin levels were measured to evaluate hepatocellular injury.

Statistical analysis

Differences between the groups were evaluated using an unpaired-t-test performed with Prism software 5.0 (Graph Pad Software, Inc., La Jolla, CA, United States). The results were considered statistically significant when $P < 0.05$.

RESULTS

Short and delayed rapamycin treatment prevents the rejection of allogeneic orthotopic liver transplantation

In this study, we used a model of DA liver transplantation in LEW recipient rats. These two rat strains differ in their major histocompatibility antigens. Allogeneic transplantation resulted in the death of all untreated rats within 13 to 21 d due to allogeneic liver rejection (Figure 1A). Four grafted rats were sacrificed for histological examination of their transplanted livers on day 11 and exhibited the characteristic signs of acute rejection according to the BANFF classification (mean score = 8.75 ± 0.25, Figure 1B and C). Next, we evaluated whether delayed rapamycin treatment exerts immunosuppressive effects. Four rats received rapamycin at a dose of 1 mg/kg per day beginning on day 4 post-transplantation and continuing until day 30. Under these conditions, 3 out of 4 grafted animals survived over 100 d without any additional treatment, suggesting that delayed and long-term rapamycin treatment is effective (Figure 1A).

We then tested whether a shorter course of rapamycin also leads to transplant liver maintenance. Eleven grafted rats received 1 mg/kg per day of rapamycin from day 4 to day 11 (8 d) and only 2 out of 11 rats died on days 19 and 49 (Figure 1A). To evaluate immunosuppression levels in transplanted livers, 4 rats were sacrificed on day 11 at the end of the treatment period. Compared to untreated control transplanted rats, the treated rats demonstrated a dramatic reduction in their histological rejection grade (Figure 1B and C). Four additional transplanted rapamycin-treated

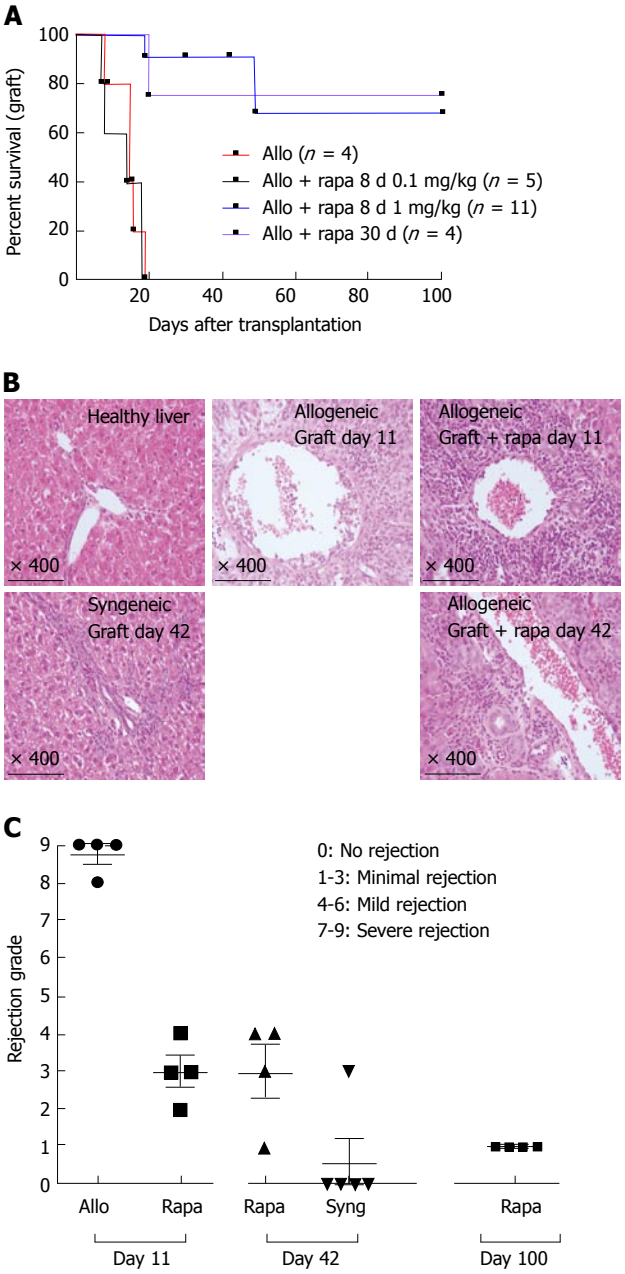


Figure 1 A short course of rapamycin prolongs liver transplantation in rats. A: Fully allogeneic liver transplantation (DA donor to LEW recipient) was performed, and the rats were given no treatment or were treated with rapamycin as follows: a short course at a dose of 1 mg/kg (oral gavage for 8 d from day 4 to day 11, *n* = 11), a short course at a dose of 0.1 mg/kg (8-d treatment from day 4 to day 11, *n* = 5) or a long course at a dose of 1 mg/kg (30-d treatment from day 4 to day 34, *n* = 4). Survival curves based on cumulative data are shown; B: H and E-stained histological sections from biopsy samples of a healthy liver and a grafted liver at the time of sacrifice on day 11 after liver transplantation (no treatment *vs* short course of rapamycin at 1 mg/kg) and on day 42 (short course of rapamycin at 1 mg/kg *vs* syngeneic); C: Histological grading of liver grafts using Banff scoring on day 11 and day 42 after transplantation, *n* = 4-5 rats/group.

rats were sacrificed on day 42 (1 mo after the end of rapamycin treatment) to compare their histological scores to those observed in syngeneic liver-transplanted animals. Although significantly higher than the scores observed in rats that did not reject their syngeneic liver transplants, the histological scores of the rapamycin-protected animals remained constant between day 11 and day 42 and were dramatically reduced compared to those of the untreated, transplanted rats (Figure 1B and C). Importantly, 4 protected animals sacrificed at day 100 did not display any histological signs of liver rejection. In addition, the blood analysis of residual rapamycin levels after 48 h of treatment administered in non-grafted control rats was under the threshold of detection of our platform, but rapamycin persisted in livers on day 21 (10 d after the end of rapamycin treatment, 6.66 ± 0.44 ng/g liver). Notably, none of the treated rats demonstrated dehiscence of either the abdominal wall or the vascular anastomosis, therefore circumventing a major side effect of the treatment on wound-healing processes. We also tested a reduced dose of rapamycin, 0.1 mg/kg, administered from day 4 to day 11. Under these experimental conditions, all animals rejected their transplanted livers with the same kinetics observed in the non-treated animals (Figure 1A). Therefore, graft acceptance following delayed, short-term treatment is dose-dependent.

We next assessed hepatocellular injury by quantifying total bilirubin, ALAT and ASAT in the blood of transplanted animals administered a short course of rapamycin on days 11 and 42. On day 11, we observed a dramatic decrease in total bilirubin in the treated rats compared to the untreated control rats (15.50 ± 4.51 μmol/L *vs* 109.5 ± 11.43 μmol/L, respectively) and decreasing trends for ALT (147.66 ± 66.02 UI/L *vs* 263.25 ± 45.88 UI/L, respectively) and AST (1257 ± 240.62 UI/L *vs* 558.25 ± 364.01 UI/L, respectively). On day 42, transaminase levels continued to decrease in rapamycin-treated rats compared to day 11, but remained higher than in rats with syngeneic liver transplants (ALAT = 609.8 ± 133.7 UI/L *vs* 152 ± 61.50 UI/L, respectively; ALAT = 130 ± 22.84 UI/L *vs* 35.50 ± 4.36 UI/L, respectively) (Figure 2). Therefore, the relationship between these biological abnormalities and the time from treatment appears to correlate with BANFF scores.

Based on our results, a delayed and short course of rapamycin effectively prevents allogeneic OLT rejection and correlates with low histological scores and long-term preserved liver function.

Allogeneic orthotopic liver tolerance under rapamycin treatment is associated with activated CD4$^+$ and CD8$^+$ regulatory T cell phenotypes and MDSC expansion

Given the *in vivo* effects of a delayed and short course of rapamycin in the allogeneic liver transplant model, we next sought to evaluate the involvement of different cell populations in these tolerance effects at the end of treatment and one month later.

We performed a flow cytometry analysis of the CD4$^+$Foxp3$^-$ (CD4conv) and CD8$^+$Foxp3$^-$ (CD8conv) T cell populations in non-dLNs and dLNs and in the spleens of grafted animals at two important time points: day 11 post-transplantation (the timing of liver

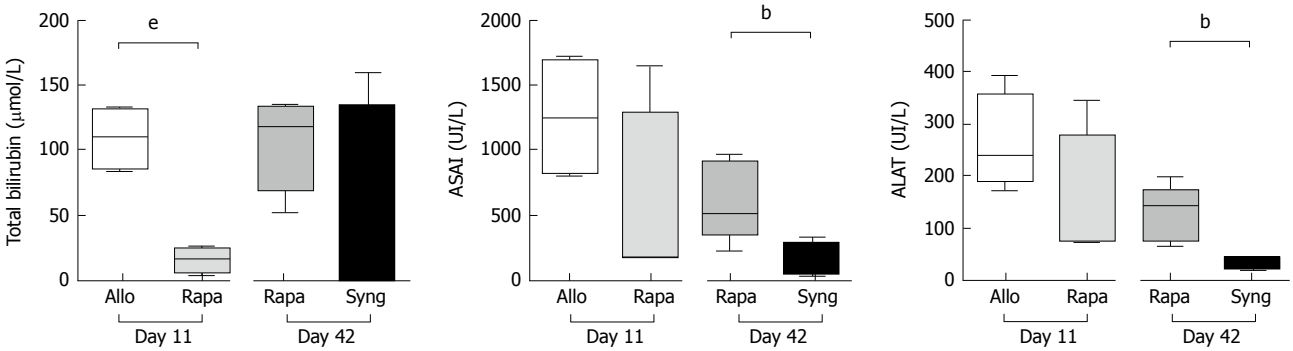


Figure 2 The protective effects of a short course of rapamycin on liver function. Fully allogeneic liver transplantation (DA donor to LEW recipient) was performed, and the rats were given no treatment or were treated with 1 mg/kg rapamycin (8-d treatment from day 4 to day 11). Total bilirubin, ALAT, and ASAT expression levels were measured in rat sera on postoperative days 11 (control *vs* rapamycin) and 42 (rapamycin *vs* syngeneic graft). The graph shows the cumulative data from 4-5 rats/group. *P*-values are indicated when the differences between the two groups of rats are significant ($^{b}P \leqslant 0.01$, $^{e}P \leqslant 0.001$). ALAT: Alanine aminotransferase; ASAT: Aspartate aminotransferase.

rejection and the end of treatment) and day 42 (when tolerance appears to be strong in the rapamycin-treated group). We did not observe any differences in the percentage or the number of CD4conv and CD8conv T cells at day 11 or day 42 (data not shown). Although we did not observe any changes in the naïve compartment on day 11, on day 42, the percentage of naïve CD4conv ($CD4^+Foxp3^-CD62L^+CD45RC^+$) T cells in dLNs and non-dLNs increased in the rapamycin-treated group compared to day 11 and was statistically significantly higher compared to the rats that received syngeneic liver transplants (Figure 3A).

We then quantified IFNγ and TNFα production by $CD4^+$ and $CD8^+$ T cells in the dLNs of grafted animals. Compared to untreated rats, rapamycin reduced the expression of IFNγ in both $CD4^+$ and $CD8^+$ T cells on day 11 post-transplantation ($CD4^+$ = 5.59% ± 1.15% *vs* 2.6% ± 0.54%, $CD8^+$ = 5.07% ± 1.58% *vs* 3.13% ± 0.35%, Figure 3B), but with weak modification of the expression of TNFα. One month after the end of the treatment, IFNγ expression in $CD4^+$ and $CD8^+$ T cells was higher in the rapamycin-treated rats compared to the rats with syngeneic grafts, but the percentage of secreting cells remained weak. Therefore, a short course of rapamycin appears to transiently inhibit the T cell inflammatory cytokine production capacity.

To identify the cellular mechanisms associated with liver acceptance, we then analyzed both $CD4^+$ and $CD8^+$ Tregs in grafted animals on day 11 and day 42. No major changes in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs were observed in the treated rats (Figure 4A). However, rapamycin treatment induced CD25 overexpression based on the mean intensity of fluorescence (MFI) on day 11. Additionally, ICOS (MFI) overexpression in $CD4^+CD25^+Foxp3^+$ Tregs observed on day 42 in rapamycin-treated rats compared to untreated rats grafted with syngeneic livers suggested the activation of $CD4^+$ Tregs 1 mo after the end of rapamycin treatment (Figure 4A). According to previous studies, the transcription factor Helios is required to maintain regulatory T cell identity[21,22]. In our model, Helios frequency in $CD4^+Foxp3^+$ cells was higher in rapamycin-treated rats one month after the end of treatment on day 42 than in syngeneic-grafted rats, suggesting a stable regulatory profile for this transcription factor.

$CD8^+Foxp3^+CD25^+$ regulatory cells are central mediators of tolerance during allogeneic heart transplantation in rats[23]. In our model, compared to untreated rats, rapamycin-treated rats demonstrated a slight increase in $CD8^+Foxp3^+CD25^+$ cells in the dLNs on day 11 and increased MFI CD25, ICOS and CTLA-4 expression in these cells on day 42, suggestive of their activated status compared to rats transplanted with syngeneic livers (Figure 4B). Additionally, $CD8^+CD45RC^{low}$ Tregs in transplanted livers are associated with organ transplant tolerance[24]. We observed a significant increase in $CD8^+CD45RC^{low}$ Tregs in the dLNs of rapamycin-treated rats on day 11.

Several recent studies have shown the beneficial effects of MDSCs during solid organ allograft rejection in humans and mice[25]. In our OLT model, we phenotypically characterized these myeloid cells in the spleens of grafted rats based on their expression of CD11b/c, their intermediate expression of CD161, and the lack of MCH class Ⅱ protein expression. Compared to untreated control rats, rapamycin treatment increased the frequency of MDSCs in the spleen on day 11 (1.603 ± 0.302% *vs* 4.085 ± 0.4596%) (Figure 5). The MDSC proportion remained relatively stable on day 42 and comparable to that of MDSCs observed in the syngeneic group on day 42. Therefore, these two subpopulations ($CD8^+CD45RC^{low}$ Tregs and MDSC) appear to be involved in tolerance during the early phase of rejection.

A short course of rapamycin induces antigen-specific tolerance

To test whether a short course of rapamycin treatment induces donor-specific tolerance, skin allografts were performed one month after liver transplantation (20 d after the end of rapamycin treatment). We grafted donor skin collected from syngeneic liver donor rats (DA) or skin collected from third-party donor rats

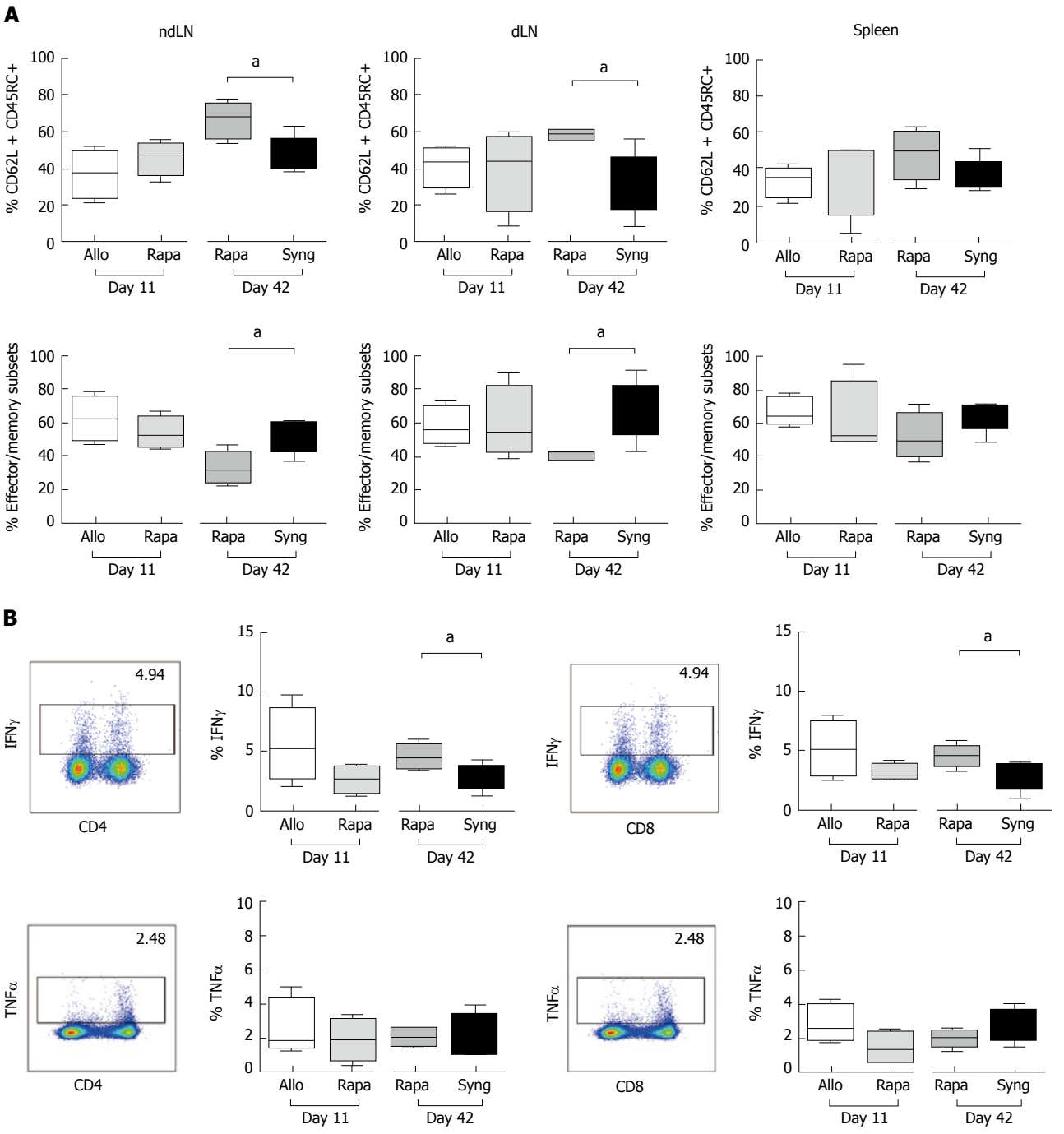


Figure 3 The effects of a short course of rapamycin treatment on naïve and effector memory cells (A) seems to transiently inhibit the T cell function (B). A: The ndLNs, dLNs and spleens of grafted animals were collected on day 11 and day 42 and conventional T cells were analyzed by flow cytometry. Naïve T cells, defined as $CD62L^{+}CD45RC^{+}$, and effector/memory cells, defined as ($CD62L^{-}CD45RC^{-}$) + ($CD62L^{+}CD45RC^{-}$) + ($CD62L^{-}CD45RC^{+}$), were analyzed in $CD4^{+}Foxp3^{-}$ and $CD8^{+}Foxp3^{-}$ T cell populations. The results are expressed as the mean +/- SEM of the percentages of effector/memory and naïve T cells, respectively. P-values are indicated when the differences between the two groups of rats are significant ($^{a}P \leqslant 0.05$). The graph shows the cumulative data from 4-5 rats/group; B: Quantification of IFNγ and TNFα expression by $CD4^{+}$ and $CD8^{+}$ T cells in the dLNs of grafted animals after PMA/ionomycin stimulation for 4 h. The graph shows the cumulative data from 4-5 rats/group. The results are expressed as the mean +/- SEM. *P*-values are indicated when the differences between the two groups of rats are significant ($^{a}P \leqslant 0.05$).

(neither donor nor recipient, F344) onto rapamycin-treated rats. In control LEW rats grafted with both DA and F334 skin, we observed no signs of rejection on day 7. On day 18, all skin transplants were rejected. In rapamycin-treated rats that did not reject their DA liver transplants, we observed the first signs of F344 skin rejection starting on day 7 and all skin grafts were fully rejected on day 10. Conversely, no signs of rejection were observed for DA-grafted skin on day 7 and black hairs from DA donor skin were detectable on protected animals until day 60 (Figure 6A and B).

DISCUSSION

Since the establishment of a small-animal liver transplant model[26], different preclinical studies have

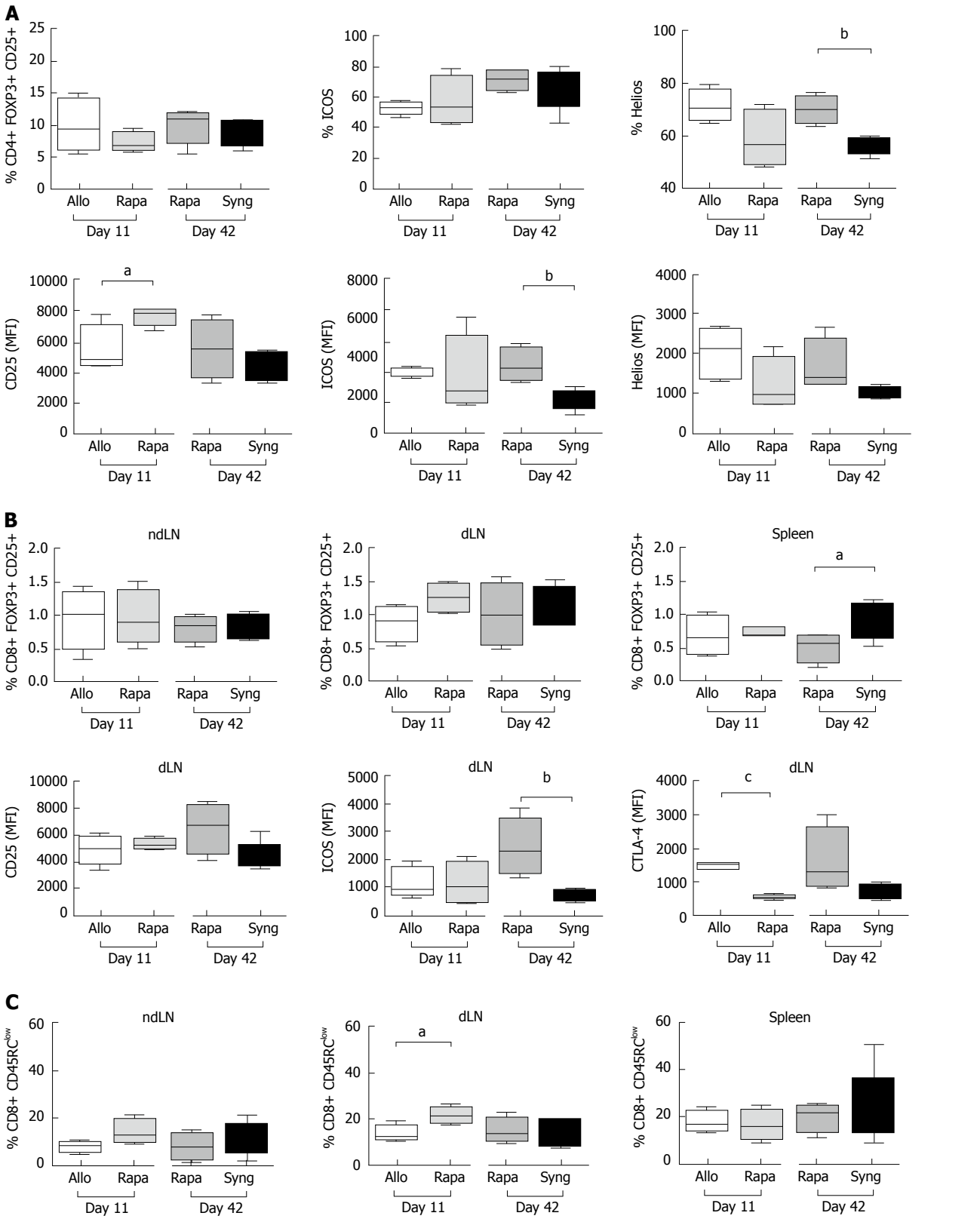


Figure 4 The effects of a short course of rapamycin treatment on CD4$^+$ and CD8$^+$ regulatory T cell phenotypes. A: The dLNs of grafted animals were collected on day 11 and day 42 and regulatory CD4$^+$ T cells were analyzed by flow cytometry. Different activation markers were studied (CD25$^+$, ICOS$^+$, Helios$^+$) after gating on CD25$^+$FOXP3$^+$ among CD4$^+$ cells. The results are expressed as the mean +/- SEM of the percentages of CD25$^+$, ICOS$^+$, and Helios$^+$ or the MFI values for CD25, ICOS, and Helios; *n* = 4-5 rats/group. *P*-values are indicated when the differences between the two groups of rats are significant ($^aP \leqslant 0.05$, $^bP \leqslant 0.01$). MFI, mean fluorescence intensity; B: The ndLNs, dLNs and spleens of grafted animals were collected on day 11 and day 42 and the percentages of CD25$^+$Foxp3$^+$ regulatory CD8$^+$ T cells were analyzed by flow cytometry. The MFI values of the activation markers (CD25, ICOS, and CTLA-4) are shown for the dLNs. The results are expressed as the mean +/- SEM. *P*-values are indicated when the differences between the two groups of rats are significant ($^aP \leqslant 0.05$, $^bP \leqslant 0.01$, $^cP \leqslant 0.0001$); C: The percentages of CD45RC^{low} cells among CD8$^+$ cells in the dLNs, ndLNs and spleens of grafted rats are shown. The results are expressed as the mean +/- SEM. *P*-values are indicated when the differences between the two groups of rats are significant ($^aP \leqslant 0.05$).

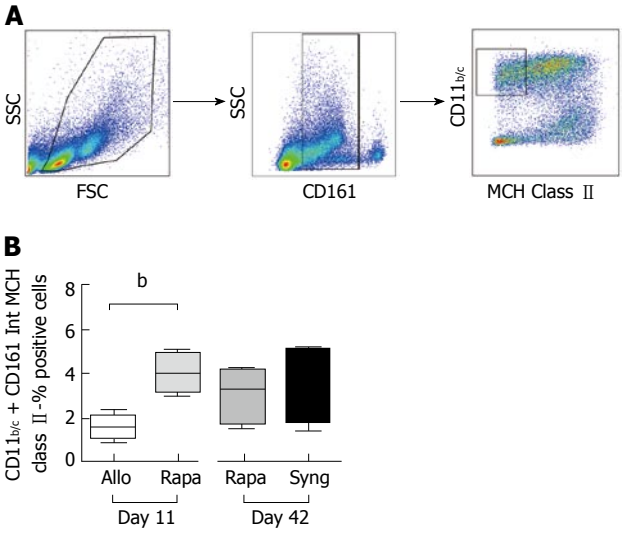


Figure 5 A short course of rapamycin increases myeloid-derived suppressor cells numbers on postoperative day 11 in allogeneic liver-grafted rats. The spleens of grafted animals were collected on day 11 and day 42, and myeloid-derived suppressor cells (MDSCs) were analyzed by flow cytometry. A: Gating strategy for MDSC analysis: CD11b/c^{+}CD161^{int}MCH class Ⅱ$^{-}$; B: Cumulative data indicating MDSC percentages. The results are expressed as the mean +/- SEM of the MDSC percentages. *P*-values are indicated when the differences between the two groups of rats are significant ($^{b}P \leqslant 0.01$).

demonstrated the feasibility of reducing or even substituting immunosuppressive drugs in favor of alternative therapies[27-29] to overcome the side effects responsible for significant morbidity and mortality[15]. In this context, we were strongly invested in the development of new pharmacological strategies to induce tolerance in transplantation. Notably, we have studied different approaches based on low-dose IL-2 administration in different pathophysiological settings. In allogeneic stem cell transplantation, we observed that low dose IL-2 +/- rapamycin treatment had no effect on GVHD, particularly in a model of xeno-GVHD using human cells[30]. In contrast, a similar protocol significantly delayed allogeneic skin graft rejection in mice. At the beginning of this work, we tried to transfer this therapeutic protocol in a model of allogeneic liver transplantation to rats considered as a less immunogenic setting. Fortuitously, in a group of control rats, we were surprised to observe that a 30-d rapamycin administration initiated at day 4 was sufficient to allow for allogeneic liver acceptance. We initially decided to delay rapamycin administration to day 4 to avoid the known side effects of the molecule when used at the time of transplantation. Additionally, the elevated grade of histopathological rejection observed as soon as day 11 led us to treat the rats at day 4 to avoid the potentially irreversible rejection process if administered too late. Due to the impressive results obtained with 30 d of treatment, we tested the potential of a reduced treatment duration. In summary, we investigated a novel rapamycin administration protocol to prevent acute liver rejection. A short (8 d) and delayed (starting on day 4 after transplantation) course of rapamycin was sufficient to control acute OLT rejection in rats and induce a state of operational tolerance. Indeed, more than 75% of grafted animals treated with 1 mg/kg rapamycin *via* oral administration survived more than 100 d without any additional immunosuppressive treatment. This is in marked contrast to previous results and represents a dramatic improvement over a previously described protocol using the same rat strains. Indeed, the authors tested the administration of rapamycin between days 0 and 7, resulting in an MST of OLT of 24 d[31]. Our results are of significant importance because they demonstrate that delayed rapamycin treatment does not jeopardize the safety of the transplanted organ. Rapamycin can also be stopped without impairing the developed graft tolerance. These results are consistent with a previous study that tested a delayed and short-course treatment with an anti-CD3 mAb[32] to obtain strong and long-term tolerance of allogeneic cardiac allografts. Therefore, in the context of transplantation, establishing a strong but time-limited allogeneic immune response prior to the initiation of a therapeutic approach may be favorable in the establishment of sustainable and specific tolerance.

We also sought to identify the immunological mechanisms underlying this long-term tolerance obtained with rapamycin. CD4^{+} Tregs have been regarded as a potential therapeutic modality for the prevention and treatment of allogeneic immune responses for a long time[33]. They also demonstrate potential for the specific targeting of cells involved in the allogeneic immune response without inducing general immunosuppression. We observed the preservation of immune potential as indicated by the capacity of tolerant rats to reject allogeneic skin grafts and accept skin grafts that originated from rats with the same background as the accepted livers. We quantified Tregs contents in the dLNs of grafted animals on day 11 (at the end of the rapamycin treatment) and one month later. In contrast to the results of a previous report[34], we did not observe a major change in the percentage of Tregs. However, CD25 overexpression on day 11 and the overexpression of ICOS in CD4^{+} Tregs on day 42 suggested the activation of these cells in rapamycin-treated rats. We also observed an increase in CD8^{+}Foxp3^{+}CD25^{+} regulatory cells, but this increase was only present on day 11 and only in the spleen. At the same time point, CD8^{+}Foxp3^{+}CD25^{+} Tregs overexpressed CD25, ICOS and CTLA4 in the dLNs of rapamycin-treated animals compared to syngeneic grafted rats, again suggesting an activated CD8^{+}Foxp3^{+}CD25^{+} Tregs phenotype[35]. CD8^{+}CD45RC^{low} Tregs[36] have also been associated with organ transplant tolerance[24,37]. We observed a significant increase in CD8^{+}CD45RC^{low} Tregs in the dLNs of rapamycin-treated rats on day 11. In murine cardiac allograft models, a short course of rapamycin leads to the recruitment of MDSCs and increases their expression of iNOS. According to Nakamura *et al*[8], even if the T cell activity is suppressed in a dose-dependent manner in vitro, monocytic MDSCs treated

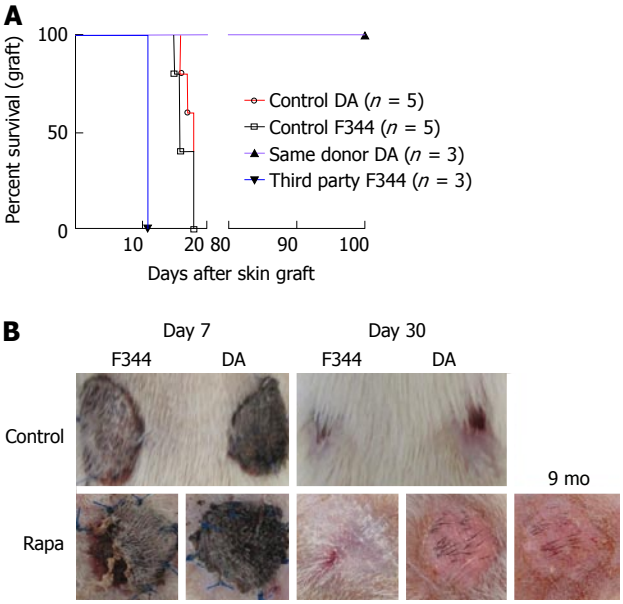


Figure 6 A short course of rapamycin induces antigen-specific tolerance. Allogeneic liver transplantations were performed (DA donor to LEW recipients), and the rats were treated with a short course of rapamycin (1 mg/kg, days 4 to 11). On day 30 after liver transplantation, skin grafts were performed with skin from the same DA donor or from third-party F344 rats. The control groups received allogeneic skin grafts from DA or F344 donors with rats (LEW) that did not undergo liver transplantation. A: Cumulative survival curves; days indicate the days post-skin graft, *n* = 3-5 rats/group; B: Representative images are shown on day 7 and day 30 after skin grafts.

with rapamycin suppress $CD4^+$ T cell proliferation more efficiently than granulocytic MDSCs treated with rapamycin. Therefore, mTOR down-regulation promotes monocytic MDSC migration in vitro and *in vivo*[38]. Altogether, our results suggest that MDSCs and $CD8^+CD45RC^{low}$ Tregs are involved in the early stages of tolerance initiation followed by sustained tolerance that involves both $CD4^+$ and $CD8^+Foxp3^+CD25^+$ Tregs.

In summary, our results provide new perspectives on research regarding the use of rapamycin in OLT. Notably, the precise mechanisms of tolerance remain to be identified by sequential depletion of the different regulatory cell subtypes to confirm their potential roles in graft acceptance. Additionally, if rapamycin should be reintroduced early in the therapeutic arsenal for the prevention of liver transplant rejection in humans, several steps must be taken. First, the main concern in using early rapamycin involves hepatic artery thrombosis. Consequently, the efficacy of our protocol remains to be tested in an additional model of OLT with consideration of this central parameter in a large animal model for example. Finally, the compatibility of early rapamycin administration should be evaluated with the gold standard of liver rejection prevention that relies on FK506 or CNI administration. Such a comparative study deserves specific experiments that would allow for a direct comparison of the clinical effects of these two type of molecules and also for the identification of the mechanisms of tolerance that take place depending on the molecule used. This information would even support therapeutic decisions consistent with the mechanisms of tolerance identified.

COMMENTS

Background

Solid organ transplantation remains the last option for vital organ failure. Even though the control of the immune response in the recipient is now better controlled, the side effects of the immunosuppressive approach are still present and the therapeutic approach needs perpetual improvement. Current immunosuppression treatments following solid organ transplantation are based on calcineurin inhibitors (CNIs). However, reducing the use of CNIs by combining or replacing them with immunosuppressive drugs that exert different mechanisms of action remains the primary strategy to lower the incidence of adverse events.

Research frontiers

The main challenge in transplantation is to identify therapeutic protocols that allow for organ specific tolerance with reduced or absent side effects.

Innovations and breakthroughs

Our data revealed that (1) rapamycin as mono-therapy allows for long-term allogeneic liver protection in rats; (2) this treatment was slightly delayed as opposed to rats treated at day 0; and (3) an 8-d treatment was as efficient as a 3-wk treatment to induce long-term tolerance. We also identified two populations of immune cells (MDSC and CD8+CD45low) that were increased in tolerant rats. These results reveal notable new perspectives by identifying the precise mechanisms at the origin of this state of tolerance.

Applications

These results support the development of similar therapeutic approaches in large animal models.

Terminology

Allogeneic orthotopic liver transplantation (OLT) is performed with a liver from a donor with a different genetic background from the recipient. A syngeneic graft is performed within the same genetic background.

Peer-review

An interesting and readable manuscript to explore a delayed and short course of rapamycin initiated on day 4 following allogeneic OLT in rats resulted in the survival of grafted rats for more than 100 d. This study is very clear, and the manuscript is well written. The main flaws of the study are its limited translational components.

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ORIGINAL ARTICLE

Basic Study

Adipose-derived stromal cells resemble bone marrow stromal cells in hepatocyte differentiation potential *in vitro* and *in vivo*

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Abstract

AIM

To investigate whether mesenchymal stem cells (MSCs) from adipose-derived stromal cells (ADSCs) and bone marrow stromal cells (BMSCs) have similar hepatic differentiation potential.

METHODS

Mouse ADSCs and BMSCs were isolated and cultured. Their morphological and phenotypic characteristics, as well as their multiple differentiation capacity were compared. A new culture system was established to induce ADSCs and BMSCs into functional hepatocytes. Reverse transcription polymerase chain reaction, Western blot, and immunofluorescence analyses were performed to identify the induced hepatocyte-like cells. CM-Dil-labeled ADSCs and BMSCs were then transplanted into a mouse model of CCl_4-induced acute liver failure. Fluorescence microscopy was used to track the transplanted MSCs. Liver function was tested by an automatic biochemistry analyzer, and liver tissue histology was observed by hematoxylin and eosin (HE) staining.

RESULTS

ADSCs and BMSCs shared a similar morphology and multiple differentiation capacity, as well as a similar phenotype (with expression of CD29 and CD90 and no expression of CD11b or CD45). Morphologically, ADSCs and BMSCs became round and epithelioid following hepatic induction. These two cell types differentiated into hepatocyte-like cells with similar expression of albumin, cytokeratin 18, cytokeratin 19, alpha fetoprotein, and cytochrome P450. Fluorescence microscopy revealed that both ADSCs and BMSCs were observed in the mouse liver at different time points. Compared to the control group, both the function of the injured livers and HE staining showed significant improvement in the ADSC- and BMSC-transplanted mice. There was no significant difference between the two MSC groups.

CONCLUSION

ADSCs share a similar hepatic differentiation capacity and therapeutic effect with BMSCs in an acute liver failure model. ADSCs may represent an ideal seed cell type for cell transplantation or a bio-artificial liver support system.

Key words: Adipose-derived stromal cells; Bone marrow stromal cells; Cell differentiation; Hepatocyte differentiation

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Core tip: We investigated whether mesenchymal stem cells from adipose-derived stromal cells (ADSCs) and bone marrow stromal cells (BMSCs), have similar hepatic differentiation potential. We found that adipose-derived stromal cells resemble bone marrow stromal cells in their hepatocyte differentiation potential *in vitro* and *in vivo*. Because ADSCs are obtained more easily and less invasive than BMSCs, ADSCs might be more suitable seed cells for cell transplant or liver tissue engineering. We also developed a new protocol of preparing mouse BMSCs and established a new hepatic induction system.

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INTRODUCTION

Liver transplantation remains the only effective treatment for liver failure. However, its clinical application is limited by donor organs and immune rejection. To this end, hepatocyte transplantation and a bio-artificial liver support system are two potential surrogate complementary therapies for patients with liver failure. Hepatocyte-like cells can be induced from mesenchymal stem cells (MSCs) for xenotransplantation and have been demonstrated to perform hepatocyte functions in preclinical animal studies[1,2]. However, the best type of MSC has not yet been investigated, and therefore it is important to screen for an ideal seed cell type for cell transplantation or a bio-artificial liver support system.

MSCs are non-hematopoietic multipotent stem cells that can be isolated from multiple tissues, such as bone marrow, adipose tissue, cord blood, and amniotic fluid[3-5]. Bone marrow stromal cells (BMSCs) are the most extensively studied and fertile stem cell source used in regenerative medicine and liver tissue engineering[6,7]. More recently, adipose-derived stromal cells (ADSCs) were identified as another promising and extensively studied stem cells for use, especially in liver tissue engineering[8]. Although both ADSCs and BMSCs can be induced into hepatocyte-like cells[9-12], there is no uniform culture system to evaluate their hepatic differentiation potential. Therefore, it is necessary to establish an ideal system to improve the efficiency of hepatic induction.

In this study, we developed a novel culture protocol for mouse BMSCs and optimized the hepatic differentiation system. We compared the morphological and phenotypic characteristics, as well as the multi-differentiation capacity of BMSCs and ADSCs *in vitro*. We also compared therapeutic effect of BMSCs and ADSCs following transplantation into a mouse model of acute liver failure.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice (3 d old and 6 wk old; Charles River, Beijing, China) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China (Beijing). All studies were conducted after approved by the Ethics Committee of the Animal Facility of Chinese PLA General Hospital and complied with the guidelines for the care of laboratory animals. Mice were housed in cages in a controlled environment (25 ℃ and a 12 h light/dark cycle) and fed standard mouse chow and tap water, and were observed every day in our animal facility.

Isolation and purification of adipose-derived stromal cells and bone marrow stromal cells

The isolation and purification of ADSCs were performed as previously described[13]. Fibrous tissue was excluded and adipose tissue was minced into pieces of < 1 mm^3 and digested in 1 mg/mL collagenase I for 1 h at 37 ℃. The cell suspension was filtered through a 100 μmol/L cell strainer and centrifuged at 300 × *g* for 5 min. ADSCs were plated at a density of 5 × 10^5/cm^2 with alpha minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and cultured in a humidified incubator at 37 ℃ and 5% CO_2. Cells were harvested after reaching a 90% confluence with 0.25% trypsin-EDTA (Gibco, American). Cells in passages 2-4 were used for subsequent experiments.

A new method was established to isolate mouse BMSCs as follows: 3-d-old male BALB/c mice were sacrificed by cervical dislocation and soaked in 75% alcohol for 5 min. The tibia and fibula were isolated under sterile conditions and washed twice with phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin. Muscle and fibrous tissue were excluded. Tibias and fibulas were minced into pieces of < 1 mm^3 and washed once with α-MEM, cultured directly by incubation with α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37 ℃ and 5% CO_2. After 72 h, half of the medium was changed and the bone chips were kept. After reaching a 50% confluence, cells were harvested with 0.25% trypsin-EDTA and seeded as the first passage. At each passage, cells were diluted 1:3-4 every two days. BMSCs at passages 2-4 were used for subsequent experiments.

Measurement of adipose-derived stromal cells and bone marrow stromal cells proliferation

For the cell proliferation assay, 2 × 10^3 viable ADSCs and BMSCs were seeded in triplicate onto a 96-well plate. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Beyotime, China). Plates were placed in a humidified incubator at 37 ℃ until the cells adhered to the plate. Next, 10 μL of the CCK-8 solution was added to each well and plates were incubated for another 2 h at 37 ℃ prior to reading the absorbance at 450 nm on a microplate reader. The assay was repeated every day at the same time for 10 d.

Flow cytometry

Passage 2 and 3 ADSCs and BMSCs were trypsinized and incubated with fluorescein isothiocyanate-conjugated CD45 and CD90, and phycoerythrin-conjugated CD11b and CD29 antibodies for 30 min at 4 ℃, followed by two washes with PBS. Fluorescent-labeled cells were analyzed on a flow cytometer.

Differentiation assays

For adipogenic differentiation, cells were seeded at 1 × 10^4/cm^2 on 12-well plates. When cells adhered to the plate, the expansion medium (α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin) was replaced with adipogenic induction medium containing 10^{-6} mmol/L dexamethasone (Dex), 0.5 μmol/L isobutylmethylxanthine, 200 μmol/L indomethacin, and 5 μg/mL (wt/v) insulin, and the cells were incubated for 8 d. Cells cultured in a base medium of α-MEM supplemented with 10% (v/v) FBS served as a negative control. Adipogenic differentiation was assessed by Oil-Red-O staining.

For osteogenic differentiation, cells were seeded at 5 × 10^3/cm^2 on 12-well plates. When cells adhered to the plate, the expansion medium was replaced with osteogenic induction medium containing 10^{-7} mmol/L Dex, 10 mmol/L β-glycerol phosphate, and 50 μmol/L ascorbate-2-phosphate. Cells cultured in a base medium of α-MEM supplemented with 10% FBS were used as a negative control. Cells were incubated for 3 wk and osteogenic differentiation was assessed by Alizarin Red staining.

Hepatic differentiation was achieved following a one-step procedure using mouse ADSCs and BMSCs. ADSCs and BMSCs (passage 3) were seeded at 5 × 10^3/cm^2 onto 24-well culture dishes in expansion medium. When cells adhered to the plate, the expansion medium was replaced with hepatocyte culture medium (HCM; DMEM containing 10% FBS) supplemented with 50 ng/mL hepatocyte growth factor (HGF), 25 ng/mL fibroblast growth factor 4 (FGF4), 30 ng/mL oncostatin M (OSM), 20 ng/mL epidermal growth factor (EGF), 25 ng/mL acidic fibroblast growth factor (aFGF), 10 ng/mL basic fibroblast growth factor (bFGF), 10^{-6} mmol/L Dex, 1 × insulin-transferrin-selenium (ITS), 2 mmol/L ascorbic acid (Vc), and 50 μmmol/L nicotinamide (Vpp). Differentiation medium (1 mL) was added to each 24-well culture dish and changed every 3 d. Afterwards, cells were cultured for 10 d in HCM. Undifferentiated cells served as negative controls and the HepG2 cell line served as a positive control.

Reverse transcription polymerase chain reaction

On day 10 of hepatic differentiation, total RNA was isolated from ADSCs and BMSCs with Trizol reagent

(Sigma-Aldrich, St. Louis, MO, United States), reverse-transcribed into first-strand cDNA using oligo (dT) primer, and amplified with 35 cycles (95 ℃, 10 min; 58 ℃, 1 min; and 72 ℃, 5 min) of PCR using 10 pmmol/L of specific primers. Upon completion of the PCR, products were examined by 2% agarose gel electrophoresis. Actin was used as an internal standard (30 cycles of amplification were performed). To compare the differences in expression levels of albumin (ALB), alpha fetoprotein (AFP), cytokeratin 18 (CK-18), CK-19, and glucose-6-phosphate (G-6-P) between undifferentiated and differentiated ADSCs and BMSCs, the products were quantified on an image analyzer (Uvitec, Warwickshire, United Kingdom). The primer sequences used are listed in Supplement Table 1.

Western blot analysis

Western blot analysis was performed as previously described[6]. In brief, total cellular proteins were prepared and quantified by the Bradford method. A total of 80 μg of lysates was electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Immoblin-P, Millipore, Bedford, MA, United States). Membranes were blocked with 5% fat free milk powder at room temperature for 2 h and incubated overnight with polyclonal rabbit anti-mouse CYP1A1 or CK-19, goat anti-mouse ALB, AFP, or CK-18 (1:1000; Santa Cruz Biotechnology, Dallas, TX, United States), or anti-β-actin antibody (Santa Cruz Biotechnology) at 4 ℃ overnight. After three washes of 15 min in Tris-buffered saline containing Tween 20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody, HRP-conjugated rabbit anti-goat IgG antibody (Zhongshan Jinqiao, Beijing, China), or goat anti-mouse IgG antibody for 2 h at room temperature. The membranes were washed again in TBST. Enhanced chemiluminescence reagent was added and monitored for color development.

Immunofluorescence

Cultured cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature, washed twice with PBS, and permeabilized with 1% Triton X-100 (Sigma-Aldrich) for 20 min at room temperature. Cells were then incubated with blocking solution consisting of PBS and 10% normal goat serum NGS at room temperature for 2 h. For immunofluorescence staining, primary antibodies (1:200; Santa Cruz Biotechnology) against ALB, AFP, CK-18, CK-19, or CYP1A1 were used. Following incubation with the primary antibodies overnight at 4 ℃, cells were incubated with secondary antibodies for 2 h at 37 ℃. Subsequently, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and photographed with a structured illumination fluorescence microscope.

Transplantation

Six-week-old BALB/c mice were used for the transplantation experiment. An acute liver failure model was established by administering one dose of CCl_4. Mice received intraperitoneal injection of 100 μL/20 g body weight of olive oil containing 10 μL CCl_4 24 h before MSC transplantation. On day 0, mice underwent MSC transplantation at a concentration of 1×10^6 cells per mouse (0.2 mL cell suspension was injected *via* the tail vein). As a control, PBS-transplanted CCl_4-treated mice and non-transplanted olive oil-treated mice were used. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured on days 1, 2, 3, and 7 after transplantation. Liver tissue samples were harvested at the indicated time points after cell transplantation, fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (HE). Frozen sections (8 μm thick) were used for fluorescence observations.

Statistical analysis

Data are presented as the mean ± SD. ALT and AST levels were analyzed by Student's *t* test using SPSS software version 19.0 (SPSS Inc., Chicago, IL, United States). Significance for all statistical analyses was defined as $P < 0.05$.

RESULTS

Cellular morphology of adipose-derived stromal cells and bone marrow stromal cells

Cells were observed under an inverted microscope. On the second day of primary culture, few ADSCs were observed. On the third day, the number of cells increased (the cell density was approximately 60%) and, on the fourth day, the cells exhibited a spiral-shaped structure and reached a 90% confluence. Cells were passaged at 1:3 (Supplement Figure 1A). On the second day of primary culture (BMSCs), few fibroblast-like cells had "climbed out" around the bone fragments. The number of cells increased over time and, on the fourth day, BMSCs reached a 50% confluence and were passaged at 1:1 (Supplement Figure 1A).

From passages 1-4, the two types of MSCs were long and spindle-shaped and showed no difference in morphology (Supplement Figure 1B).

Cell proliferation ability

The cell number of the second and third generations of the two cell types was determined over time and a growth curve was drawn. The two cell types showed active proliferative capacity, with an S-shaped growth curve. During the first three days, cells grew slowly. Between days 4 and 7, the two cell types grew rapidly and entered a logarithmic growth period. Cells

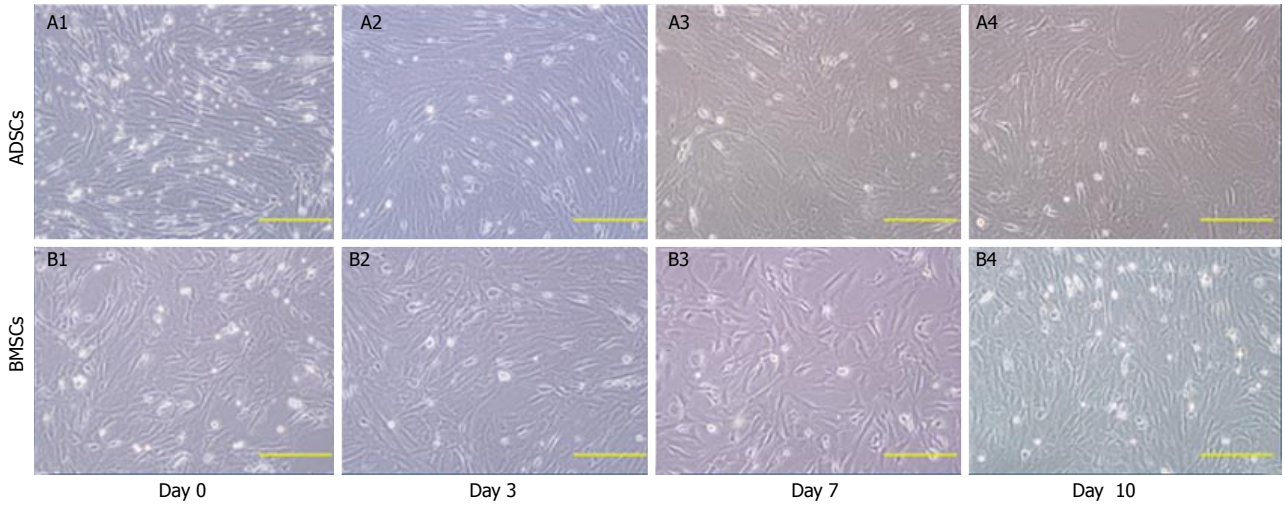


Figure 1 Morphological changes of adipose-derived stromal cells and bone marrow stromal cells following hepatic differentiation. After hepatic differentiation, the cellular morphology changed gradually and, on day 10 after differentiation, the induced cells were polygonal in shape. ADSCs: Adipose-derived stromal cells; BMSCs: Bone marrow stromal cells.

stopped growing at day 8 and entered the growth plateau phase (Supplement Figure 1C). There was no significant difference between the two types of adult stem cells in the same generation, and there was no significant difference between the same kinds of adult stem cells in the different generation (Supplement Table 1).

Phenotypic characterization of adipose-derived stromal cells and bone marrow stromal cells

ADSC and BMSC cell surface markers were analyzed by flow cytometry. Both ADSCs and BMSCs expressed the stem cell-associated surface markers CD90 and CD29, but did not express CD11b or CD45 (Supplement Table 2). The two cell types had a similar proliferative ability and stem cell capacity, consistent with our previous study.

Multiple differentiation ability of adipose-derived stromal cells and bone marrow stromal cells

The adipogenic and osteogenic differentiation of ADSCs and BMSCs was evaluated at passages 2 and 3. Small and round vacuoles began to appear in the cytoplasm on the third day of induction. On the 8th day, a large number of lipid droplets appeared in the majority of the induced cells and the cells became round, oval, or polygonal. Adipogenic differentiation was identified by Oil-Red-O staining, which stained the lipid vacuoles bright red.

After three weeks of induction, cells aggregated in some areas and formed a multilayered, nodular structure known as a bone nodule. Osteogenic differentiation was identified by Alizarin Red staining, which stained the bone nodules red. Oil-Red-O and Alizarin Red images of such cells were shown in our previous study.

Hepatic differentiation of adipose-derived stromal cells and bone marrow stromal cells

Before differentiation, both ADSCs and BMSCs exhibited fibroblast morphology with spindle cell bodies. After differentiation, the cell morphology changed dramatically. On day 4 after hepatic differentiation, cells became round in shape. On day 7, the number of oval and polygonal cells increased, and on day 10, the induced cells exhibited a clear polygonal shape (Figure 1). The expression of several hepatic genes was examined after hepatic differentiation by RT-PCR. Uninduced cells served as a negative control. On day 10 after hepatic differentiation, the expression of ALB, AFP, CK-18, CK-19, and G-6-P was significantly enhanced (Figure 2A).

The expression of ALB, AFP, CYP1A1, and CK-18 in hepatocyte-like cells was confirmed by Western blot analysis (Figure 2B). The expression of ALB, AFP, CYP1A1, and CK-18 increased continuously in the two types of hepatocyte-like cells compared with undifferentiated ADSCs and BMSCs, suggesting that these two types of MSCs were successfully induced into hepatocyte-like cells *in vitro*.

On day 10 after differentiation, immunofluorescence staining showed that the differentiated hepatocyte-like cells expressed the hepatocyte markers ALB, CK-19, CK-18, AFP, and CYP1A1 (Figure 2C), whereas the undifferentiated ADSCs and BMSCs (Figure 2C) did not express the hepatocyte-related markers.

Transplanted cells were found in damaged livers

To investigate whether the CM-Dil-labeled MSCs localized to the injured liver, animals were sacrificed on days 1, 2, 3 and 7 following treatment. In the ADSC and BMSC groups, red fluorescent cells were detected in the injured livers. In addition, the largest number of cells was detected on day 3 after transplantation and

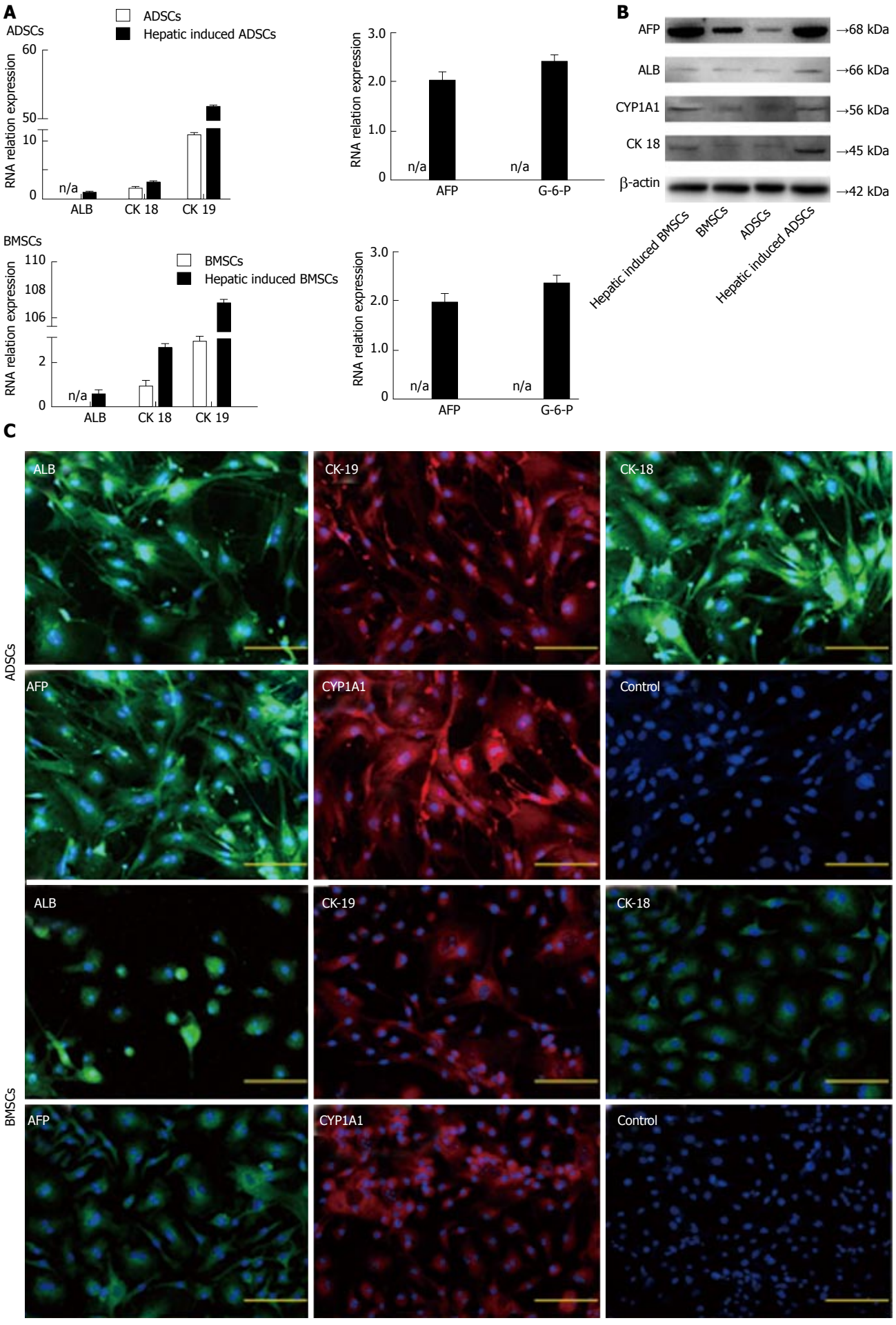

A
ADSCs
Hepatic induced ADSCs
RNA relation expression
60
50
10
0
n/a
ALB
CK 18
CK 19
BMSCs
Hepatic induced BMSCs
110
108
106
2
0
3.0
2.0
1.0
AFP
G-6-P
B
AFP
ALB
CYP1A1
CK 18
β-actin
→68 kDa
→66 kDa
→56 kDa
→45 kDa
→42 kDa
Hepatic induced BMSCs
BMSCs
ADSCs
Hepatic induced ADSCs
C
ALB
CK-19
CK-18
AFP
CYP1A1
Control
ADSCs
BMSCs

Figure 2 RT-PCR, western blot analysis and immunofluorescence staining of hepatocyte-related markers following hepatic induction. A: Gene expression of albumin (ALB), alpha fetoprotein (AFP), cytokeratin 18 (CK-18), CK-19, and glucose-6-phosphate (G-6-P) was significantly enhanced in hepatocyte-like cells on day 10 following hepatic differentiation. Undifferentiated cells served as a negative control; B: Western blot analysis of hepatocyte-related markers in adipose-derived stromal cells (ADSCs), bone marrow stromal cells (BMSCs), and hepatocyte-like cells; C: Immunofluorescence staining of hepatocyte-related markers in ADSCs, BMSCs, and hepatocyte-like cells. On day 10 after differentiation, immunofluorescence staining revealed that the differentiated hepatocyte-like cells expressed the hepatocyte markers ALB, CK-19, CK-18, AFP and CYP1A1. Scale bar: 200 μm.

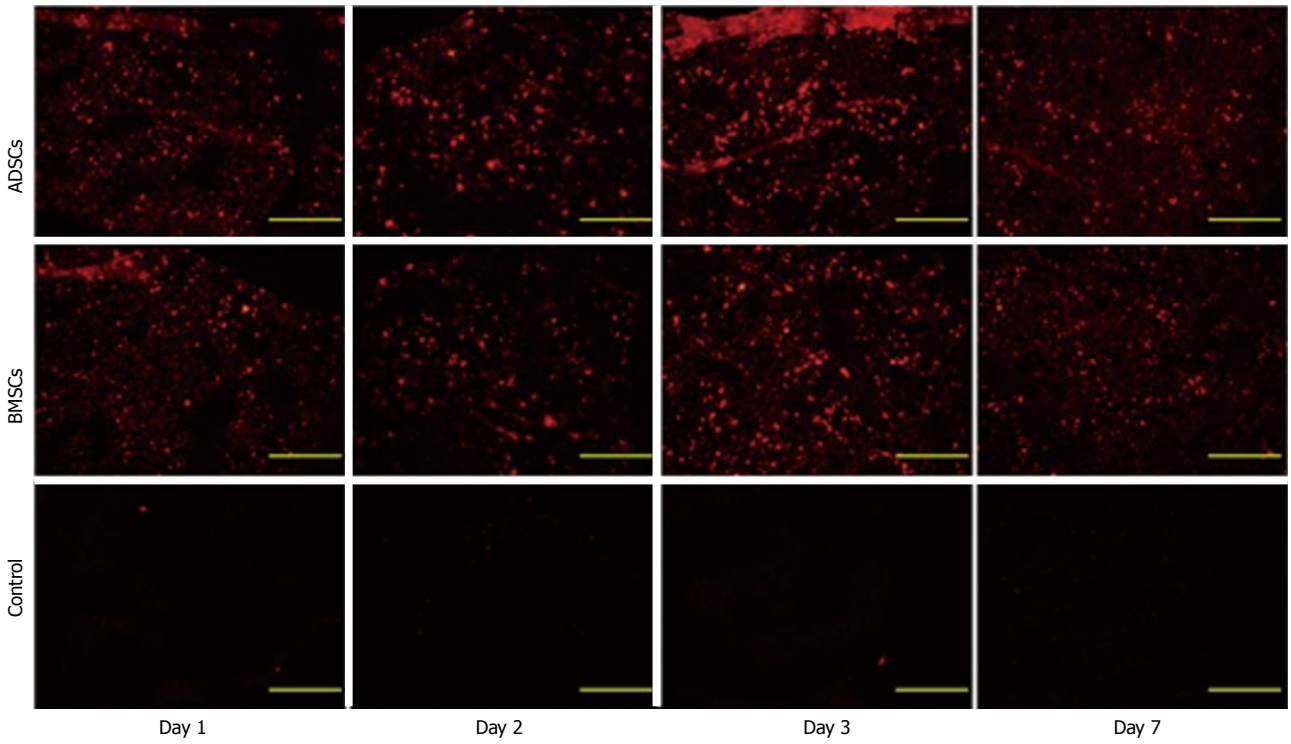


Figure 3 Tracing transplanted cells in a mouse model of acute liver failure. Red fluorescent cells were detected in the injured livers under fluorescence microscopy on days 1, 2, 3 and 7 following the transplantation. ADSCs: Adipose-derived stromal cells; BMSCs: Bone marrow stromal cells.

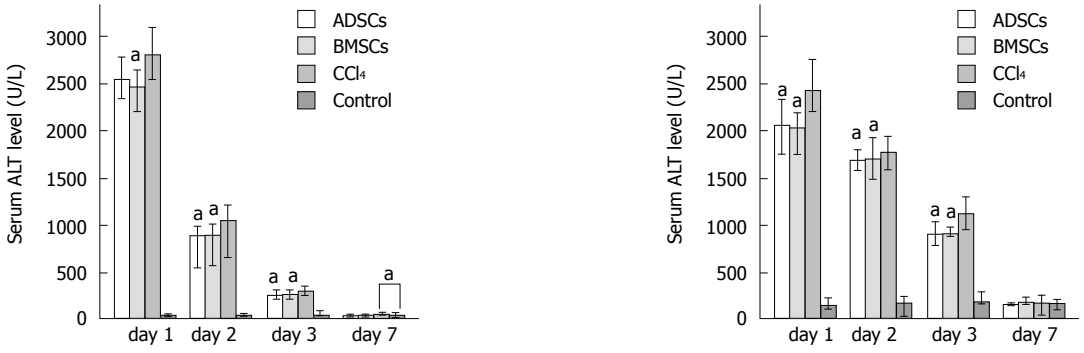


Figure 4 Analysis of liver function in CCl_4-treated mice following mesenchymal stem cell transplantation. Comparison of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the experimental and CCl_4 groups. Hepatic function nearly recovered on day 7 ($^aP < 0.05$ compared to the CCl_4 group). ADSCs: Adipose-derived stromal cells; BMSCs: Bone marrow stromal cells.

gradually decreased on day 7 (Figure 3). These results suggest that cells transplanted *via* the intravenous route can be integrated in the liver parenchyma during the early stage of transplantation.

Both adipose-derived stromal cells and bone marrow stromal cells improved liver function

To address whether ADSCs and BMSCs can regenerate the injured mouse liver, these cell types were transplanted into liver-injured mice. Twenty-four hours after CCl_4 injection, mice exhibited serious liver injury. Biochemical parameters, including ALT and AST, were measured and stem cells were transplanted. The therapeutic abilities of the two experimental groups were compared at different time points. Following transplantation, the ALT and AST levels of the

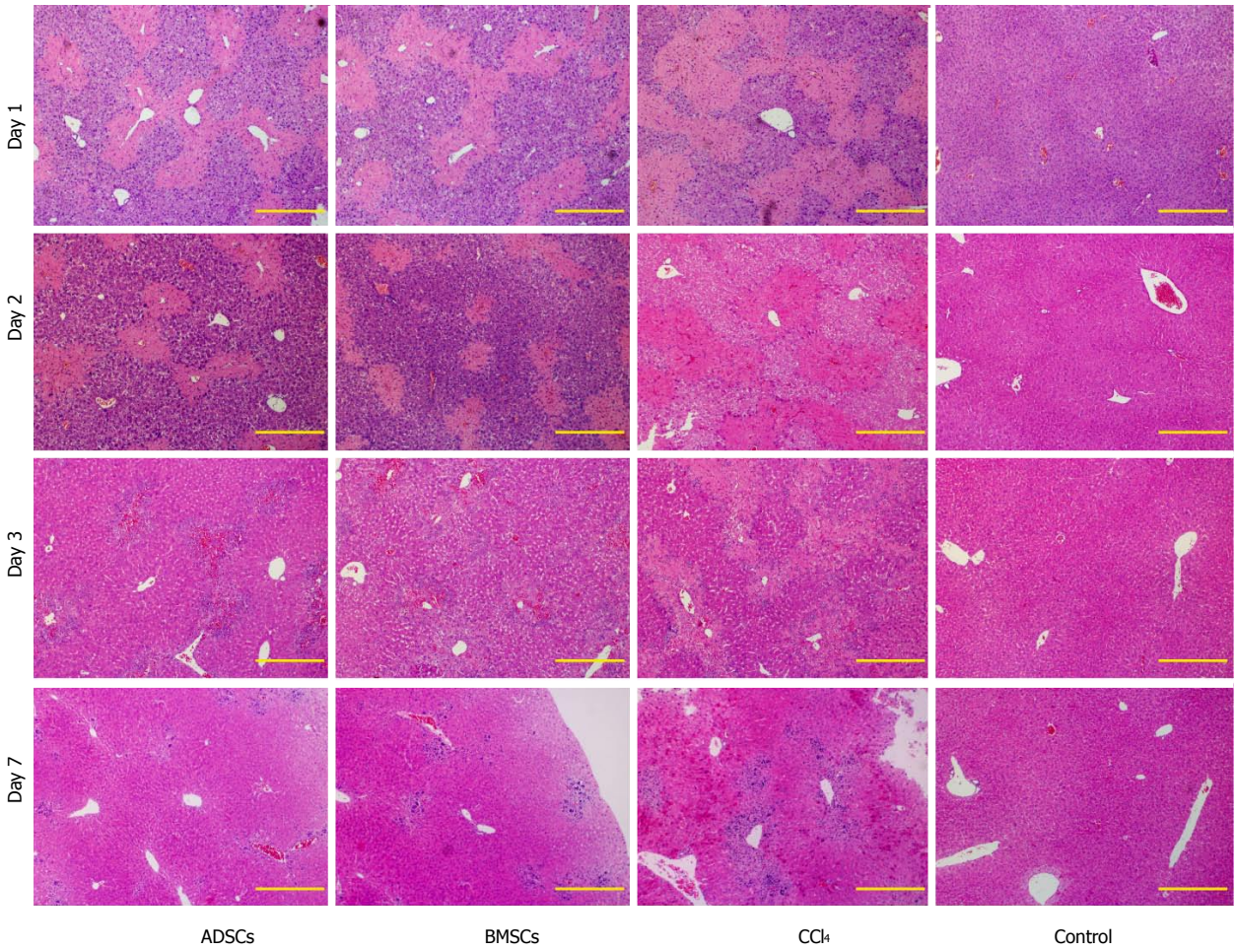


Figure 5 Hematoxylin and eosin staining in CCl_4-treated mice following mesenchymal stem cells transplantation. HE staining of liver tissue on different days following MSC transplantation (100 × magnification). The MSC groups showed smaller congested areas than the CCl_4 group. ADSCs: Adipose-derived stromal cells; BMSCs: Bone marrow stromal cells; HE: Hematoxylin and eosin; MSC: Mesenchymal stem cells; Scale bar: 200 μm.

experimental groups decreased significantly compared with the control group (Figure 4). HE staining revealed that the degree of injury was much weaker in the injured transplanted mice than in the injured, non-transplanted mice (Figure 5). On day 7, all of the experimental groups recovered histologically. However, damaged areas were still observed in the CCl_4 group. These observations indicated that ADSCs and BMSCs possessed similar repairing abilities against CCl_4-induced liver injury.

DISCUSSION

MSC transplantation is a promising alternative therapy for some liver diseases.

Availability of a large number of functional hepatocytes is essential for cytotherapy. However, primary hepatocyte cultures have been hindered by their short life span and the rapid loss of hepatic function *in vitro*[14]. MSCs have become a promising type of seed cell for liver transplantation for their multiple differentiation potential, high proliferation capacity, and plastic adherence properties. However, it is difficult to determine the ideal seed cells from different types of MSCs. In the present study, we established a novel isolation and culture protocol for mouse BMSCs that could provide a large number of BMSCs in a short period of time and share a similar multiple differentiation ability with ADSCs. We also established a new HCM that not only shortens the induction time but also produces a large number of hepatocyte-like cells. With the same induction system, ADSCs resembled BMSCs in term of hepatocyte differentiation potential both *in vitro* and *in vivo*.

A large quantity of ADSCs can be easily obtained by digesting adipose tissue derived from mice, rats, and humans with collagenase I[15]. However, it is difficult to obtain a large quantity of pure mouse BMSCs. We analyzed the conditioned medium method of Sun *et al*[16] and the compact bone method of Zhu *et al*[17], and developed a novel and reliable isolation and culture method that provides a large number of BMSCs in a short period of time. Compared to the method of Sun *et al*[16], the period required to obtain purified cells was shortened to 10-11 d. Compared to the method of Zhu *et al*[17], we omitted the digestion step of collagenase II. Additionally, the passage time was shortened to 2 d (compared to Zhu's twice per week). Compared to the

method for the isolation and culture of mouse BMSCs from bone marrow[18], our protocol not only shows less contamination of hematopoietic lineage cells, but also obtains a large number of BMSCs in a short period of time. The number of BMSCs obtained from five mice can reach 10^7 in 10-11 d. We believe that the reason for the rapid proliferation of cells is that neonatal mice exhibit better cell viability. Therefore, we developed a novel and easy method to isolate mouse BMSCs from tibia and fibula bone fragments.

Among the various hepatic differentiation systems, a one-step induction method has been most extensively applied[19]. Various cytokines and chemicals can be used to induce MSCs into hepatocyte-like cells. Current studies have reported that HGF, FGF4, EGF, OSM, Dex, aFGF, bFGF, DMSO, Vpp, and Vc can be applied for the hepatic differentiation of ADSCs[10,20]. Additionally, HGF, FGF4, EGF, OSM, Dex, ITS, trichostatin A (TSA), and L-glutamine have been applied for the hepatic differentiation of BMSCs[21-24]. In the present study, we used multiple factors, including HGF, FGF4, OSM, aFGF, bFGF, Dex, Vpp, ITS, and Vc, to develop a new induction system to promote the differentiation of MSCs. This hepatic induction system induced ADSCs and BMSCs into hepatocyte-like cells in only 10 d. However, a previously reported one-step induction system usually took more than two weeks[25-26]. This hepatic induction system shortens the induction time compared to the previously reported one-step system. Therefore, we consider it as an optimized one-step hepatic induction system.

We also identified induced hepatocyte-like cells from ADSCs and BMSCs in terms of morphology, as well as gene and protein expression. Our results revealed a similar hepatic differentiation potential of these two cell types *in vitro*. *In vivo*, we established an animal model of acute liver failure and proved that ADSCs and BMSCs engrafted into the injured liver showed similar therapeutic effects in the improvement of liver function. The results in our study suggest that BMSCs and ADSCs may serve as ideal seed cells for treating liver injury.

In conclusion, we developed a new protocol for the preparation of mouse BMSCs and established a new hepatic induction system. Our findings proved that ADSCs resembled BMSCs in the hepatic differentiation potential *in vitro* and *in vivo*. ADSCs can be obtained more easily and are less invasive than BMSCs. Therefore, ADSCs may be a more suitable seed cell type for use in cell transplantion or liver tissue engineering. In future experiments, we will explore the roles of ADSCs in liver tissue engineering.

COMMENTS

Background

Liver transplantation remains the only effective treatment for liver failure. However, its clinical application is limited by donor organs and immune rejection. To this end, hepatocyte transplantation and a bio-artificial liver support system are two potential surrogate complementary therapies for patients with liver failure. Hepatocyte-like cells can be induced from mesenchymal stem cells (MSCs) for xenotransplantation and have been demonstrated to perform hepatocyte functions in preclinical animal studies. However, the best type of MSCs has not yet been investigated and therefore it is important to screen for an ideal seed cell type for use in cell transplantation or a bio-artificial liver support system.

Research frontiers

MSCs are non-hematopoietic multipotent stem cells that can be isolated from multiple tissues, such as bone marrow, adipose tissue, cord blood, and amniotic fluid. Bone marrow stromal cells (BMSCs) are the most extensively studied and fertile stem cell source used in regenerative medicine and liver tissue engineering. More recently, adipose-derived stromal cells (ADSCs) were identified as another promising and extensively studied stem cells for use, especially in liver tissue engineering. Although both ADSCs and BMSCs can be induced into hepatocyte-like cells, there is no uniform culture system to evaluate their hepatic differentiation potential. Therefore, it is necessary to establish an ideal system to improve the efficiency of hepatic induction.

Innovations and breakthroughs

A new protocol for the preparation of mouse BMSCs and a new hepatic induction system were established. This study proved that ADSCs resembled BMSCs in the hepatic differentiation potential *in vitro* and *in vivo*. ADSCs can be obtained more easily and are less invasive than BMSCs. Therefore, ADSCs may serve as a more suitable seed cell type for use in cell transplantion or liver tissue engineering.

Applications

ADSCs are likely to serve as suitable seed cells for use in cell transplantion or liver tissue engineering.

Terminology

MSC transplant is a promising alternative therapy for some liver diseases. Availability of large number of functional hepatocytes is essential for cytotherapy. Both ADSCs and BMSCs can be induced into hepatocyte-like cells. Therefore, both ADSCs and BMSCs may be suitable seed cells for use in cell transplantion or liver tissue engineering.

Peer-review

In this paper, the authors investigated a novel method of generating differentiated hepatocytes from two sources of mesenchymal stem cells and tested their ability to recover liver damage in an experimental mouse model. The paper thus adressed an important question as stem cell therapy could help overcome current limitations of treatment for metabolic liver diseases and liver injury.

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ORIGINAL ARTICLE

Basic Study

Fecal microbiota transplantation prevents hepatic encephalopathy in rats with carbon tetrachloride-induced acute hepatic dysfunction

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Author contributions: Wang WW performed the research, analyzed the data, wrote and revised the paper; Zhang Y, Li J and You N performed the research; Zheng L participated in research design; Li J designed the research and participated in the revision of the paper; all authors have read and approved the final manuscript.

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Institutional animal care and use committee statement: All experiments on animals were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, 8th edition, 2011). All procedures involving animals were reviewed and approved by the Animal Care and Research Committee of the Second Affiliated Hospital of Third Military Medical University.

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Abstract

AIM

To investigate whether fecal microbiota transplantation (FMT) prevents hepatic encephalopathy (HE) in rats with carbon tetrachloride (CCl_4)-induced acute hepatic dysfunction.

METHODS

A rat model of HE was established with CCl_4. Rat behaviors and spatial learning capability were observed, and hepatic necrosis, intestinal mucosal barrier, serum ammonia levels and intestinal permeability were determined in HE rats receiving FMT treatment. Furthermore, the expression of tight junction proteins (Claudin-1, Claudin-6 and Occludin), Toll-like receptor (TLR) 4/TLR9, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α was examined.

RESULTS

FMT improved rat behaviors, HE grade and spatial learning capability. Moreover, FMT prevented hepatic

necrosis and intestinal mucosal barrier damage, leading to hepatic clearance of serum ammonia levels and reduced intestinal permeability. The expression of TLR4 and TLR9, two potent mediators of inflammatory response, was significantly downregulated in the liver of rats treated with FMT. Consistently, circulating pro-inflammatory factors such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α were remarkably decreased, indicating that FMT is able to limit systemic inflammation by decreasing the expression of TLR4 and TLR9. Importantly, HE-induced loss of tight junction proteins (Claudin-1, Claudin-6 and Occludin) was restored in intestinal tissues of rats receiving FMT treatment.

CONCLUSION

FMT enables protective effects in HE rats, and it improves the cognitive function and reduces the liver function indexes. FMT may cure HE by altering the intestinal permeability and improving the TLR response of the liver.

Key words: Fecal microbiota transplantation; Hepatic encephalopathy; Cognitive function; Intestinal permeability; Toll-like receptor

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Core tip: In this article, we first established a rat model of hepatic encephalopathy, and then carried on the fecal microbiota transplantation (FMT). Our results suggest that FMT can serve as a kind of new method for the treatment of hepatic encephalopathy, probably better than VSL#3.

Wang WW, Zhang Y, Huang XB, You N, Zheng L, Li J. Fecal microbiota transplantation prevents hepatic encephalopathy in rats with carbon tetrachloride-induced acute hepatic dysfunction. *World J Gastroenterol* 2017; 23(38): 6983-6994 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/6983.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.6983

INTRODUCTION

Hepatic encephalopathy (HE) is a common and serious disorder with a wide spectrum of neuropsychiatric abnormalities due to chronic and acute liver dysfunction[1]. HE is associated with poor quality of life and increased mortality, thus representing a major healthcare burden in patients with liver cirrhosis[2,3]. The estimated one-year survival rate is 42% after the first episode of overt HE, and the three-year survival rate is 23%[1,4,5]. Therefore, HE with cirrhosis is considered to have a poor prognosis for subjects who do not undergo liver transplantation. The death rate is substantially lower for patients who receive a transplant. The survival rate is more than 70% in the first 5 years after transplantation[1,6].

Although the pathogenesis of HE remains incompletely understood, gut-derived neurotoxin ammonia that accumulates in the central nervous system (CNS) is the most frequent precipitating factor[7-11]. In cases of normal hepatic function, ammonia enters the portal circulation of the liver and is subsequently converted to urea through the urea cycle[1].

Gut microbiota has emerged as a critical factor in the development of HE. Dysbiosis or an altered gut microbiota population promotes a systemic pro-inflammatory milieu, resulting in neuro-inflammation and ultimately neuronal dysfunction including HE[12-17]. Lactulose and lactitol, which target gut microbiota, have been demonstrated to be effective as first-line therapies for both acute and chronic HE. However, lactulose and lactitol have significant gastrointestinal side effects[18,19]. Furthermore, approximately 20% of patients with chronic liver failure and HE have been found to be non-responsive to lactulose treatment. A systemic review demonstrated that lactulose and lactitol failed to impart any survival benefit to cirrhotic patients with HE[20].

Probiotics (*e.g.*, VSL#3, a clinically tested probiotic formula consisting of Lactobacilli, Bifidobacteria, and Streptococci) contain living beneficial bacteria and have been shown to improve mental status and cognitive functioning. Therefore, probiotics may prevent HE recurrence by decreasing urease-producing bacteria populations and ammonia absorption while also increasing hepatic ammonia clearance[21]. Although probiotics are thought to have no adverse effects when used as long-term therapeutics, they are not widely utilized clinically due to the potential risk of introducing live bacteria into immunosuppressed patients. Therefore, the use of probiotics for cirrhotic patients with HE cannot be currently recommended, and rigorous clinical evaluation in randomized controlled trials is required[22-25].

Given the limitations of current HE therapy, research efforts are now aimed at improving existing treatments or developing novel therapies. Fecal microbiota transplantation (FMT) is rapidly being accepted as a viable, safe, and effective treatment for chronic gastrointestinal infections, recurrent inflammatory bowel diseases (IBD) and Clostridium difficile infection (CDI)[26-29]. It has also gained attention for its therapeutic potential for cardiometabolic, autoimmune, and other extra-intestinal conditions that were not previously considered to be associated with the intestinal microbiota[30-32]. However, whether FMT has a healing effect on HE has not been investigated.

In this study, we present *in vivo* evidence that FMT is effective for improving HE symptoms as evidenced by increased rat motor activity, spatial learning and memory following FMT. Moreover, FMT reduces systematic inflammation, leading to restored hepatic and intestinal dysfunction. Mechanically, FMT reduced liver expression of Toll-like receptor (TLR) 4 and

TLR9, which are two critical factors implicated in the accumulation of ammonia and regulating the levels of circulating pro-inflammatory mediators; therefore, FMT decreased liver inflammation and damage. Additionally, FMT recovered the expression of tight junction proteins such as Claudin-1, Claudin-6 and Occludin, resulting in attenuated intestinal permeability. Taken together, our study demonstrates for the first time the efficacy of FMT in the treatment of rats with HE and reveals the mechanisms underlying this process. Notably, we also provide an experimental basis for the potential use of FMT in HE patients.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 240-270 g were used for the experiments. Rats were fed regular chow and water ad libitum in cages placed in a room with a 12-h light/dark cycle at constant humidity and temperature (25 ℃). All of the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by The National Academy of Sciences and published by The National Institutes of Health.

Preparation of donor fecal material

The fecal material was collected and isolated as previously reported[33,34]. Briefly, approximately 30 g of fecal material was collected from a healthy female donor in a non-menstrual period with no history of hypertension, diabetes or cytomegalovirus, hepatitis or HIV infection. The volunteer also must not have presented with fever, abdominal pain, diarrhea, constipation, *etc*. In addition, the volunteer must not have used antibiotics, antiviral drugs and any drugs that may affect the function of the gut and bacteria within 2 wk of fecal matter donation. The volunteer was told to eat a liquid diet the day before the bacteria were extracted, and the fecal matter was then passed through 2.0-, 1.0-, 0.5- and 0.25-mm stainless steel laboratory sieves (WS Tyler, Mentor, OH) to remove undigested food and smaller particulate material. The resulting material passing through the 0.25-mm sieve was centrifuged at 6000 *g* for 15 min and homogenized in 150 mL of sterile normal saline. Glycerol (85%) was added to obtain a final concentration of 10%. The resulting fecal bacteria were resuspended and quantified by calculating the optical density (OD) relative to that of *Enterococcus faecalis* (*E. faecalis*) stored at -80 ℃.

Experimental design

The rat model of HE was established as previously described. Briefly, rats were given subcutaneous injections of 5 mL/kg of CCl_4 solution (a mixture of CCl_4 and peanut oil at a ratio of 2:3) twice a week and were fed 5% alcohol in drinking water with a week normal complete diet for 9 consecutive weeks. Control animals received normal saline. Nine weeks after injection, the rats were randomized into five groups (four rats per group) to receive saline, low-dose FMT (containing 2.7 billion fecal bacteria), moderate-dose FMT (containing 5.4 billion fecal bacteria), high-dose FMT (containing 7.1 billion fecal bacteria) or probiotics (VSL#3200 μL/d) for 3 wk. Each capsule of VSL#3 contained 112.5 billion freeze-dried bacteria (Streptococcus thermophilus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, and Lactobacillus bulgaricus) suspended in corn starch dissolved in 8.33 mL phosphate buffered saline (PBS). The rats received 200 μL/d of this solution (containing 2.7 billion CFU) *via* intestinal intubation. Prior to introducing an intestinal tube, rats were anesthesized and incised to allow duodenal exposure. Then a small cut was made in the duodenum, allowing a PE50 tube for implantation, followed by stitching the cut. The tube was made to pass into the air and stuffed with sterilized cotton for further administration.

Observations and measurement of rat behaviors

Rat behaviors were observed one week before or after administrations and recorded in an open field. Behaviors include mortality, body weight, motor activity, stools, hematochezia and infection. During the activity measurements, the animals had no access to food or chow. All studies were performed under strictly standardized conditions in a dark room for 30 min. The numbers of total movements, ambulatory movements, and vertical movements were separately recorded to reflect the motor activities of rats with hepatic failure. The motor activities were defined as zero in dead mice.

Morris water maze

HE rats were evaluated for spatial learning and memory capabilities using a Morris water maze as described previously. Two training trials a day were conducted on 3 consecutive days during the 8th week of the study. The experimental apparatus consisted of a cylindrical water tank (145 cm in diameter, 60 cm in height) filled with water maintained at 21 ± 1℃. The water was made opaque with black ink. A platform (10 cm in diameter) was submerged 2 cm below the water surface and placed at the midpoint of one quadrant. Room lights illuminated the pool, and the visual cues around the room (window, cabinets, furniture) were kept consistent. A video camera was placed above the center of the pool and connected to a video tracking system. During each training session, the rats were placed in the pool at a specified starting position and allowed to swim freely until they found the Morris water maze. The time required to escape (escape latency) was recorded. Rats that found the platform within 120 s were allowed to remain on it for 20 s

and were then returned to their home cages. If a rat did not reach the platform within 120 s, it was gently guided there by the experimenter, and allowed to stay on it for 20 s. The test was performed again at 12 wk to assess spatial cognitive function.

Histology examination of liver injury and intestinal damage

For histological examination, liver and distal ileum samples were fixed in 4% paraformaldehyde, dehydrated using graded ethanol, and then embedded in paraffin. The paraffin blocks were sectioned and stained with hematoxylin and eosin (HE) using standard histological techniques.

Measurement of intestinal permeability

Intestinal permeability was determined by Evans blue staining as previously described. Rats were anesthetized and transcardiacally perfused with Evans Blue dye. Colonic tissue was removed and maintained in formamide solution at 60℃ for 24 h, followed by centrifugation at 5000 r/min for 20 min. Optical density at 632 nm was measured and calculated.

Liver functional tests and serum ammonia level detection

Biochemical parameters were measured using standard clinical methods, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), total bilirubin (TBIL) and direct bilirubin (DBIL). Blood samples (0.5 mL) were collected from the portal and tail veins and analyzed with an automatic biochemical analyzer.

Enzyme-linked immunosorbent assay (ELISA) analysis of circulating pro-inflammatory mediators and stool proteins

For detection of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, serum samples from each individual group were collected and separated from portal and tail veins, followed by analysis with ELISA kits according to the manufacturer's instructions (Sangon, Shanghai). For Calprotectin and alpha-1-antitrypsin (A1AT) detection, equal weight stools from each group were collected and dissolved in PBS and then separated by centrifugation for ELISA testing according to the manufacturer's instructions (Sangon, Shanghai).

Western blot analysis

Protein expression was determined in lysates of liver and intestinal tissues using rabbit anti-TLR4, anti-TLR9, anti-Claudin-1, anti-Claudin-6 and anti-Occludin antibodies (Abcam) diluted in blocking buffer (5% milk in 0.2% Tween 20/TBS, 4℃, overnight). The results were normalized to GAPDH expression (mouse anti-GAPDH, Abcam).

Statistical analysis

SPSS statistical software, version 19.0, was used for statistical analyses. The Pearson χ^2 test or Fisher's exact test was used to compare qualitative variables and Student's *t*-test was used for comparisons of quantitative variables. All statistical tests were two-sided. $P < 0.05$ was considered statistically significant

RESULTS

FMT significantly alleviates behaviors of rats with chronic liver failure-induced HE

Chronic liver failure-induced HE was induced in rats by treatment with carbon tetrachloride-alcohol as previously described. All rats treated for 9 wk showed similar symptoms of HE, such as impaired spontaneous movement, cachexia and somnolence, when compared with saline-treated control mice. Histological examination verified the successful establishment of the rat HE model. The rats were then randomly divided into five groups including the model group, the probiotic-treated group (termed VSL) and the FMT treated groups across three doses (termed FMT-L, FMT-M and FMT-H for the low, mid and high FMT doses, respectively). Infections were observed during the experimental period; one rat each in the VSL, FMT-L, FMT-M and FMT-H groups died from infection. Data from these rats were excluded from the final statistical analysis. The behaviors of each group were observed clinically until the end of the experiment and were compared for mortality rate and the clinical grade by the scoring method represented in Table 1 and as previously described[35,36]. Significant weight loss and decreased appetite were observed in the control group relative to the other treated groups, whereas rats treated with either probiotics or any of the three FMT doses, but not the model group, started to increase food intake and gain weight during the first week. The feeding behavior of rats in the high-dose FMT treatment group normalized during the second week (Figures 1 and 2). Together, the data presented here demonstrate that FMT enables the healing of chronic liver failure-induced HE.

FMT markedly improves learning and memory functioning in the rat HE model

The Morris water maze was used to evaluate the spatial learning of rats from different treatment groups. The Morris water maze test lasted six days. The first five days of acquisition training with an invisible platform were followed by 4 d of reversal training with an invisible platform. A probe trial was then carried out with no escape platform. Finally, four trials were conducted using the visible platform.

In contrast to the control group, the total travel distance was significantly decreased in the model group ($P = 0.014$), whereas the VSL or FMT treated

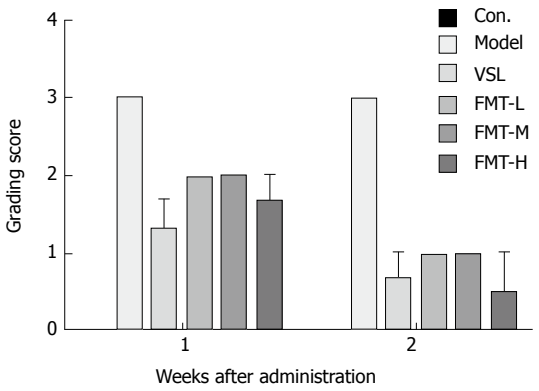


Figure 1 Animal behavior score after fecal microbiota transplantation. FMT: Fecal microbiota transplantation.

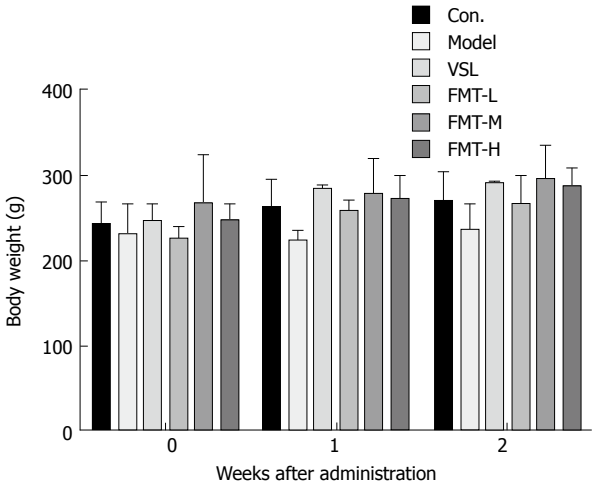


Figure 2 Fecal microbiota transplantation treatment enables body weight gain. FMT: Fecal microbiota transplantation.

rats showed longer travel distances compared with the model group ($P < 0.003$, Figure 3A). The swim speed was remarkably reduced in the model group, whereas it was markedly increased in rats that received probiotics or FMT (Figure 3B). The VSL and FMT groups exhibited twice as many line crossings than did the model group ($P < 0.05$, Figure 3C). Although crossing times increased along with FMT dose, this relationship did not reach statistical significance among FMT-L, FMT-M and FMT-H. Collectively, these observations indicate that FMT is capable of improving learning and memory deficits in the rat HE model.

FMT restores hepatic function in a dose-dependent manner

Since FMT effectively improved the behavior and spatial cognitive capability of rats with HE, we next determined whether FMT affected rat hepatic function in the HE model. It was noted that FMT improved hepatic function in a dose-dependent manner as revealed by hematoxylin and eosin staining.

Hepatic function was examined by liver histology and serological parameters including ALT, AST, ALB, TBIL and DBIL. All of these parameters were significantly elevated in the model group ($P < 0.0001$), which was indicative of severe liver damage. However, rats that received probiotics or FMT showed reduced levels of ALT, AST, ALB, TBIL and DBIL ($P < 0.0001$) compared with the model rats (Figure 4A-E). Notably, the FMT-H group revealed a marked decrease in these parameters when compared with FMT-L and FMT-M groups ($P < 0.0002$), suggesting a dose-dependent improvement in hepatic function. Moreover, liver hepatomegaly and severe liver disease were clearly observed on the liver surfaces of the model group whose livers also turned brown, indicating liver damage. On the contrary, liver necrosis of probiotic-treated rats was dramatically relieved. When treated with FMT, the livers showed decreased necrosis as the concentration of FMT increased (Figure 4F). However, the liver color was still brown. In agreement with altered liver morphology, liver histology analysis suggested that inflammation and spotty or patchy necrosis were observed in the model group, whereas none of these manifestations were observed in the control group. Livers from rats that received probiotics or FMT showed less necrotic areas than did the model rats (Figure 4G-L).

FMT prevents intestinal mucosal barrier dysfunction

The intestinal barrier controls physical and biochemical activities to maintain a balance with the external environment. Patients with liver cirrhosis develop a series of alterations in the intestinal barrier associated with the severity of liver disease that ultimately increase intestinal permeability. We therefore examined whether FMT could protect intestinal integrity in the HE model. As showed in Figure 5A, the intestinal permeability tripled in the model group compared with the control group ($P < 0.0001$), as detected by Evans blue extravasation method. FMT treatment remarkably decreased the intestinal permeability ($P < 0.0001$), whereas the VSL-treated group appeared to have even less intestinal permeability. Histological observations suggested mild edema and mucosal separation in the model group. FMT-treated HE rats displayed decreased edema, mucosal damage and inflammatory infiltration. Similar effects were observed when the HE rats were treated with probiotics (Figure 5F). Intestinal permeability was not different between the VSL and FMT treatment groups, irrespective of dose. Taken together, these findings support a protective role for FMT in maintaining intestinal integrity and attenuating the mucosal barrier dysfunction induced by HE. In accordance with altered intestinal permeability, expression ofA1AT in the stool was elevated in the model rats, which was indicative of excessive gastrointestinal protein loss. A1AT levels decreased in the rats that received probiotics or FMT, suggesting improved intestinal permeability and reduced gastrointestinal protein loss (Figure 5B).

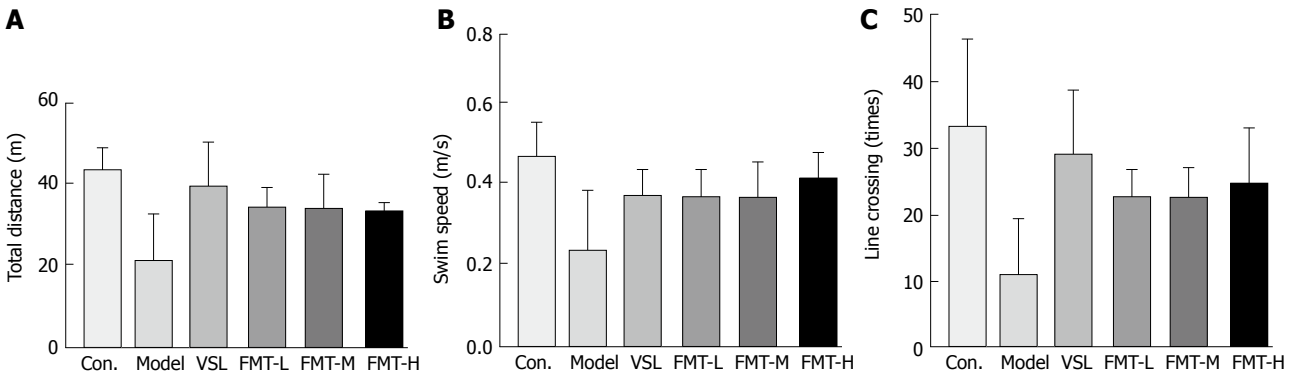


Figure 3 Cognitive function before and after VSL#3 or fecal microbiota transplantation treatment. A: FMT treatment increases rat total swim distance as revealed by the Morris water maze; B: Rats that received FMT showed improved swim speed; C: The number of line crossings in the FMT group was significantly increased relative to that of the model group. FMT: Fecal microbiota transplantation.

Table 1 Animal behavior scoring

Clinical score	Definition
0	Normal behavior
1	Mild lethargy
2	Decreased motor activity, poor gesture control, diminished pain perception
3	Severe ataxia, no spontaneous righting reflex
4	No righting reflex, no reaction to pain stimuli
5	Death

Elevated Calprotectin levels in the stool, which are commonly used in the clinic to measure the intestinal inflammation index, were found to be reversed by probiotics and FMT, implying attenuated intestinal inflammation (Figure 5C). Overall, the data presented here indicate that FMT preserves intestinal mucosal barrier function.

FMT attenuates serum ammonia levels and the systemic proinflammatory response

It is well known that ammonia serves as the most important precipitating factor leading to HE. In addition, accumulating evidence has demonstrated that inflammation, including systemic inflammation, neuroinflammation and endotoxemia, acts concomitantly with ammonia to drive HE pathogenesis in cirrhotic patients. We next determined whether FMT affects serum ammonia levels and proinflammatory responses. Serum ammonia levels and proinflammatory cytokines including IL-1β, IL-6 and TNF-α were detected using Berthelot reaction and ELISA assay. The mean ammonia level from the portal vein was 12.37 μmol/L in the control group, which was increased to 36.28 μmol/L in HE rats. When the HE rats were given probiotics, the mean serum ammonia level decreased to 22 μmol/L. FMT therapy resulted in a notable decline in ammonia levels ($P < 0.0001$). In particular, high-dose FMT administration was almost as effective as probiotic treatment in decreasing ammonia levels. Moreover, similar effects were observed when the ammonia level was evaluated from the tail vein, indicating that FMT enables effective clearance of serum ammonia and ultimately improves clinical symptoms (Figure 6A). Consistent with the decreased serum ammonia concentration, circulating levels of proinflammatory mediators such as IL-1β, IL-6 and TNF-α were elevated in the model group and reduced in groups that received probiotics or FMT treatment ($P < 0.0001$, Figure 6B-D). Significant differences in proinflammatory mediator levels were observed among the groups that received different FMT doses ($P < 0.002$). The data described here demonstrate a systematic relief of HE severity in rats, and it was clear that systemic inflammation, but not ammonia, was strongly correlated with increasing grades of HE.

FMT protects HE progression through distinct mechanisms

Although FMT has been shown to be effective in a rat model of HE, leading to improved hepatic and intestinal function and impaired systemic inflammation, the underlying mechanisms of these processes have not been investigated. Liver TLRs are reported to be important determinants of HE severity and are intimately related to arterial ammonia concentration and levels of circulating pro-inflammatory mediators[37-39]. Additionally, tight junction proteins (Occludin and Claudins) are implicated in the regulation of intestinal permeability[40,41].

Therefore, we examined the protein expression levels of TLR4 and TLR9 in the liver tissues, and the expression levels of Claudin-1, Claudin-6 and Occludin were analyzed in the intestinal tissues (Figure 7). As expected, liver dysfunction stimulated the expression of TLR4 and TLR9 in the liver, which was downregulated in liver tissue from rats treated with probiotics and FMT, indicating that FMT can limit systemic inflammation by decreasing the expression of TLR4 and TLR9. TLR4 and TLR9 are potent mediators that promote the expression of pro-inflammatory factors such as IL-6 and TNF-α. Accordingly, the expression of Claudin-1, Claudin-6 and Occludin was lost in the intestinal tissues from the model rats,

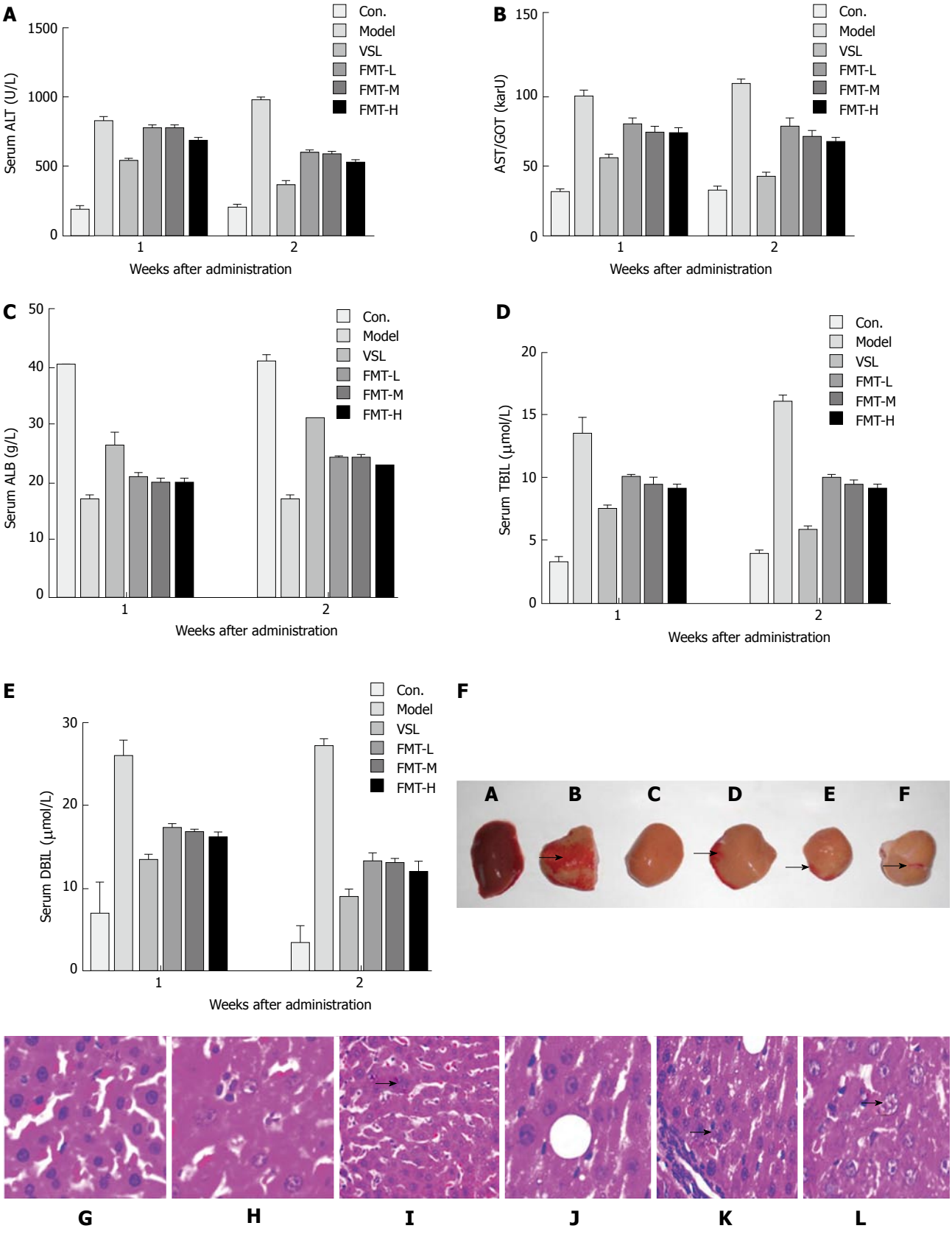


Figure 4 The changes of hepatic function and hepatic pathology before and after treatment with VSL#3 or fecal microbiota transplantation. A-E: Serological parameter examinations suggested improved hepatic function. Blood samples were collected from the indicated groups at the described time points and analyzed using an automatic biochemistry analyzer; F: Rats treated with FMT showed attenuated liver damage (arrow); J-L: FMT administration decreased CCl_4-induced liver necrosis in rats. I: Following VSL#3 treatment, liver dikaryocytes (arrow) were visible; K, L: Following FMT, liver cells with red nucleolus were visible (arrow). FMT: Fecal microbiota transplantation.

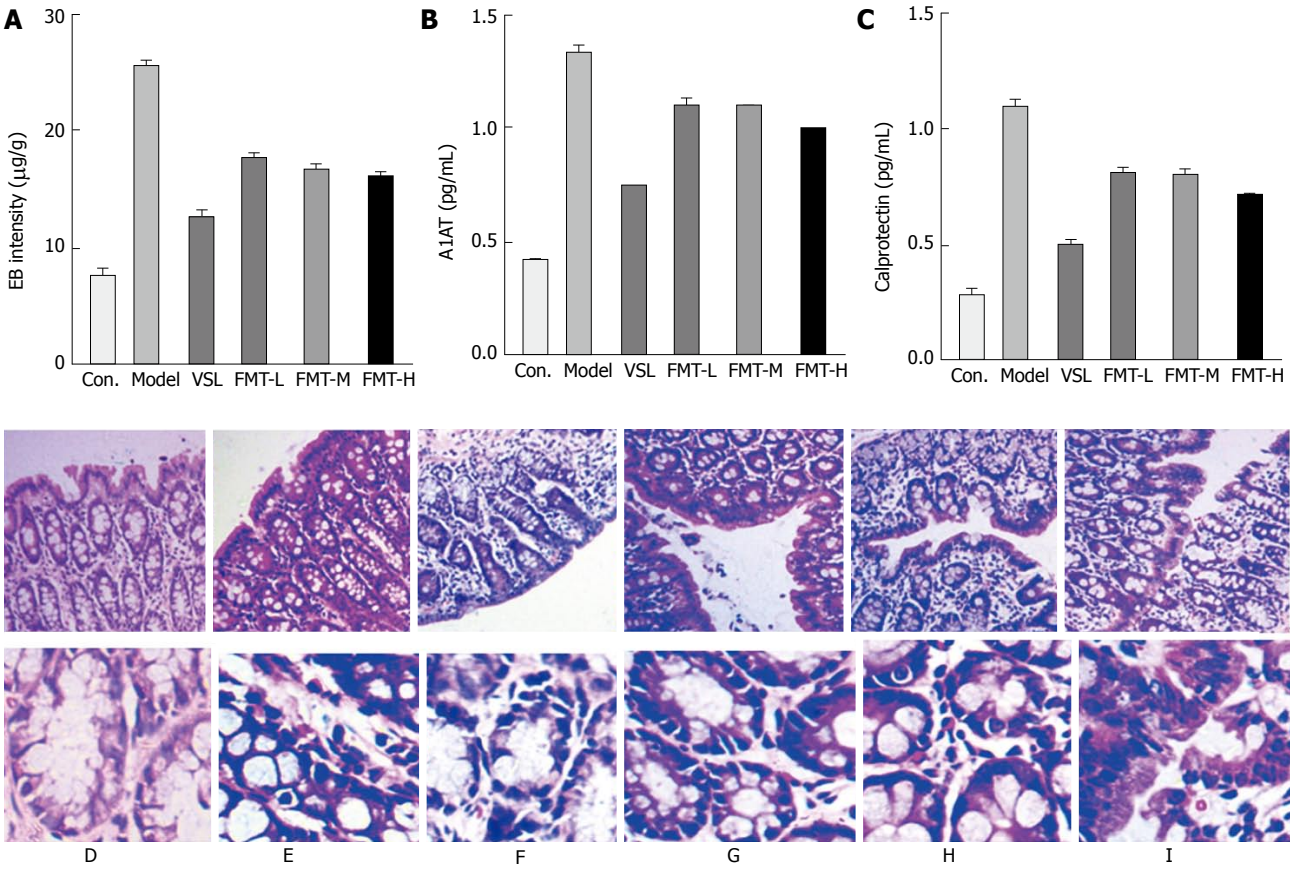


Figure 5 Expression of proteins related to intestinal permeability and intestinal wall stained with hematoxylin and eosin. A: FMT reduced intestinal permeability in HE rats as evidenced by Evans blue staining; B: Stool A1AT levels in rats were decreased following FMT treatment, which was indicative of improved intestinal permeability and reduced gastrointestinal protein loss. The stools in the indicated groups were collected and diluted in PBS and analyzed by ELISA; C: Stool Calprotectin levels in rats with HE were decreased following FMT treatment, which was indicative of reduced intestinal inflammation. Stools in the indicated groups were collected and diluted in PBS and analyzed by ELISA; D-I: Five-micron thick tissue sections were obtained and stained with hematoxylin and eosin for microscopic examination. The data shown are representative of hematoxylin and eosin staining in the indicated groups, but there were no differences among the tissues. FMT: Fecal microbiota transplantation.

suggesting impaired tight junction function. Tight junction protein loss was reversed by probiotic as well as FMT treatment. High dose FMT appeared to induce more expression of Claudin-1, Claudin-6 and Occludin than did other doses of FMT (Figure 6A and B). In summary, these data demonstrate that FMT delays HE progression in rats by reducing the liver expression of TLR4 and TLR9 and triggering tight junction protein expression, resulting in attenuated systemic inflammation and decreased intestinal permeability.

DISCUSSION

HE is a potentially reversible spectral neuropsychiatric complication caused by acute and chronic liver diseases, significantly affecting the prognosis. Although decades of clinical practice have demonstrated that nonabsorbable disaccharides, such as lactulose or lactitol, are effective for approximately 80% of HE patients, little survival benefit in cirrhotic HE patients has been observed following treatment with these agents. Probiotics, thought to have a therapeutic effect with no adverse effects, are not widely used clinically due to the potential risk of introducing live bacteria[42]. Acute liver organ shortage and increased morbidity have expanded research efforts aimed at improved treatment[28,43].

FMT is the introduction of a fecal suspension derived from a healthy donor into the gastrointestinal tract of a diseased individual. The clinical application of FMT has grown significantly within the last decade and is now gaining mainstream acceptance as a valuable, low cost procedure with apparently safe, and readily available materials, especially for the treatment of recurrent or refractory CDI[26,28,29]. Whether FMT is effective for HE patients has not yet been investigated. Here we provide experimental evidence that FMT has potent protective effects in improving motor activity in a rat model of HE and was comparable to probiotic administration. A significant negative correlation was found between the FMT dose and behavioral score. In addition, FMT has been shown to enhance spatial learning and memory as revealed by the Morris water maze assay. Since FMT effectively improves behavior and spatial cognitive capabilities, we next determined whether it affected hepatic function in

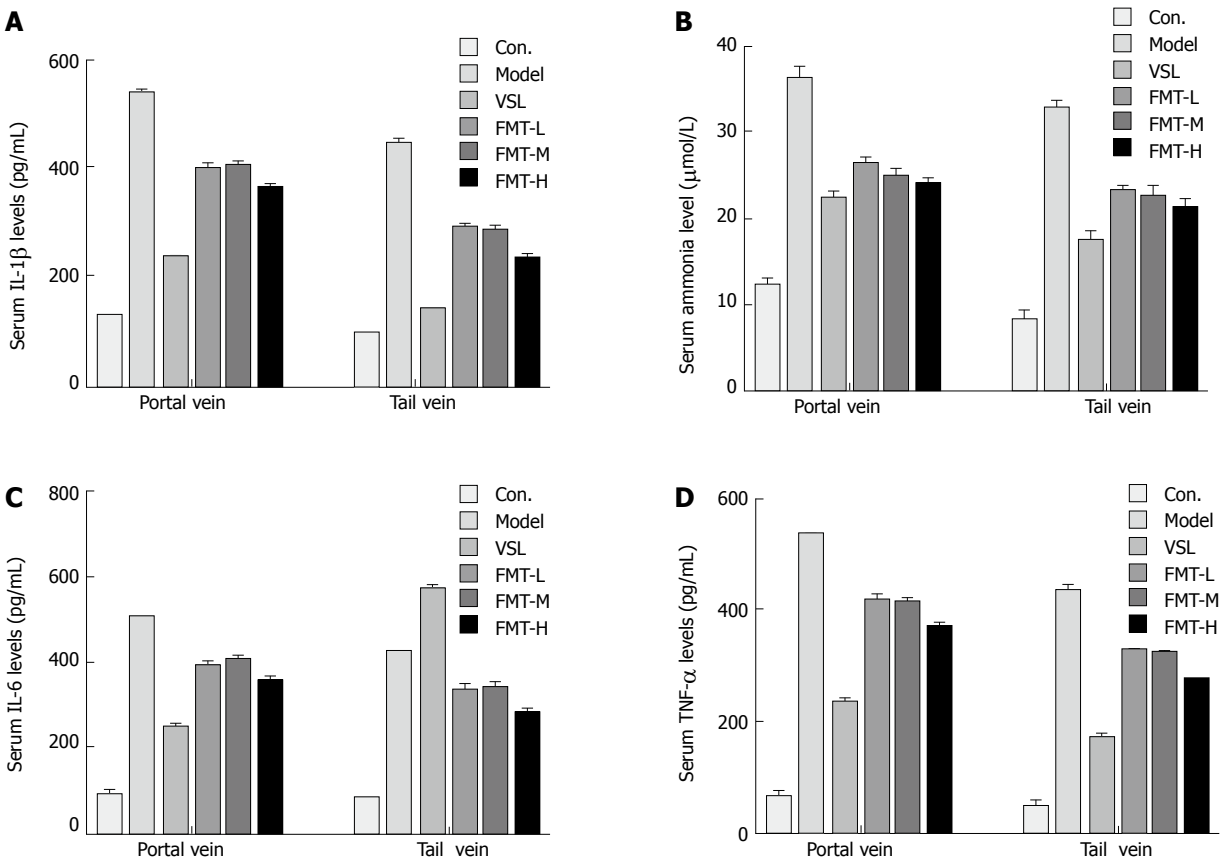


Figure 6 The changes of ammonia levels and inflammatory cytokines in plasma from the portal or tail veins. A: Plasma ammonia levels decreased significantly upon FMT treatment. Blood samples were collected from the portal and tail veins in the indicated groups and analyzed with an automatic biochemistry analyzer; B: Decreased circulating IL-1β was observed in the FMT groups. Blood samples were collected from the portal and tail veins in the indicated groups and analyzed by ELISA; C: Serum IL-6 was reduced in the FMT-treated groups. Blood samples were collected from the portal and tail veins in the indicated groups and analyzed by ELISA; D: Serum TNF-α was downregulated in the FMT-treated groups. Blood samples were collected from the portal and tail veins in the indicated groups and analyzed by ELISA. FMT: Fecal microbiota transplantation.

the rat HE model. Hepatic function was examined by liver histology and serological parameters. Rats that received FMT showed reduced levels of ALT, AST, ALB, TBIL, and DBIL ($P < 0.0001$) compared with the model rats. Notably, the FMT-H group revealed a marked decrease in these parameters when compared with the FMT-L and FMT-M groups ($P < 0.0002$). Moreover, liver necrosis in the FMT-treated rats was dramatically relieved compared with the model rats, suggesting a dose-dependent improvement in hepatic function. However, in general, the levels of cognitive function of rats that received FMT were altered more than other indicators.

Alterations in the intestinal barrier were associated with the severity of liver disease, leading to increased intestinal permeability[44-46]. We therefore examined whether FMT could protect against the intestinal permeability induced by HE. FMT treatment remarkably decreased the intestinal permeability ($P < 0.0001$), whereas histological observations suggested that FMT treatment in HE rats displayed decreased edema, mucosal damage and inflammatory infiltration. In accordance with decreased intestinal permeability, A1AT levels in the stool were reduced following FMT, suggesting improved intestinal permeability and reduced gastrointestinal protein loss. Elevated Calprotectin levels in the stool were also found to be reversed by FMT, implying that FMT prevents intestinal mucosal barrier dysfunction. Furthermore, FMT was found to attenuate serum ammonia levels and impair systematic inflammation as demonstrated by reduced proinflammatory cytokines including IL-1β, IL-6 and TNF-α, indicating a systematic relief of HE severity in rats. It was clear that systemic inflammation, but not ammonia, was strongly correlated with increasing grades of HE. Taken together, the data in present study clearly show a potent healing effect for FMT in the rat HE model. Next we tried to determine the possible mechanisms underlying the protective functions of FMT. It has been widely accepted that an impaired gut-liver-brain axis in patients with liver disease is the leading cause of complications including HE. In HE patients, bacterial translocation and increased intestinal permeability are frequently found, the latter of which promotes bacterial translocation, such as migration of microbes or their products including

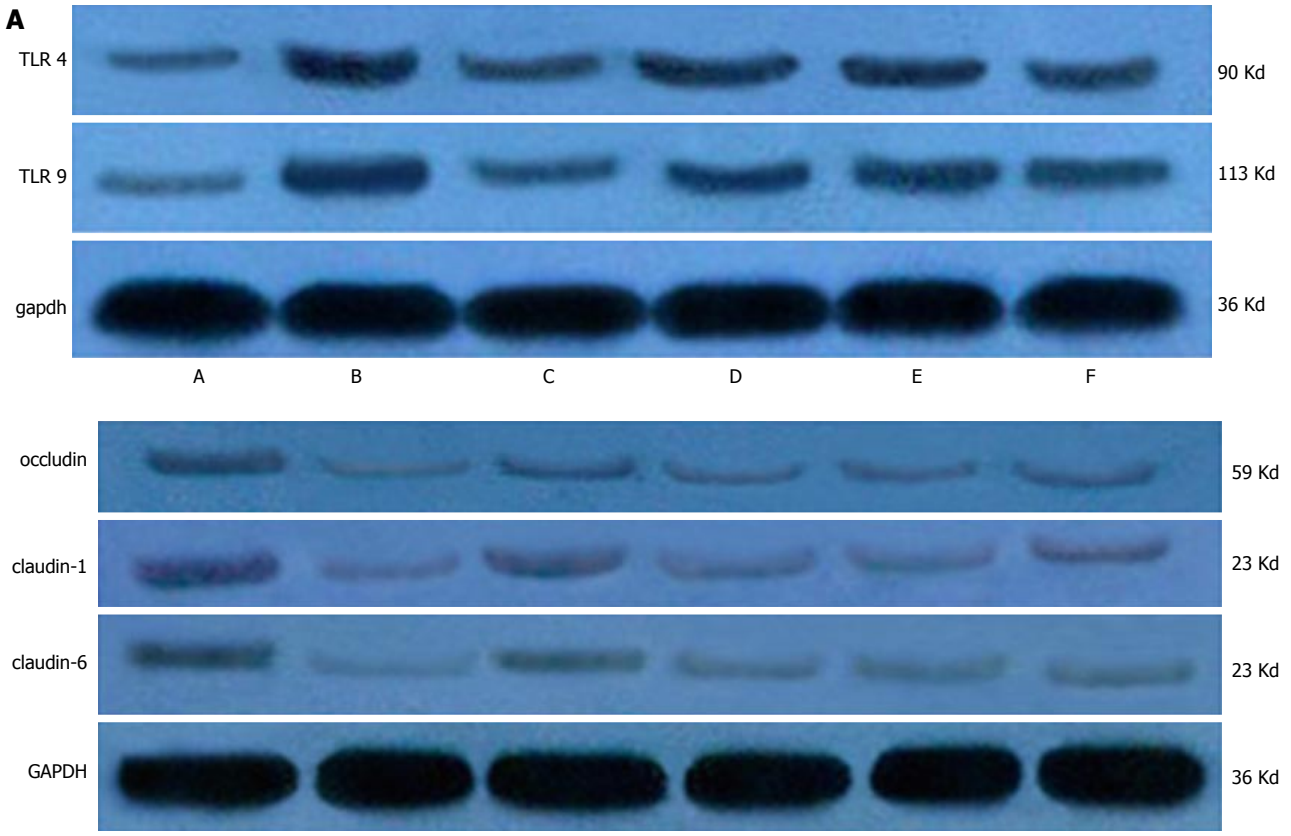


Figure 7 The expression of tight junction proteins in intestinal wall and Toll-like receptors. A: FMT induced the downregulation of liver TLR4 and TLR9 expression in a dose-dependent manner; B: FMT improved intestinal permeability by restoring the expression of Claudin-1, Claudin-6 and Occludin. FMT: Fecal microbiota transplantation.

pathogen-associated molecular patterns (PAMPs), the natural ligands for TLRs[40]. Liver expression of TLR4 and TLR9 is a critical determinant of HE severity, triggers the inflammation cascade and is intimately related to arterial ammonia concentration and circulating proinflammatory mediators such as IL-1β, IL-6 and TNF-α. We therefore evaluated the levels of liver TLR4 and TLR9 and circulating IL-1β, IL-6 and TNF-α in the HE model and FMT-treated rats.

Our data here suggest that VSL treatment efficiently reduced circulating IL-1β and TNF-α but not IL-6, a well-established independent risk factor for HE that was negatively associated with cognitive functions in HE patients[47-49]. Serum IL-6 was more evidently decreased in the FMT group than the VSL group in our study, which was consistent with more improved spatial learning and motor activity, suggesting that FMT is more effective than VSL, at least partially. Lata *et al*[50] also reported the limited effectiveness of VSL for HE.

Note that our study was the first to provide experimental data for potential use of FMT for HE, yet more accurate investigations are supposed to be done for optimized usage of FMT. Instead, VSL is commercially available with more detailed study.

FMT significantly reduced the liver expression levels of TLR4 and TLR9 and proinflammatory mediators, suggesting that FMT improves HE symptoms by impairing liver inflammation and reducing systemic inflammation. Importantly, the expression of tight junction proteins Claudin-1, Claudin-6 and Occludin was increased in the intestinal tissues of rats that were given FMT, indicating improved intestinal mucosal barrier function. The limitation of this study was the similar infection rate observed between the probiotics group and the FMT group. Our experiment still cannot fully explain whether FMT is superior to VSL#3 or not, which requires more experimental or clinical evidence.

Collectively, our study provided experimental evidence for the first time that supports an FMT-enabled protective role in treating HE rats and revealed the mechanisms behind FMT function, thus providing a basis for potential clinical application of FMT in HE patients.

COMMENTS

Background

Hepatic encephalopathy (HE) is a disease of liver dysfunction, and there is currently no effective therapy for treatment of this condition. To all knowledge, gut microbiota has emerged as a critical factor in the development of HE. Fecal microbiota transplantation (FMT) has been confirmed to treat some diseases. However, the effect of FMT on HE has not been researched deeply.

Research frontiers

Previous clinical evidence has shown that many diseases of the digestive tract, including liver diseases, have intestinal flora abnormalities, while FMT

can improve the composition and quantity of intestinal bacteria to achieve therapeutic purposes.

Innovations and breakthroughs

This is the first experimental study to confirm that FMT can be used to treat hepatic encephalopathy, and it also reflects the superiority of FMT relative to VSL#3.

Applications

According to the results of the article, FMT has the potential to become a new method for treating hepatic encephalopathy. At the same time, our results suggest that FMT has special advantages in some areas, providing more possibilities for further searching for new targets for treatment of hepatic encephalopathy.

Terminology

FMT is the introduction of a fecal suspension derived from a healthy donor into the gastrointestinal tract of a diseased individual. Initially as a treatment for Clostridium difficile infection, it has been shown to be effective in many diseases of the human body.

Peer-review

This is a very interesting paper about FMT for HE. The authors demonstrated that FMT has good effects in improving intestinal barrier function, by regulating the expression of tight junction proteins (Claudin-1, Claudin-6 and Occludin). Therefore, FMT has a protective role in treating HE.

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ORIGINAL ARTICLE

Basic Study

Mitofusin-2 mediated mitochondrial Ca^{2+} uptake 1/2 induced liver injury in rat remote ischemic perconditioning liver transplantation and alpha mouse liver-12 hypoxia cell line models

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Abstract

AIM

To investigate the protective mechanism of mitofusin-2 (Mfn2) in rat remote ischemic perconditioning (RIC) models and revalidate it in alpha mouse liver-12 (AML-12) hypoxia cell lines.

METHODS

Sprague-Dawley rats were divided into three groups (n = 6 each): sham, orthotopic liver transplantation and RIC. After operation, blood samples were collected to test alanine aminotransferase and aspartate aminotransferase. The liver lobes were harvested for histopathological examination, western blotting (WB) and quantitative real-time (qRT)-PCR. AML-12 cell lines were then subjected to normal culture, anoxic incubator tank culture (hypoxia) and anoxic incubator tank culture with Mfn2 knockdown (hypoxia + Si), and data of qRT-PCR, WB, mitochondrial membrane potential (ΔΨm), apoptosis, endoplasmic reticulum Ca^{2+} concentrations and mitochondrial Ca^{2+} concentrations were collected.

RESULTS

Both sham and normal culture groups showed no injury during the experiment. The RIC group showed amelioration of liver function compared with the orthotopic liver transplantation group (P < 0.05). qRT-PCR and WB confirmed that Mfn2-mitochondrial Ca^{2+} uptake 1/2 (MICUs) axis was changed (P < 0.005). In AML-12 cell lines, compared with the hypoxia group, the hypoxia + Si group attenuated the collapse of ΔΨm and apoptosis (P < 0.005). The endoplasmic reticulum Ca^{2+} decrease and mitochondrial Ca^{2+} overloading observed in the hypoxia group were also attenuated in the hypoxia + Si group (P < 0.005). Finally, qRT-PCR and WB confirmed the Mfn2-MICUs axis change in all the groups (P < 0.005).

CONCLUSION

Mfn2 participates in liver injury in rat RIC models and AML-12 hypoxia cell lines by regulating the MICUs pathway.

Key words: Remote ischemic per-conditioning; Ischemia-reperfusion injury; Ca^{2+}; Mitofusin-2; Mitochondrial Ca^{2+} uniporter

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Core tip: Compared to the orthotopic liver transplantation, the remote ischemic perconditioning (RIC) model can significantly improve liver functions. But, knowledge of its mechanism remains largely unknown. This research is the first to prove the protective mechanism of the mitofusin-2-mitochondrial Ca^{2+} uptake 1/2 axis by affecting the metabolism of intracellular calcium in the RIC model of liver transplantation and to revalidate it in alpha mouse liver-12 hypoxia cell lines.

Liang RP, Jia JJ, Li JH, He N, Zhou YF, Jiang L, Bai T, Xie HY, Zhou L, Sun YL. Mitofusin-2 mediated mitochondrial Ca^{2+} uptake 1/2 induced liver injury in rat remote ischemic perconditioning liver transplantation and alpha mouse liver-12 hypoxia cell line models. *World J Gastroenterol* 2017; 23(38): 6995-7008 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/6995.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.6995

INTRODUCTION

Liver transplantation (LT) has become an important countermeasure for end-stage liver disease[1]. Ischemia-reperfusion injury (IRI) is one of the severe complications of LT, leading to graft dysfunction and thus increasing the morbidity and mortality[2-4]. Our previous studies confirmed that ischemia preconditioning (IPC) can effectively alleviate IRI by reducing hepatic enzymatic leakage, leukocyte infiltration and apoptosis formation[5,6]. However, because of its traumatic nature and ethical reasons, its application has failed to develop extensively[7]. Remote ischemic perconditioning (RIC) was first proposed by Przyklenk in 1993, and compared with IPC, it is a temporary ischemic treatment for distant organs and plays a protective role without damaging the target organs[8]. We have optimized the application of RIC and proved that the PI3K/Akt/eNOS/NO pathway could reduce the damage[9,10].

Mitofusin-2 (Mfn2), localized in the outer membrane of the mitochondria, maintains the network stability of mitochondria by participating in mitochondrial fusion and fission[11]. Ca^{2+} is an important intracellular signaling pathway, involved in important pathophysiological functions such as maintenance of biological potential, cell growth/proliferation regulation and apoptosis regulation[12-15]. Vasington *et al*[16] discovered mitochondrial Ca^{2+} uptake 1/2 (MICUs) in 1962. They used two Ca^{2+}-binding EF hands to sense Ca^{2+} concentration, thereby maintaining mitochondrial Ca^{2+} stability, which were considered as "gatekeepers" for mitochondrial Ca^{2+} influx[17-20]. Furthermore, they are mediated by the mitochondrial Ca^{2+} uniporter (MCU) and form an MCU protein complex together with MCU, MICU-1 and MICU-2[21,22]. In our previous

work, we also observed that Mfn2 induced apoptosis in HCC cells due to down-regulation of the MICUs gatekeepers[11].

There is little information about relationships between the protective effect of RIC and the Mfn2-MICUs axis during LT. Ca^{2+} plays an important role in apoptosis. In our previous RIC experiment, compared with the orthotopic liver transplantation (OLT) group, the RIC group showed decreased apoptosis. In the present study, we hypothesized and verified whether RIC model *via* Mfn2-MICUs axis has a protective effect for LT. As it is hard to perform gene knockout operations using primary cells, genetically engineered rats are expensive and the experiment is time-consuming, we designed the use of alpha mouse liver-12 (AML-12) hypoxia cell lines to simulate the RIC model and to revalidate it to prove our hypothesis.

MATERIALS AND METHODS

Rat sham, OLT and RIC models

All experimental protocols were conducted in accordance with the Animal Research: Reporting *in vivo* Experiments (ARRIVE) guidelines (http://www.nc3rs.org/ARRIVE). Adult male Sprague-Dawley rats weighing 250-300 g were chosen, placed in standard environment, and allowed *ad libitum* access to food and water. Thirty rats (including 12 donors) were randomly allocated to three groups (n = 6 in each group): sham, OLT and RIC. The following operations were performed: After fasting for 12 h, all rats were anesthetized for 3 h by intraperitoneal injection with 4% chloral hydrate (Shanghai Guchen Biological, China).

In the OLT group, the modified method of Kamada was adopted[23]. Donor liver was obtained, and cold physiological saline containing 25 U/mL heparin was used to perfuse the graft liver through portal vein and stored in 0-4 ℃ cold saline for about 45 min before the graft was transplanted into the recipient. After the suprahepatic inferior vena cava and portal vein were anastomosed by cuff method, the liver was reperfused along with the ligation of the hepatic artery. Then, the infra-hepatic inferior vena cava was connected, and common bile duct was reconstructed by tying the duct over a stent. Physiological saline (1 mL) was injected *via* the dorsal penile vein of the recipient after operation in order to prevent acid-base imbalance.

In the RIC group, the method used in our previous study was adopted. In the recipients who underwent OLT, hindlimb ischemia (using a standard tourniquet appending 1 kg weight to both legs) and reperfusion was performed for three 5-min cycles starting at the anhepatic phase[10] (Figure 1). After recovery from anesthesia, the rats allowed free access to sterile water and standard diet.

In the sham group, the abdomen was opened after 70 min, and then closed in order to acquire the mean time of the total ischemia in the OLT/RIC group (2-min warm ischemia, 45-min cold ischemia, and 70-min total ischemia).

Collection of liver lobe and blood samples

Sham group rats were euthanized after anesthesia. After 3 h of blood flow into the recipient's portal vein, euthanasia was performed in OLT and RIC groups. Blood samples were drawn from the portal vein and then centrifuged for 15 min at 3000 × g, and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured to assess liver function (7600 automatic analyzer; Hitachi, Japan). Some of the liver lobes were kept in formalin solution for histopathological examination, and the other parts of the lobes were used for western blotting (WB) and PCR studies.

Quantitative real-time (qRT)-PCR

qRT-PCR was used to detect the level of mRNA expression (Table 1). Total RNA was extracted from the previous phase of the experiment (liver lobes and cell lines) using Trizol reagent (Thermo Fisher Scientific, United States) and PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Japan) for reverse transcription to cDNA. Following the protocol, we performed qRT-PCR in a total volume of 10 μL with 7900Fast (Applied Biosystems Inc, United States) to test the efficiency of small interfering (si)RNA in the hypoxia + Si group. The mRNA levels of Mfn2, MCU, MICU-1 and MICU-2 in the three model groups of sham, OLT and RIC and three cell line groups of normal culture (NC), hypoxia and hypoxia + Si were compared.

WB

WB was used to detect the level of protein expression of Mfn2, MCU, MICU-1, MICU-2 and β-actin. Total proteins were extracted from the previous phase of the experiment (liver lobes and cell lines). Cells and tissues (the liver tissues had first been ground in liquid nitrogen) were transfected after incubation in RIPA lysis buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific) for 1 h on ice and then underwent ultrasonic oscillation crushing (Shanghai Experimental Instruments, China) for three 5-s cycles on ice. After centrifugation (14000 × g, 4 ℃, 15 min), the supernatant was collected for protein concentration measurement by Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, United States).

The denatured protein samples (30 μg/10 μL) were separated by SDS-PAGE (Invitrogen, United States) and transferred to polyvinylidene fluoride membranes. Then, the membranes were blocked with 5% non-fat milk (BD Biosciences, United States) dissolved in 30 mL Tris-buffered saline with Tween (TBST) for 2 h at room temperature. Using primary antibodies against β-actin/Mfn2 (1:1000; Abcam, United States),

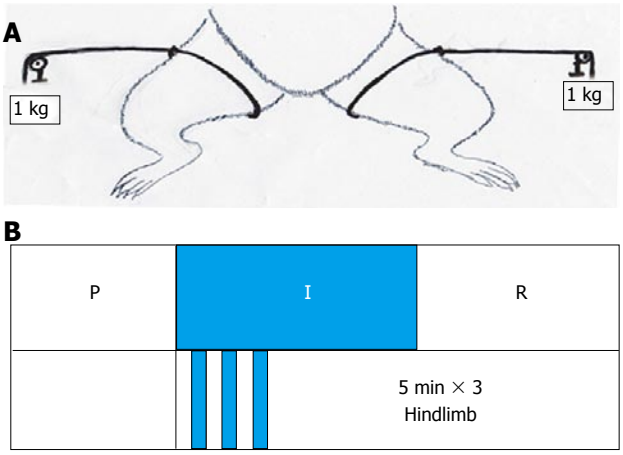


Figure 1 Remote ischemic perconditioning models. A: Tourniquet was used for ligature of each hindlimb with 1 kg additional weight; B: P - Perfusion phase, I - Ischemic phase, R - Reperfusion phase. The remote ischemic perconditioning group underwent orthotopic liver transplantation with hindlimb ischemia and reperfusion for three 5-min cycles starting at the beginning of the anhepatic phase.

MCU/MICU-1 (1:1000; Cell Signaling Technology, United States) and MICU-2 (1:1000; Santa Cruz Biotechnology, United States), the membranes were incubated overnight at 4 ℃.

After agitation washing of the membranes for three 5-min cycles with TBST, appropriate secondary horseradish peroxidase-linked antibodies (1:2000; all purchased from Abcam), including goat anti-mouse IgG for total Mfn2, goat anti-rabbit IgG for β-actin and MCU/MICU-1/MICU-2, were used to incubate the membranes for 1 h at room temperature. An ECL kit (Pierce Biotechnology) was used to acquire enhanced chemiluminescence.

Cell lines and culturing

The mouse liver hepatocyte cell line AML-12 [established from hepatocytes from a male mouse (CD1 strain, line MT42) non-tumorigenic], preserved by our laboratory, was cultured according to the ATCC guideline, with 10% fetal bovine serum (Gibco, United States) added in a 90% 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Hangzhou Genom Biochemical and Pharmaceutical, China) with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium and 40 ng/mL dexamethasone (all purchased from Sigma-Aldrich). All cells were cultured in 5% CO_2 at 37 ℃ in a moderately moist environment.

Cells were assigned to three groups (NC, hypoxia and hypoxia + Si) in the logarithmic phase, and less than 70% had good conditions. The NC group cells were cultured using the normal protocol. The hypoxia group cells were first cultured for 3 h in a humidified atmosphere containing 2% O_2, 5% CO_2 and 93% N_2 at 37 ℃ in a noxic incubator tank (Thermo Fisher Scientific), and then cultured for another 3 h in normal atmosphere as NC group cells to simulate the IRI process. The hypoxia + Si cells were first used in siRNA experiments (Table 2; Shanghai GenePharma Biochemical and Pharmaceutical, China) to knock down the Mfn2 expression. The same culturing was performed in the hypoxia group. Cells were collected for subsequent experiments.

Table 1 Primer sequences for qRT-PCR

Target gene	Primer sequence
Rat-*β-Actin*	Forward: 5'-ACGGTCAGGTCATCACTATCG-3'
	Reverse: 5'-GAGGTCTTTACGGATGTCAACG-3'
Rat-*Mfn2*	Forward: 5'-GAAGAAGAGTGTCAAGACCGTG-3'
	Reverse: 5'-CAGGCAAAACTTATCAATCCAG-3'
Rat-*Mcu*	Forward: 5'-GACCCTGAACGATGTGAAGAC-3'
	Reverse: 5'-TTCTCCGCTTTCCTGCTAAT-3'
Rat-*MICU-1*	Forward: 5'-GACTAAGCGGAGACTGATGTTG-3'
	Reverse: 5'-GAGATTCTGCGTGAGCCTTC-3'
Rat-*MICU-2*	Forward: 5'-ACAAAGCCTCACTCTGGGT-3'
	Reverse: 5'-TCACTGTTGGTTCCTGGTATT-3'
Mus-*β-Actin*	Forward: 5'-CATTGCTGACAGGATGCAGAAGG-3'
	Reverse: 5'-TGCTGGAAGGTGGACAGTGAGG-3'
Mus-*Mfn2*	Forward: 5'-CAAGTGTCCGCTCCTGAAG-3'
	Reverse: 5'-CCACCAGCACAAACACATC-3'
Mus-*Mcu*	Forward: 5'-GAGCCGCATATTGCAGTACGGT-3'
	Reverse: 5'-AAACACGCCGACTGAGTCAGAG-3'
Mus-*MICU-1*	Forward: 5'-GAAGTGTCCAGCCGTGAAGGAA-3'
	Reverse: 5'-TGGTGTGGAGTAGGCTCGGATT-3'
Mus-*MICU-2*	Forward: 5'-GGAAGACTTTGCTATCGCCATGC-3'
	Reverse: 5'-GGTGTCCAGAAGATTGTCCGAG-3'

Mfn2: Mitofusin-2; *Mcu*: Mitochondrial Ca^{2+} uniporter; *MICU*: Mitochondrial Ca^{2+} uptake.

Table 2 Primer sequences for siRNA

Target gene	Primer sequence
Mus-*Mfn2*	Sense strand: 5'-CUGCGAAUUAAGCAGAUUATTdTdT-3'; Anti-sense strand: 5'-UAAUCUGCUUAAUUCGCAGTTdTdT-3'

Mfn2: Mitofusin-2.

Mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was quantified using a JC-1 kit (Beijing Beyotime Institute of Biotechnology, China). JC-1 was used to cultivate the cells for 30 min, which was measured by flow cytometry (FCM) in the channels of FITC (green) and PE-A (red) of the FC500 flow cytometer (Cytomics, United States). Furthermore, cells were cultured for 30 min using JC-1 and for 15 min using Hoechst-33342 (Beijing Beyotime Institute of Biotechnology), and observed under confocal microscopy (FV1000; Olympus, Japan) to confirm the results obtained from FCM.

Apoptosis

Cell apoptosis was assayed using the Annexin V/Propidium Iodide Apoptosis (AV/PI) Detection Kit (BD Biosciences) and measured by FCM. Furthermore, cells were treated with 7.5 μmol/L of the CellEvent Caspase

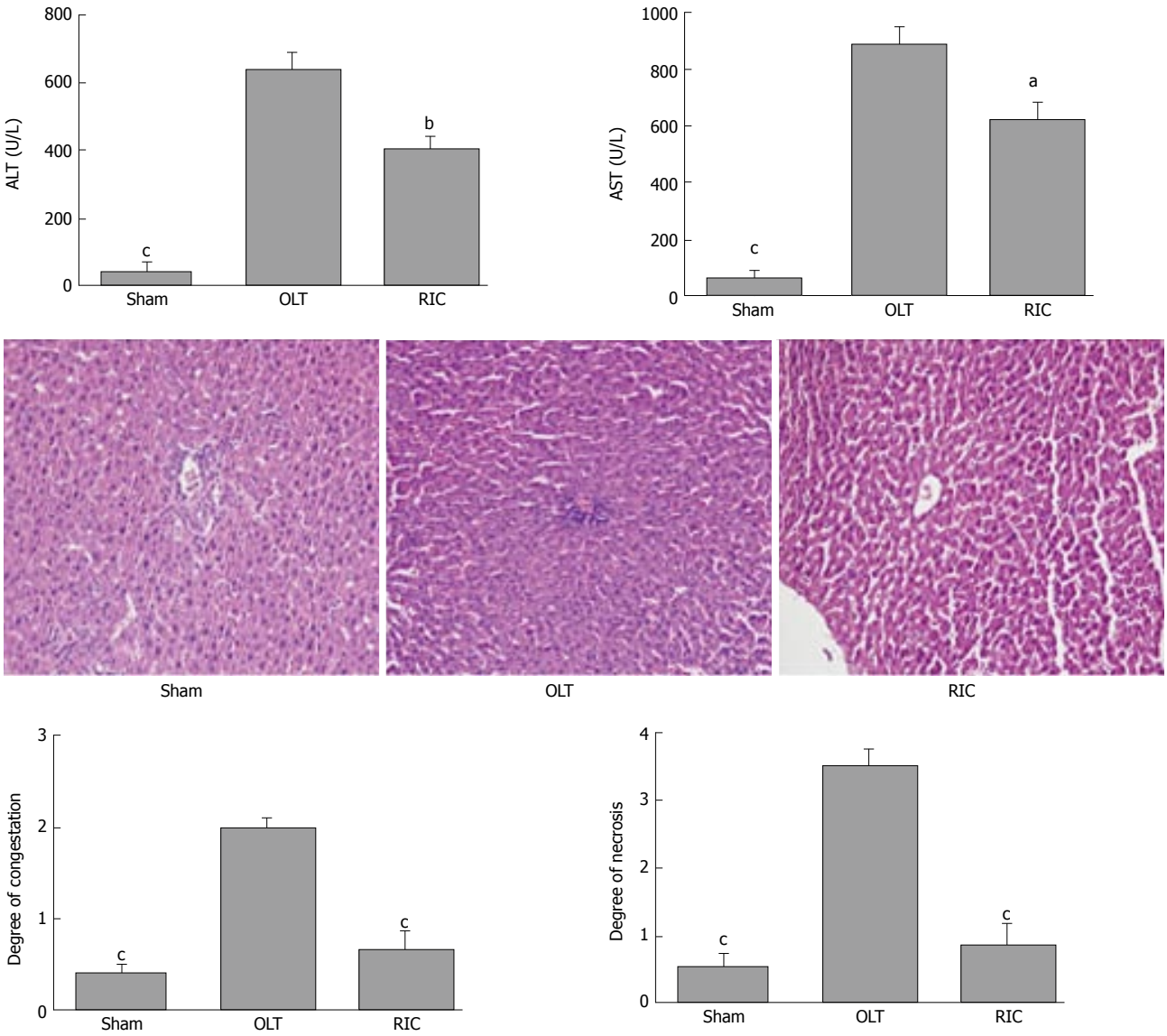


Figure 2 Remote ischemic perconditioning models model improves the liver graft function. A: Results of ALT and AST in the three model groups; B: Liver histopathological presentations (hematoxylin and eosin stain, original magnification × 200); C: Results of hepatocyte necrosis and congestion of the sinusoids and central vein in histopathologic examination. Data represent mean ± SEM for 6 rats per group. ALT: Alanine aminotransferase; AST: aspartate aminotransferase; OLT: Orthotopic liver transplantation; RIC: Remote ischemic perconditioning. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

3/7 Green Detection Reagent (Invitrogen, United States) and 10 nmol/L tetramethylrhodamine methyl ester perchlorate (Invitrogen), and observed under confocal microscopy to confirm the results obtained from FCM.

Measurement of mitochondrial Ca^{2+} concentrations

Rhod-2 acetoxymethyl ester (Rhod-2, AM; GeneCopoeia, United States) was used to measure the mitochondrial Ca^{2+} concentrations. According to our previous experience, the fluorescence value of Rhod-2 AM approximately represented the fluorescence value of mitochondrial Ca^{2+}[11,24]. The day before the experiment, 1 x 10^5 cells were plated in 10-mm confocal dishes (Nest Biotechnology, China). After overnight incubation, the growth medium was removed from the cell plates, and added with 500 μL medium with 4 μM Rhod-2 AM and 0.1% Pluronic® F-127 (GeneCopoeia) and allowed to incubate at 37 ℃ in humidified air (containing 5% CO_2) for 2 h. Next, D-Hanks balanced salt solution (Hangzhou Genom Biochemical and Pharmaceutical, China) was used to wash the cells three times, followed by addition of 500 μL Hoechst-33342 and 200 nmol/L MitoTracker Green (Beijing Beyotime Institute of Biotechnology), and allowed to incubate in the same environment for 30 min of cultivation. Confocal microscopy confirmed that the vast majority of Rhod-2 AM fluorescence was associated with the mitochondria. Furthermore, cells were incubated in 96-well plates with Rhod-2 AM and F-127 in the same process and observed by VarioskanFlash (Thermo Fisher Scientific) with the illumination at excitation 552 nm and emission 581 nm to confirm the results obtained from confocal

microscopy.

Quantification of endoplasmic reticulum (ER) Ca^{2+} concentrations

We used the FLUOFORTE® Calcium Assay (Enzo Life Sciences, United States) to detect ER Ca^{2+} concentrations. The day before the experiment, 5 × 10^4 cells were plated per well in 96-well assay plates (Corning, United States). After overnight incubation, the growth medium was removed from the cell plates and 100 μL FLUOFORTE® Dye-Loading Solution (prepared by Enzo's protocol, containing 0.5 mmol/L EGTA to eliminate Ca^{2+} concentrations in the medium) was added to each well, and the cells were incubated at room temperature for 45 min. The illumination at excitation 490 nm and emission 525 nm was observed by VarioskanFlash for quantification of cytosolic Ca^{2+} concentrations at baseline. Then, 5 μM thapsigargin (Thermo Fisher Scientific) was added to each measurement well to detect the total values, including cytosolic baseline and Ca^{2+} released from the ER, by VarioskanFlash with the same parameters. SkanIt software (Thermo Fisher Scientific) was used to measure the ER Ca^{2+} concentrations.

Statistical analysis

The results were expressed as mean ± SEM. One-way analysis of variance was used for comparisons among three groups and the *t*-test was used for comparison between the two groups. All statistical analyses and related statistical diagramming were performed using the GraphPad Prism software (ver. 5.0 for Windows; GraphPad Software Inc., United States). $P < 0.05$ was considered statistically significant.

RESULTS

RIC model improves liver graft function

According to the literature and our previous research, RIC does not cause muscle and remote organ function damage, so this experiment focused on liver function[9,10]. In the present study, the sham group showed no injury during the experiment. Compared with the OLT group, ALT and AST levels were lower in the RIC group (ALT: $P = 0.0029$, AST: $P = 0.0121$; Figure 2A). Liver histopathological examinations showed that the RIC group had fewer sinusoids, central vein congestion and hepatocyte necrosis than the OLT group ($P < 0.001$; Figure 2B and C).

RIC model lowers expression of Mfn2 and alters MICUs expression

Expression of Mfn2 and MICUs was measured by WB (Figure 3A) and qRT-PCR (Figure 3B). Taking the OLT group as the reference, both the sham and RIC groups showed lower expression of Mfn2 (WB: sham *vs* RIC: $P < 0.001$; qRT-PCR: sham $P < 0.001$, RIC $P = 0.0348$). The MICUs expression was also altered. Compared with the OLT group, both the sham and RIC groups showed higher expression of MICU-1 (WB: sham *vs* RIC, $P < 0.001$; qRT-PCR: sham $P = 0.0005$, RIC $P = 0.0030$) and similar expression of MICU-2 (WB: sham $P = 0.0011$, RIC $P = 0.0033$; qRT-PCR: sham $P = 0.0255$, RIC: $P = 0.0137$). However, we did not observe any significant changes in the expression of MCU (WB: sham $P = 0.1044$, RIC $P = 0.9243$; qRT-PCR: sham $P = 0.0005$, RIC $P = 0.1507$).

Mfn2 knockdown alters MICUs expression in AML-12 hypoxia cell lines

The NC group was cultured in normal environment. The hypoxia group followed our protocol to simulate the IRI process. In the hypoxia + Si group, siRNA was used to knock down Mfn2 expression (WB and qRT-PCR; Figure 4A) and culturing was performed by the same protocol as in the hypoxia group. Expression of MICUs was measured by WB and qRT-PCR. Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups showed higher expression of MICU-1 (WB: NC $P = 0.0001$, hypoxia + Si $P = 0.0010$; qRT-PCR: NC $P = 0.0026$, hypoxia + Si $P = 0.0430$) and similar expression of MICU-2 (WB: sham $P < 0.0001$, hypoxia + Si $P = 0.0020$; qRT-PCR: sham $P = 0.0004$, RIC $P = 0.0137$). We observed the change in expression of MCU by WB (sham: $P = 0.0158$, RIC: $P = 0.0040$), but did not obtain significant findings by qRT-PCR (sham: $P = 0.2597$, RIC: $P = 0.2420$) (Figure 4B and C).

Mfn2 knockdown attenuates the collapse of ΔΨm in AML-12 hypoxia cell lines

We first used the JC-1 kit to measure the ΔΨm in AML-12 hypoxia cell lines cultured by FCM with our protocol. JC-1 fluorescence shifted from red (JC-1 aggregates bound to mitochondria) to green (JC-1 monomers bound to cytoplasm), indicating a decline of ΔΨm. Taking the hypoxia group as the reference, the green to red fluorescence ratio was lower in the NC and hypoxia + Si groups (FCM: NC $P = 0.0329$, hypoxia + Si $P = 0.0391$; Figure 5A and C). We obtained the same result by confocal microscopy as that obtained from FCM using the JC-1 kit and Hoechst-33342 (Figure 5B).

Mfn2 knockdown attenuates apoptosis in AML-12 hypoxia cell lines

The Annexin V/Propidium Iodide Apoptosis Detection Kit was used to detect cell apoptosis by FCM. Annexin V fluorescence reflects early cell apoptosis while propidium iodide fluorescence reflects late apoptosis and dying cells. Taking the hypoxia group as the reference, the ratio of apoptosis cells was lower in the NC and hypoxia + Si groups (FCM: NC $P = 0.0092$, hypoxia + Si $P = 0.0369$; Figure 6A and C). We obtained the same result by confocal microscopy as that obtained from FCM using the caspase 3/7

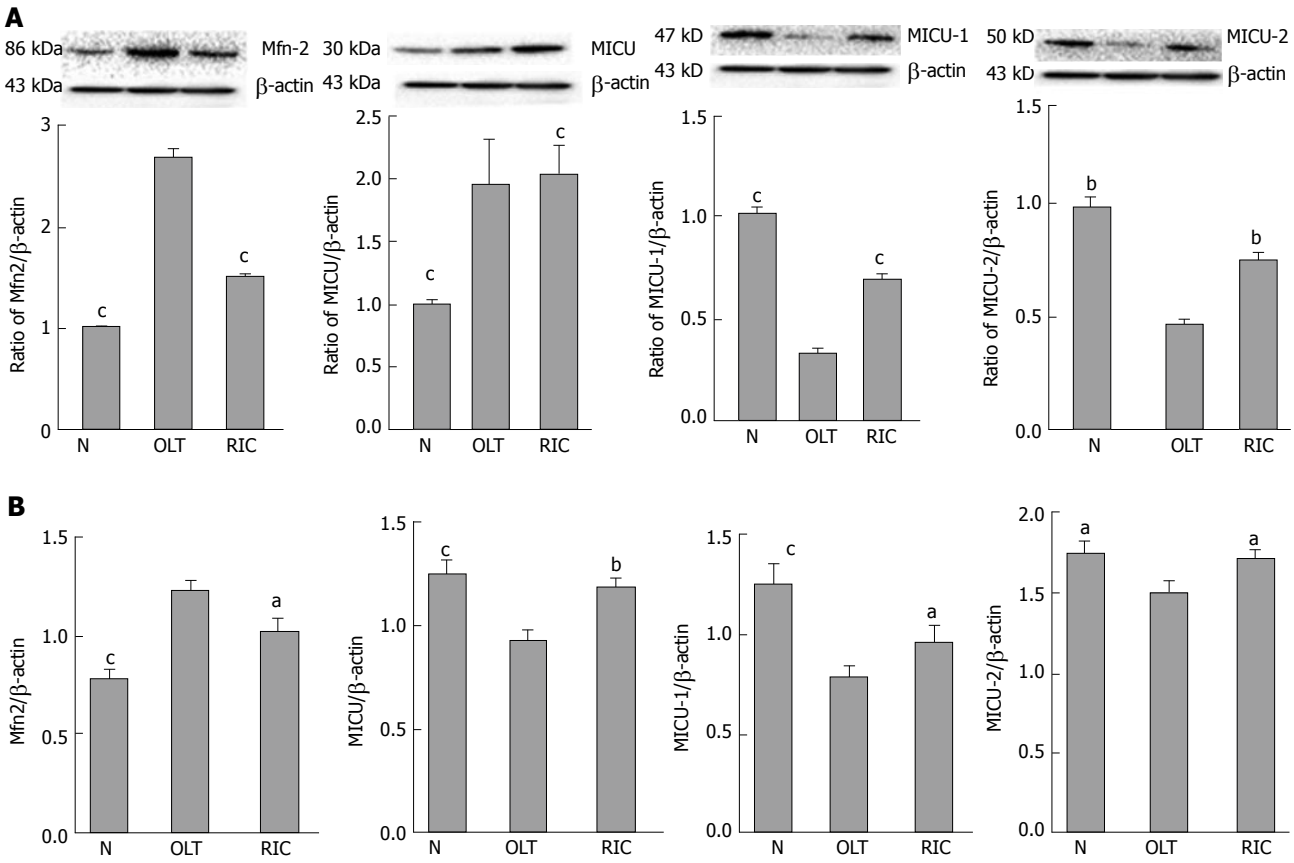


Figure 3 Remote ischemic perconditioning models model causes low expression of Mfn2 and alters MICUs expression. Expression of Mfn2 and MICUs was measured by WB (A) and qRT-PCR (B). Both sham and RIC groups showed lower expression of Mfn2 and altered MICUs expression, but no significant change was observed in expression of mitochondrial Ca^{2+} uniporter. Mfn2: Mitofusin-2; MICUs: Mitochondrial Ca^{2+} uptake 1/2; OLT: Orthotopic liver transplantation; qRT-PCR: Quantitative real-time-PCR; RIC: Remote ischemic perconditioning; WB: Western blotting. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

detection kit (green) and tetramethylrhodamine methyl ester perchlorate (red) (Figure 6B).

Mfn2 knockdown relieves mitochondrial Ca^{2+} overloading

We used Rhod-2 AM to detect mitochondrial Ca^{2+} concentrations by confocal microscopy and VarioskanFlash. Rhod-2 AM, Hoechst-33342 and MitoTracker Green were used to confirm that the fluorescence value of Rhod-2 AM approximately represented the fluorescence value of mitochondrial Ca^{2+} (Figure 7A). Then, VarioskanFlash was used to analyze the illumination at excitation 557 nm and emission 581 nm and detect mitochondrial Ca^{2+} concentrations (Figure 7B). Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups relieved mitochondrial Ca^{2+} overloading (NC *vs* hypoxia + Si: $P < 0.0001$).

Mfn2 knockdown relieves endoplasmic reticulum Ca^{2+} decrease

We used the FLUOFORTE® Calcium Assay to detect ER Ca^{2+} concentrations by analyzing the illumination at excitation 490 nm and emission 525 nm with VarioskanFlash. The cytosolic baseline was measured first, and then thapsigargin (Ca^{2+} was released by inhibiting endoplasmic reticular Ca-ATPase) was added to detect the total values, including cytosolic baseline and Ca^{2+} release from the ER. SkanIt software was used to measure the ER Ca^{2+} concentrations. Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups showed higher ER Ca^{2+} concentrations (NC *vs* hypoxia + Si: $P < 0.0001$) (Figure 7).

DISCUSSION

The incidence of liver cancer is increasing and liver cancer currently represents the sixth most frequent type of cancer worldwide[25]. LT has become an important countermeasure for end-stage liver diseases, and numerous research studies have been carried out to improve LT outcome by reducing the damages, such as IPC and RIC[26,27]. We have demonstrated in our previous studies that the RIC model protects the liver against IRI, causing no muscle or remote organ function damage[9,10], but the mechanism is not yet fully understood.

Mitochondrial Ca^{2+} plays an important role in intracellular homeostasis[28]. Mfn2 maintains the network stability of mitochondria by participating in

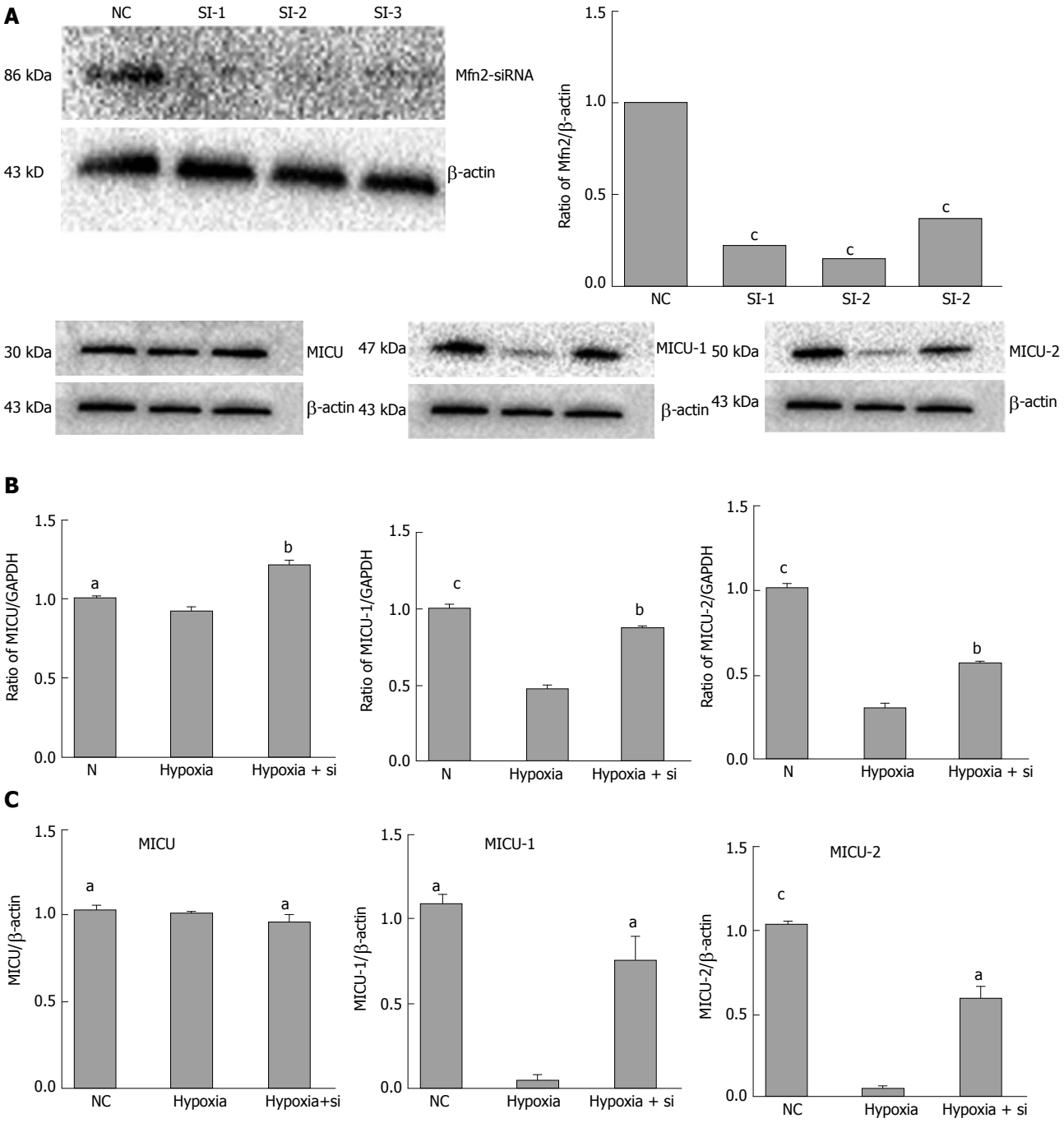


Figure 4 Mfn2 knockdown alters MICUs expression in AML-12 hypoxia cell lines. A: Mfn2 knockdown was tested by WB and qRT-PCR. Expression of MICUs in AML-12 hypoxia cell lines was measured by WB (B) and qRT-PCR (C). Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups showed higher expression of MICU-1 and similar expression of MICU-2; MCU altered expression in WB, but no significant change in qRT-PCR. AML: Alpha mouse liver; MCU: Mitochondrial Ca^{2+} uniporter; Mfn2: Mitofusin-2; MICU: Mitochondrial Ca^{2+} uptake; NC: Normal culture; qRT-PCR: Quantitative real-time-PCR; WB: Western blotting. $^{a}P < 0.05$; $^{b}P < 0.01$.S $^{c}P < 0.001$.

mitochondrial fusion and fission[29]. MCU, MICU-1 and MICU-2 form the mitochondrial calcium uniporter complex. Along with the increase in intracellular Ca^{2+}, Ca^{2+} combined with EF-hand of MICUs promotes the opening of MCU pores and leads to an increase in the mitochondrial Ca^{2+}[30,31]. Furthermore, excessive intake of Ca^{2+} can increase mitochondrial permeability transition, thereby inducing pathophysiological phenomena such as apoptosis[32]. Previous studies have shown that Mfn2 provides a convenient pathway for Ca^{2+} transport from mitochondria to ER in intracellular high Ca^{2+} environment[33-37]. However, there is little information about relationships between the protective effect of RIC and the Mfn2-MICUs axis during LT.

We, therefore, designed and performed the present RIC experiment (Figure 1) and found that, compared with the sham group, LT (including RIC) could cause liver damage and change the Mfn2-MICUs axis at the same time. While the RIC model reduced liver damage, Mfn2 expression was lower and MICUs

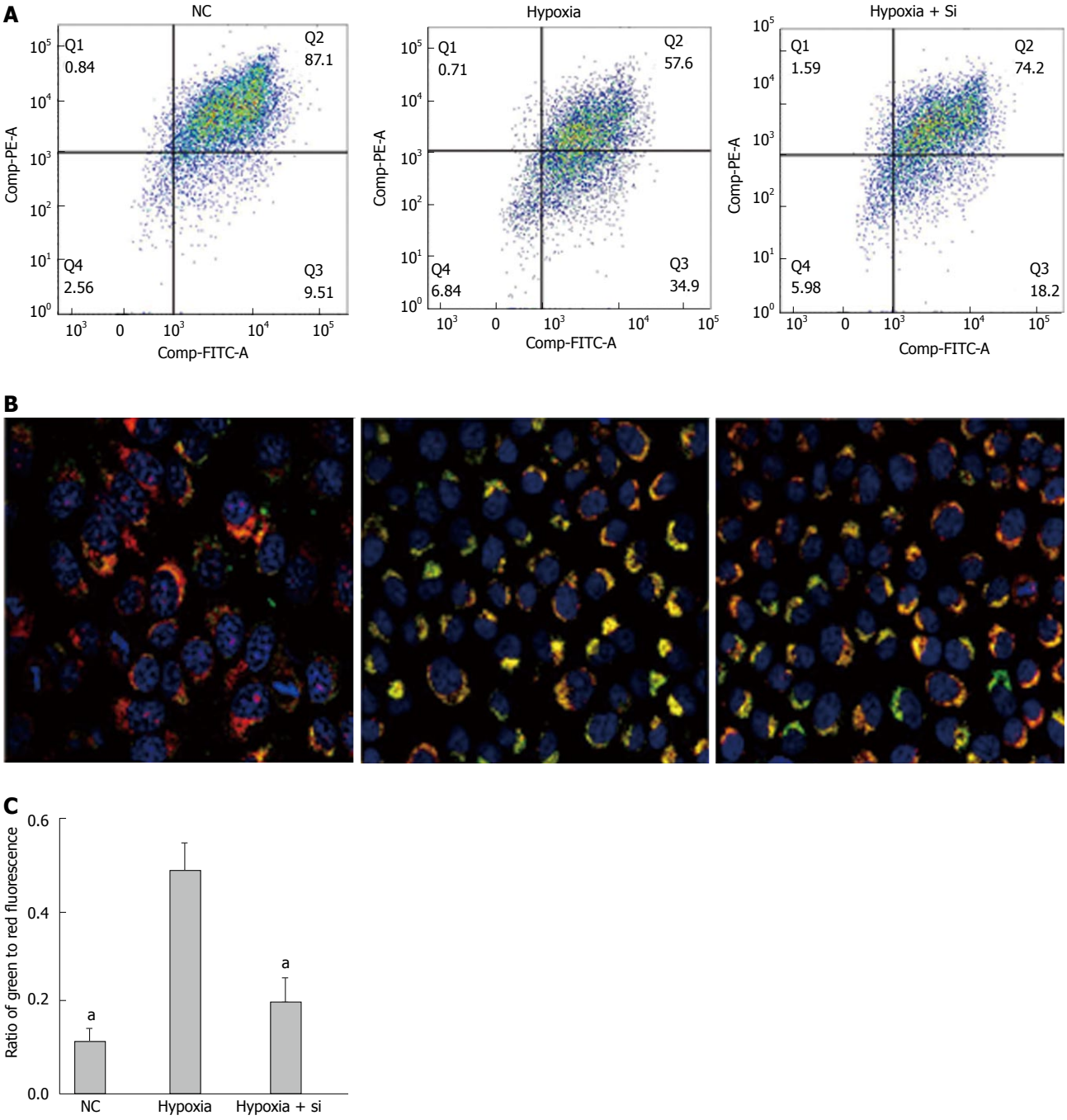


Figure 5 Mfn2 knockdown attenuates the collapse of ΔΨm in AML-12 hypoxia cell lines. JC-1 kit and Hoechst-33342 were used to measure the ΔΨm by flow cytometry (A and C) and confocal microscopy (B). Collapse of ΔΨm in the hypoxia group was attenuated both in the NC and hypoxia + Si groups. Mfn2: Mitofusin-2; NC: Normal culture; ΔΨm: Mitochondrial membrane potential. [a]$P < 0.05$.

expression was higher than in LT (Figures 2 and 3). These results demonstrated that the RIC model might exert its protective effect for LT *via* the Mfn2-MICUs axis.

To further verify our findings, we decided to knock down the Mfn2 expression by siRNA. Since genetically engineered mice are expensive, the experiment is time-consuming, and it is hard to perform gene knockout operations using primary cells, we designed the use of AML-12 hypoxia cell lines to simulate a RIC model. After determining the Mfn2 knockdown efficiency (Figure 4A), we proved that the hypoxia + Si group alleviated the hypoxia damage and showed higher expression of MICU-1 and MICU-2 compared with the hypoxia group (Figure 4B and C). Revalidating the same result from the RIC experiment provides the basis for our future mechanism studies.

Depolarization of mitochondrial membrane potential is usually associated with early apoptosis. According to the JC-1 kit instructions, in the absence of apoptotic cells, JC-1 aggregates are bound to mitochondria and red fluorescence occurs at 590 nm.

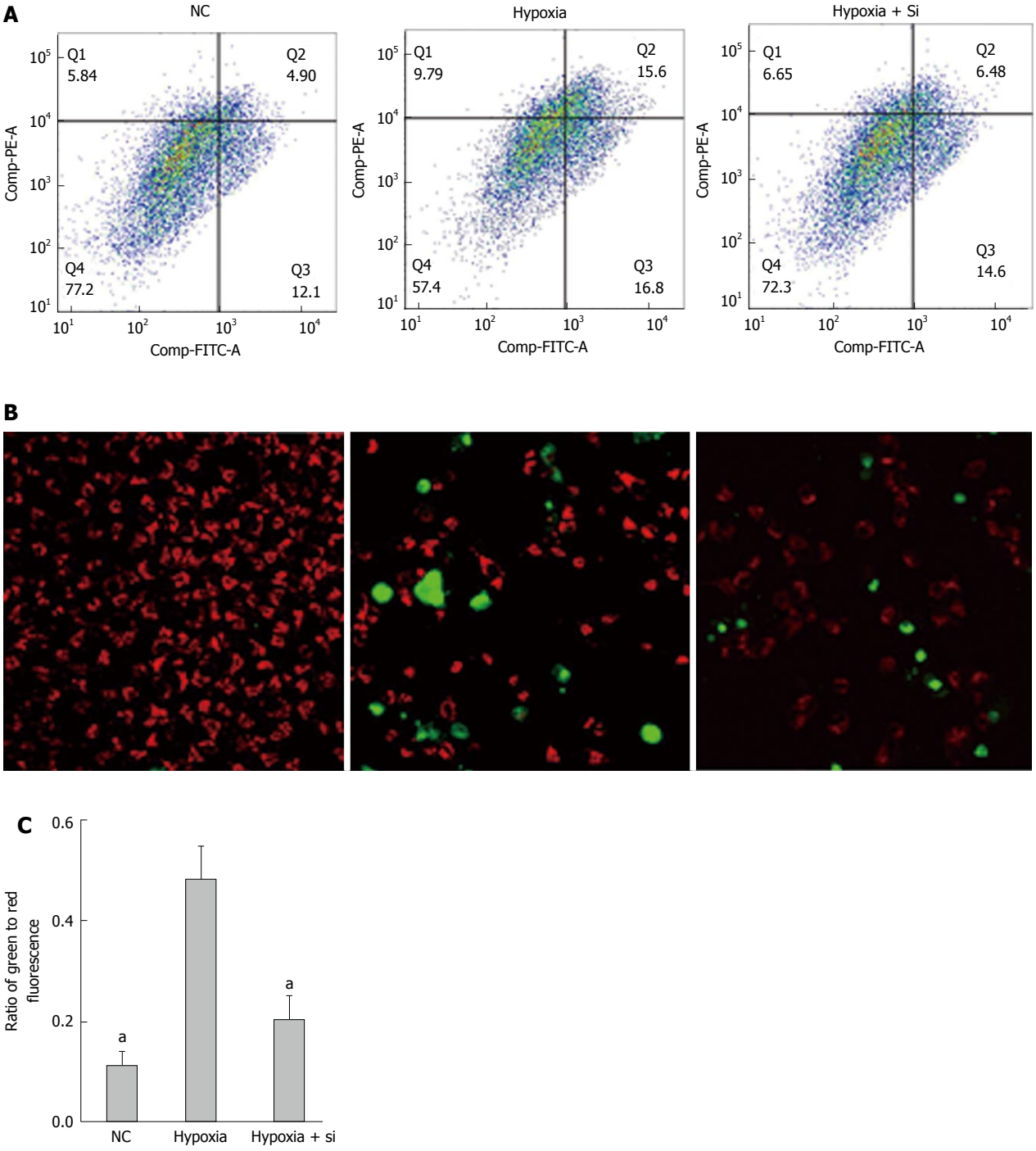


Figure 6 Mnf2 knockdown attenuates apoptosis in AML-12 hypoxia cell lines. The Annexin V/PI Apoptosis Detection Kit by flow cytometry (A and C); Caspase 3/7 detection kit and tetramethylrhodamine methyl ester perchlorate by confocal microscopy (B) used to detect apoptosis. The ratio of apoptosis cells was lower in both the NC and hypoxia + Si groups. AML: Alpha mouse liver; Mfn2: Mitofusin-2; NC: Normal culture. $^{a}P < 0.05$.

Whereas in apoptotic cells, JC-1 monomers are bound to cytoplasm and green fluorescence occurs at 530 nm. The green to red fluorescence ratio then indicates the collapse of ΔΨm. In the present study, we used the JC-1 kit to measure the ΔΨm in hypoxia cell lines by FCM, and found that the green to red fluorescence ratio was lower in the NC and hypoxia + Si groups (Figure 5A and C). The sham group showed no injury during the experiment. Compared with the hypoxia group, the hypoxia + Si group had less collapse of ΔΨm. For more intuitive observation, we used JC-1 and Hoechst-33342 to repeat the experiment by confocal microscopy, and revalidated the same result from FCM (Figure 5B). The results illustrated that Mfn2 knockdown lowered the mitochondrial membrane potential in hypoxia cells.

Apoptosis is a programmed cell death controlled by genes, which is designed to maintain homeostasis[38].

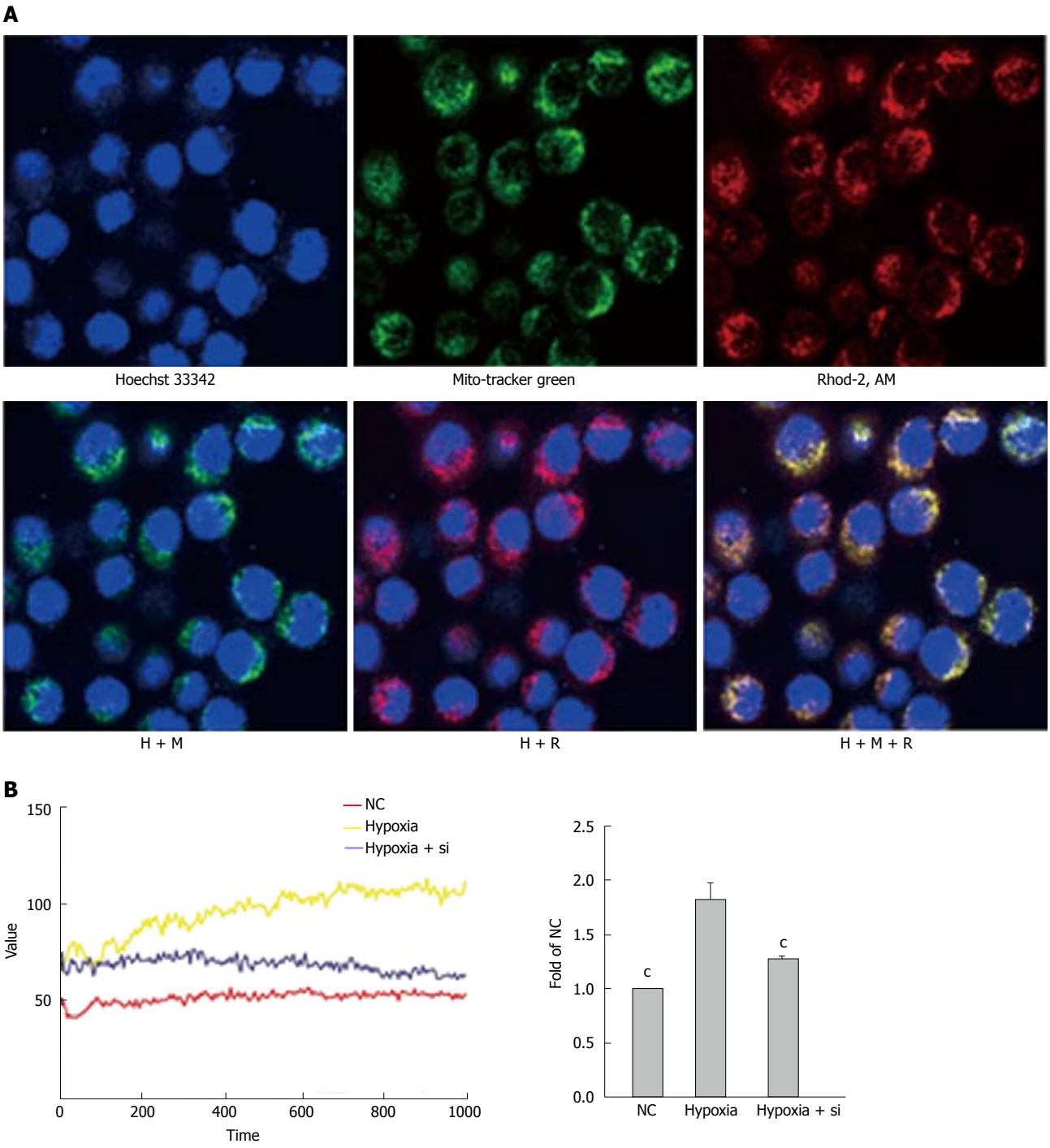


Figure 7 Mfn2 knockdown relieves mitochondrial Ca^{2+} overloading. Rhod-2 AM was used to detect mitochondrial Ca^{2+} concentrations by confocal microscopy and VarioskanFlash. A: Confocal microscopy confirmed that the vast majority of Rhod-2 AM fluorescence was associated with the mitochondria; B: VarioskanFlash was used to detect Rhod-2 AM to determine the fluorescence of mitochondrial Ca^{2+} concentrations. Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups relieved mitochondrial Ca^{2+} overloading. Mfn2: Mitofusin-2; NC: Normal culture; Rhod-2 AM: Rhod-2 acetoxymethyl ester. $^{c}P < 0.001$.

In the early stage of apoptosis, phosphotidylserine can be translocated from the inside of the cell membrane to the outside of the cell membrane and combined with annexin V (FITC). In late apoptotic and dead cells, the membranes are permeable to propidium iodide (PE-A). According to the staining status of the cells, we can distinguish the apoptosis status (annexin V/propidium iodide positive or negative). In the present study, we found that the ratio of cell apoptosis was lower in the NC and hypoxia + Si groups (Figure 6A and C). The sham group showed no injury during the experiment, indicating that compared with the hypoxia group, the hypoxia + Si group had fewer cells undergoing apoptosis. We used the caspase 3/7 detection reagent (green) and tetramethylrhodamine methyl ester perchlorate (red) to repeat the experiment by confocal microscopy and revalidate the same result from FCM (Figure 6B). The results demonstrated that Mfn2

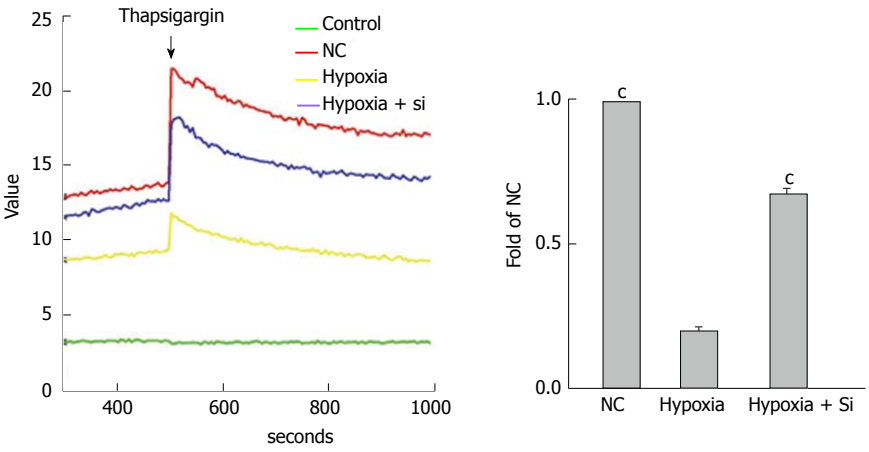


Figure 8 Mfn2 knockdown relieves endoplasmic reticulum Ca^{2+} decrease. FLUOFORTE® Calcium Assay and thapsigargin were used to detect ER Ca^{2+} concentrations by VarioskanFlash. Taking the hypoxia group as the reference, both the NC and hypoxia + Si groups showed higher ER Ca^{2+} concentrations. ER: Endoplasmic reticulum; Mfn2: Mitofusin-2; NC: Normal culture. $^{c}P < 0.001$.

knockdown reduced the apoptosis in hypoxia cells.

As mentioned earlier, whether IRI in LT or hypoxia in cells leads to injury should be verified. Mfn2 provides a convenient pathway for Ca^{2+} transport from mitochondria to ER in intracellular high Ca^{2+} environment. Excessive uptake of Ca^{2+} caused mitochondrial pathogenesis and affected 'gatekeeper' expression, thus leading to a higher Ca^{2+} concentration. In the present study, after confirming that the Mfn2-MICUs were altered in both rat models and cell models, we continued to validate Ca^{2+} changes at the mitochondrial level. We used Rhod-2 AM to analyze the mitochondrial Ca^{2+} concentration (Figure 7A), and VarioskanFlash to analyze the illumination of Rhod-2 AM to get the fluorescence of mitochondrial Ca^{2+} concentrations. We found that the hypoxia + Si group relieved mitochondrial Ca^{2+} overloading compared with the hypoxia group (Figure 7B). This indicated that Mfn2 knockdown reduced the mitochondrial Ca^{2+} overload by Mfn2-MICUs axis in cell models.

The ER is an important organelle of Ca^{2+} storage, the stored Ca^{2+} in ER is mainly released through the inositol 1, 4, 5-trisphosphate (InsP3) receptor and ryanodine receptor pathways[13]. In the present study, we investigated the relationships between ER Ca^{2+} concentrations and mitochondrial Ca^{2+} overloading. After the cytosolic Ca^{2+} concentration at baseline was quantified by VarioskanFlash using the FLUOFORTE® Calcium Assay, we added thapsigargin to release the ER Ca^{2+} and performed repeat measurement. The hypoxia + Si group showed relief of the ER Ca^{2+} decrease compared with the hypoxia group (Figure 8). This result proved that Mfn2 knockdown relieved the ER Ca^{2+} decrease by affecting the Mfn2-MICUs axis, thus altering the mitochondrial Ca^{2+} status in cell models.

The MCU protein complex is a highly selective calcium channel consisting of three components: MCU, MICU-1 and MICU-2. The complex can sense Ca^{2+} level and determine whether it passes through the mitochondrial membrane[19]. In our experiment, we failed to observe the consistency of MCU expression changes in rat and cell models. According to the literature, ER was mediated by the InsP3 receptor, Mfn2 showed no effect on expression of InsP3R, but when specific inhibitors of InsP3 receptor were used, the decline of ER Ca^{2+} induced by Mfn2 no longer occurred, suggesting that InsP3R Ca^{2+} release pathways affected the ER Ca^{2+} efflux[11]. The specific mechanism in InsP3R and IRI/cell hypoxia injury is not clear. Nevertheless, our research provides a clear clue that Mfn2 down-regulated expression of MICUs can affect the state of Ca^{2+} in organelles, thus playing a protective role in the two models. We will continue to apply gene-editing mouse models and specific inhibitors to conduct an in-depth research in the future.

In conclusion, we used an RIC model and confirmed that IRI was prevented by altered organelles' Ca^{2+} status *via* the Mfn2-MICUs axis, and revalidated the effect in AML-12 hypoxia cell models.

COMMENTS

Background

Remote ischemic perconditioning (RIC) alleviates the damage of ischemia-reperfusion injury (IRI). The Mfn2-MICUs axis is involved in the regulation of intracellular calcium, thereby affecting the process of apoptosis. But there is little information about the role of the mitofusin-2-mitochondrial Ca^{2+} uptake 1/2 (Mfn2-MICUs) axis in the protective effect of RIC during liver transplantation (LT).

Research frontiers

Most previous studies only confirmed the protective effect of RIC, but few showed the detailed mechanism. In the protective role of RIC, the mechanism of apoptosis related to the Mfn2-MICUs axis is worthy of being explored.

Innovations and breakthroughs

This research is the first to prove the protective mechanism of the Mfn2-MICUs axis as affecting the metabolism of intracellular calcium to reduce apoptosis in RIC of LT and revalidate it in AML-12 hypoxia cell lines.

Applications

This study showed the relationship between the protective effect of RIC and cell hypoxia injury, and that the Mfn2-MICUs axis and apoptosis are affected by the intracellular calcium homeostasis, thus laying a basic foundation for future studies.

Terminology

RIC was first proposed by Przyklenk in 1993, and it alleviates the damage of IRI in a temporary ischemic treatment for distant organs without damaging the target organs. RIC operations include remote-ischemic-preconditioning (RIPreC), remote-ischemic-postconditioning and RIPerC.

Peer-review

This is an interesting paper looking at the role of Mnf2 in liver IRI.

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ORIGINAL ARTICLE

Basic Study

Expression of annexin II in gastric carcinoma and its role in gastric cancer metastasis

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Author contributions: Lu ML and Huang H designed the research, and controlled the structure and quality of the paper; Han F and Shrestha S proposed the study and collected and analyzed the data, and wrote the first draft; Lv Hy, Nie C and Lin L enriched and improved the discussion section.

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Abstract

AIM

To investigate the expression of annexin II in gastric carcinoma and its role in the metastasis of gastric cancer.

METHODS

The expression of annexin II in 51 cases of gastric carcinoma and 24 cases of adjacent tissues was detected by immunohistochemistry. The relationship between annexin II and clinical features of gastric cancer was analyzed. Annexin II specific siRNA was used to inhibit the expression of annexin II in gastric cancer HGC-27 cells, and the effects of annexin II on the migration and secretion of matrix metalloproteinases (MMPs) were observed.

RESULTS

The positive rate of annexin II protein was 82.4% in gastric cancer tissues and 37.5% in adjacent tissues. There was significant difference between the two groups ($P < 0.01$); and the positive expression of annexin II was not related to the sex and age of the patients ($P > 0.05$). The expression of annexin II

protein was correlated with tumor size, histological differentiation, TNM stage, Lymph node metastasis and other clinical features were significantly correlated, the difference was statistically significant ($P < 0.05$). Inhibition of annexin II expression, gastric cancer HGC-27 cells migration and secretion of MMPs were significantly decreased, the difference was statistically significant ($P < 0.05$).

CONCLUSION

Annexin II is highly expressed in gastric cancer tissues, annexin II protein expression is related to tumor size, histological differentiation, TNM staging, lymph node metastasis and other clinical features were significantly correlated. Annexin II high expression can promote the invasion and metastasis of gastric cancer.

Key words: Gastric cancer; Annexin II; Metastasis; Real-time quantitative PCR; Human gastric cancer; HGC-27 cells; Matrix metalloproteinases

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Core tip: In this study, we retrospectively analyzed the expression of annexin II in 51 cases of gastric carcinoma and 24 cases of adjacent tissue to investigate the relationship between the positive expression of annexin II and the invasion and metastasis of gastric cancer.

Han F, Shrestha S, Huang H, Lv HY, Nie C, Lin L, Lu ML. Expression of annexin II in gastric carcinoma and its role in gastric cancer metastasis. *World J Gastroenterol* 2017; 23(38): 7009-7015 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7009.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7009

INTRODUCTION

Gastric cancer is one of the most common malignancies in my country, the first in the digestive tract malignant tumor, ranking the fourth in the world[1], Because of its symptoms hidden, the disease developed rapidly, when diagnosed in the late, the treatment effect is often poor, a serious threat to human health, but the early treatment of gastric cancer is good, 5 years after radical resection rate of up to 90% or more, So in-depth study of the occurrence and development of gastric cancer mechanism, For the prognosis of gastric cancer and the treatment of gastric cancer has a very important clinical significance.

Annexin Ⅱ is a calcium-dependent phospholipid-binding protein, Mainly in the form of monomer, dimer and tetramer, the study shows[2-6]: Annexin II are highly expressed in liver cancer, breast cancer, colon cancer, lung cancer and other malignant tumors, And play an important role in the occurence, development and infiltration of tumor. In this study, the expression of annexin II in 51 cases of gastric carcinoma and 24 cases of adjacent tissues was detected by immunohistochemistry, to investigate the relationship between the positive expression of annexin II and the clinical characteristics of gastric cancer. And annexin II specific siRNA was used to inhibit the expression of annexin II in gastric cancer HGC-27 cells, Detection of cell adhesion, invasion, migration, matrix metalloproteinases (MMPs) secretion and other capacity changes.

MATERIALS AND METHODS

Materials

Fifty-one cases of surgical specimens of gastric cancer were collected during the period from January 2012 to June 2014, among them, 30 males and 21 females; aged 32-78 years, mean age 58.6 ± 3.2 years; the above cases were not treated before radiotherapy, chemotherapy and other anti-tumor therapy. From the above 51 cases of gastric cancer, twenty-four patients were randomly selected from the normal tissue of the stomach. All specimens were fixed with formaldehyde, Paraffin embedded, sliced. Gastric cancer HGC-27 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology. Study has been reviewed by the appropriate ethics committee and has therefore been performed in accordance with the ethical standards laid down in an appropriate version of the Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study.

Methods

Immunohistochemical staining: Annexin II antibody was purchased from Abcam Reagent Company; Immunohistochemistry kit, DAB kit was purchased from Pikiton Reagent Company. Sliced was kept in high temperature and high pressure tissue antigen repair. The dyeing step is carried out according to the kit instructions. Positive plates were used as positive control, PBS instead of primary antibody as a negative control. The results showed that annexin II positive expression of brown matter in the cytoplasm appears brown matter. Each slice 5 representative of the high power field, Each field count 100 cells, The percentage of positive cells and the intensity of staining were scored separately. Specific criteria are as follows: (1) according to the degree of staining score: no staining for 0 points, Dyeing strength is 1 point; Medium dyeing intensity of 2 points; Strong staining intensity of 3 points. (2) Score according to the percentage of colored cells: The percentage of cells in the stained cells was < 5% for 0 points, 5%-25% for 1 points, 26%-50% for 2 points, 51%-75% for 3 points, > 75% for 4 points. (1) + (2) for the total points, The total score of 0-7 points. 0 is negative (-),

1-2 is divided into weak positive (+), 3-5 are divided into positive (++), 6-7 is divided into strong positive (+++).

Cell culture: Gastric cancer HGC-27 cells were cultured in RPMI1640 cell culture medium containing 10% fetal bovine serum and cultured in 5% CO_2, 37 ℃, saturated cell culture incubator.

Cell transfection: Gastric cancer HGC-27 cells (1×10^5 cells/mL) were seeded in 24-well plates, Cultured overnight at 37 ℃, 5% CO_2 and saturated humidity. Serum-free medium wash cells, 1 μL (20 pmol) of Annexin II siRNA was dissolved in 49 μL serum-free medium, 1 μL of liposomes was dissolved in 59 μL of serum-free medium, After keeping at room temperature for 10 min, Mix the two type of liquids, room temperature for 20 min. The mixture was then added to the 24-well plate of the cells to be transfected, Add serum-free medium to 500 μL, Placed in 37 ℃, 5% CO_2 cell incubator culture, 6 h later replaced with 10% newborn bovine serum RPMI1640 medium.

Real-time quantitative PCR: Use Trizol method to extract total RNA from cells, The total RNA samples of the extracted osteoblasts were reverse transcribed into cDNA, Using the following reaction system: 2 μg template RNA,1 μL oligo dT primer, 2 μL dNTP mixture,1 μL Ace reverse transcriptase,1 μL RNase inhibitor,4 μL 5 × RT buffer, 10 μL RNase-free water. The reaction conditions were as follows: first step 37 ℃ 30 min, The second step is 84 ℃ for 30 s. The reaction product was stored at 4 ℃. And then real -time quantitative PCR reaction. Using the following reaction system: 12.5 μL Real Master Mix (SYBR I), 2 μL template cDNA, 1 μL forward primer, 1 μL reverse primer, 8.5 μL RNase-free water. The reaction conditions are: first step 94 ℃ 5 min; second step 94 ℃ 60 s, 57 ℃ 30 s, 72 ℃ 30 s, a total of 30 cycles; the third step 72 ℃ 5 min; the last 4 ℃ end of the reaction. The experimental data were analyzed by Option Monitor V3.1 software of real-time PCR.

Cell invasion experiment: A 1:3 diluted matrigel (RMPI 1640 dilution) was added to the matrigel invasion chamber, 37 ℃ for 2 h, 2×10^4 gastric cancer cells were resuspended in 0.1% fetal bovine serum culture medium and inoculated in the chamber, RMPI 1640 medium containing 10% fetal bovine serum was added to the 24-well plate of the lower chamber, 37 ℃ for 24 h. Remove the invasion chamber, With a cotton swab gently wipe the room did not pass through the cells, formaldehyde fixed for 10 min, Hematoxylin-eosin staining, Cut the filter, the neutral gum seal, light microscopy count the number of cells through the membrane. The number of cells in the membrane was calculated by taking 10 fields under light microscope. Each independent experiment was repeated three times.

Cell migration experiments: First with a marker pen in the 6-well plate even behind a horizontal line, Cross through the hole. About 2×10^4 gastric cancer cells were seeded in 6-well plates when the cells are completely fused, the tip of the nose is as far as possible to the horizontal line behind the horizontal scratches. Wash the cells three times with PBS, wash off the cells under the cells, and then add serum-free medium. And incubated in a CO_2 incubator at 37 ℃. 0 and 24 h under inverted microscope to observe and take pictures. The scribe width was measured with Image J software and the cell mobility was calculated. Cell mobility = (0 h scratch width - 24 h scratch width)/0 h scratch width × 100%. Each independent experiment was repeated three times.

Cell gelatin zymography experiments: Cells were treated with annexin II siRNA Digest the cells and count, the corresponding conditioned medium was collected, 1500 *g* low-speed centrifugation 10 min after removal of cell debris, Take the supernatant 4 ℃ spare. And then take the protein quality samples separately with non-reduction Loading Buffer Homogeneously mixed, incubated at 55 ℃ for 5 min. Prepare 5% concentrated gum and 8% separated gel containing 0.1% gelatin. Constant pressure electrophoresis until the edge of bromophenol blue separation from the front of the separation of about 0.5 cm. The gel was removed and transferred to the elution solution, eluting twice every 30 min. Rinse with gelatin incubation once, and then placed in gelatin incubation buffer at 37 ℃ for 18 h. Dyeing solution 4 h After the rinsing of distilled water, And then take a photo by a gel image analyzer.

Statistical analysis

Each independent experiment was repeated more than three times. Student-t test was performed with SPSS 16.0 software, the results are expressed as mean ± SD, qualitative data were compared using χ^2 test, $P < 0.05$ indicating that the difference was statistically significant.

RESULTS

Expression of annexin II Protein in gastric carcinoma and its relationship with clinical characteristics of gastric cancer

Expression of AnnexinII in gastric carcinoma and adjacent tissues: Annexin II protein positive expression of cytoplasm in the yellow granular material, Mainly located in the cytoplasm (Figure 1). In 51 cases of gastric cancer, 42 cases were positive, The positive rate was 82.4% of which strong positive in 3 cases, Positive in 12 cases, Weakly positive in 27 cases, Negative in 9 cases. In 24 cases of

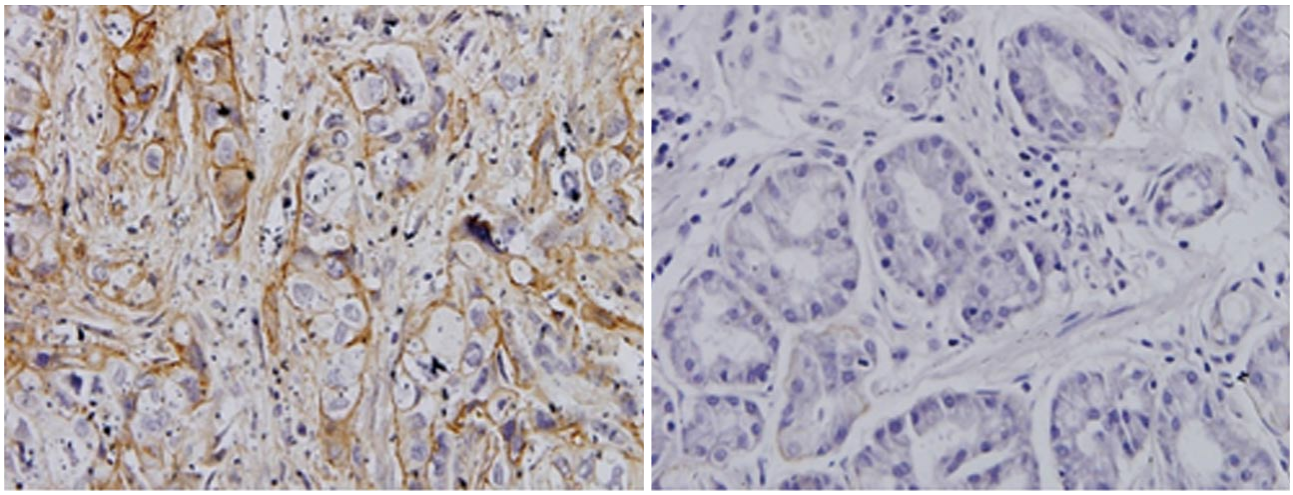


Figure 1 Immunohistochemistry to detect annexin II protein in gastric cancer tissues and adjacent tissues.

Table 1 Expression of annexin II protein in gastric carcinoma and paracancerous tissues

Group	*n*	Annexin II protein				Positive rate, %	*P* value
		-	+	++	+++		
Gastric cancer	51	9	27	12	3	82.4	
Paracancerous	24	15	6	3	0	37.5	< 0.01

paracancerous tissue, there were 9 positive expression, of which strong positive 0 cases, positive in 3 cases, weak positive in 6 cases, The positive rate was 37.5%. The expression of AnnexinII protein in gastric cancer tissues was significantly higher than that in adjacent tissues, Differences were significant (Table 1, $P < 0.01$).

Expression of annexin ii protein in patients with clinical characteristics of gastric cancer: The study found, annexin II positive expression was not related to the sex of the patient ($P > 0.05$) while annexin II protein expression was associated with tumor size, histological differentiation, TNM staging, lymph node metastasis and other clinical features were significantly correlated, the difference was statistically significant ($P < 0.05$, Table 2).

The role of annexin II in gastric cancer metastasis

SiRNA inhibits annexin II expression: The siRNA fragment targeting annexin II was transfected into human gastric cancer HGC-27 cells, the mRNA and protein expression of annexin II in human gastric cancer HGC-27 cells were detected by real time-PCR and Western blot after 24 h. And transfected unrelated interfering siRNA fragments (control siRNA) as a negative control. The results show, annexin II siRNA can significantly inhibit the transcription and protein expression of annexin II mRNA in human gastric cancer HGC-27 cells, the difference was statistically significant ($P < 0.05$; figure 2).

Table 2 Relationship between annexin II protein and clinical characteristics of gastric cancer patients

Group	*n*	Annexin II positive	Positive rate, %	*P* value
Age in yr				
≤ 60	27	23	85.2	> 0.05
> 60	24	19	79.2	
Sex				
Male	31	27	87.1	> 0.05
Female	20	15	75	
Tumor diameter in cm				
≤ 5	19	12	63.2	< 0.05
> 5	32	30	93.8	
Differentiation				
Well differentiated	15	9	60	< 0.05
Poorly differentiated	36	33	91.7	
Clinical stage				
Ⅰ + Ⅱ period	17	10	58.8	< 0.05
Ⅲ + Ⅳ period	34	32	94.1	
Lymph node metastasis				
Have	33	31	93.9	< 0.05
Not have	18	11	61.1	

Effect of annexin II on the migration ability of gastric cancer cells: Scaling experiments were performed to detect changes in cell migration ability. Compared with the control group transfected with Control siRNA, the migration ability of human gastric cancer HGC-27 cells transfected with Annexin II siRNA was significantly decreased, differences were statistically significant ($P < 0.01$; figure 3).

Effect of annexin ii on secretion of mmps in gastric cancer cells: The changes of MMPs secretion were detected by gelatin zymography. Compared with the control group transfected with Control siRNA, Human gastric cancer HGC-27 cells transfected with AnnexinII siRNAs after MMPs secretion was significantly reduced, Differences were statistically significant ($P < 0.01$; figure 4).

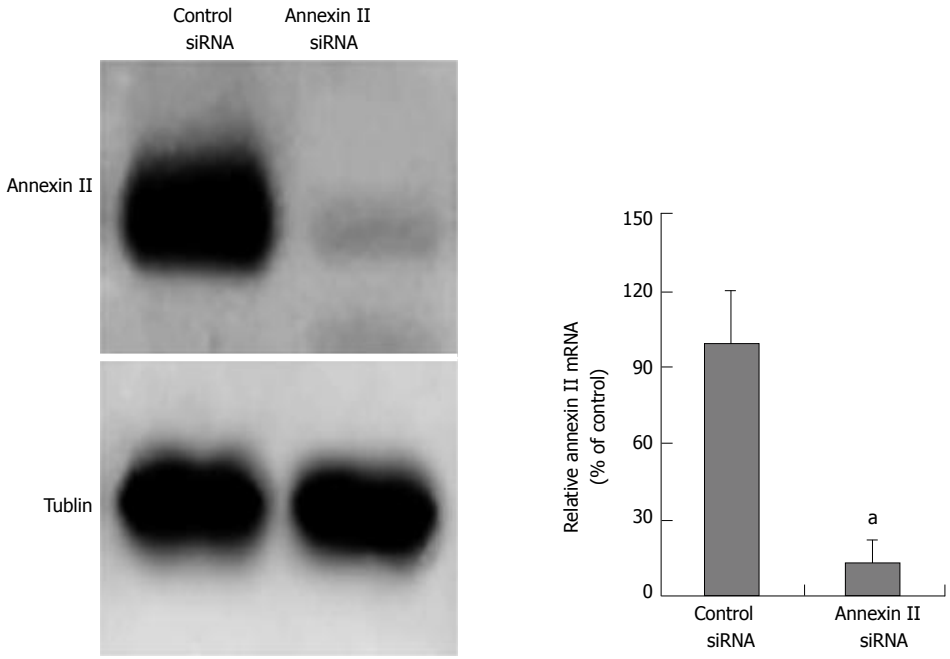


Figure 2 Effect of annexin II siRNA on annexin II protein expression in human gastric cancer HGC-27 cells. [a]*P* < 0.05. siRNA: Small interfering RNA.

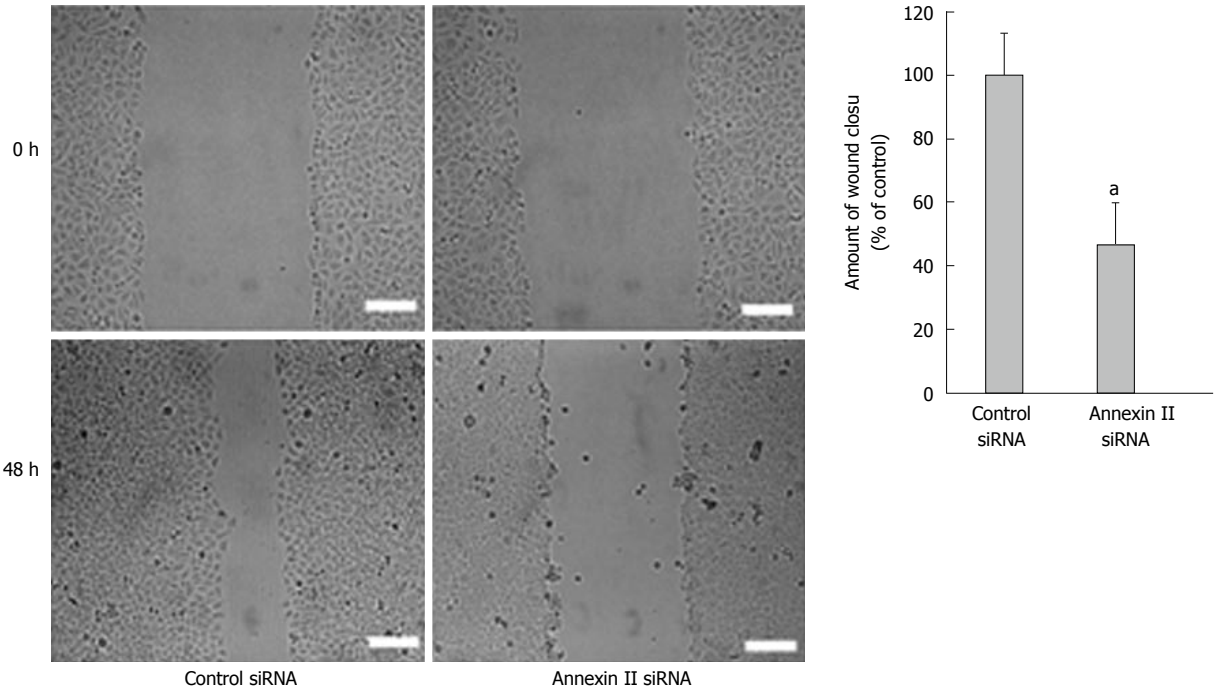


Figure 3 Effect of annexin II siRNA on the migration ability of human gastric cancer HGC-27 cells. [a]*P* < 0.05. siRNA: Small interfering RNA.

DISCUSSION

Annexins is a class of calcium-dependent phospholipid-binding proteins, which are widely present in the cell membrane, cytoplasmic or extracellular matrix. There are two basic functional domains on the structure: Highly conserved C-terminal core regions and highly variable N-terminal domains. The former has a binding site of Ca^{2+} and membrane, in the form of calcium-dependent reversible binding to cell membrane phospholipids, the latter determines annexins specific biological functions. Annexins participate in a series of activities of cells, such as endocytosis, exocytosis, cell proliferation, differentiation and apoptosis, and cytoskeleton activity, signal transduction and etc[7]. Annexin II as a member of the annexins family A subfamily, Also known as p36, AXA2, LIP2, etc. Its gene is located in 15q21-q22, containing 1.4 kb encoding gene[8,9].

In this study, the expression of annexin II in gastric

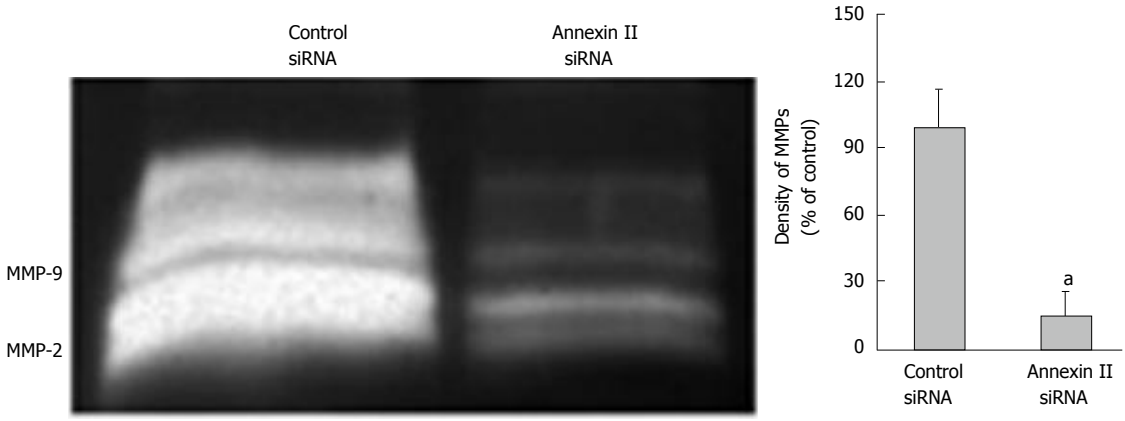


Figure 4 Effect of annexin II siRNA on secretion of matrix metalloproteinases in human gastric cancer HGC-27 cells. $^{a}P < 0.05$. MMPs: Matrix metalloproteinases; siRNA: Small interfering RNA.

cancer tissues and adjacent tissues was analyzed. The results showed that the expression of annexin II protein in gastric cancer tissues was significantly higher than that in adjacent tissues. To analyze the correlation between the expression of annexin II protein and the clinical characteristics of gastric cancer patients. The positive expression of annexin II was not related to the sex and age of the patients, while annexin II protein was expressed with tumor size, histological differentiation, TNM stage, lymph node metastasis and other clinical features were significantly correlated, the difference was statistically significant. The results suggest that annexin II protein is closely related to the malignant degree of gastric cancer.

Tumor metastasis is the most important malignant phenotype of the tumor and is the direct cause of this high mortality. Tumor metastasis is required in tumor cells themselves, host cells and extracellular matrix between the interaction can be completed, Involves a series of complex processes and steps, Including tumor cells from the primary tumor lesions, through the secretion of MMPs degradation of the extracellular matrix into the blood vessels or lymphatic vessels migrate to the distant, and then attack to the secondary organization or organ in the formation of metastases[10-14]. Therefore, the study of tumor metastasis for clinical diagnosis and treatment is important. To clarify whether annexin II in the process of gastric cancer metastasis play a role, In this study, annexin II-specific siRNA was used to inhibit annexin II expression in gastric cancer HGC-27 cells, after the expression of annexin II was reduced, gastric cancer HGC-27 cells migration, MMPs secretion and other capacity changes. The study found: In gastric cancer HGC-27 cells using annexin II- specific siRNA inhibited annexin II expression, gastric cancer cell migration, MMPs secretion and other capacity were reduced, suggesting that annexin II in the process of gastric cancer metastasis plays an important role.

In summary, annexin II in gastric cancer showed high expression, and the degree of differentiation of gastric cancer, TNM stage, lymph node metastasis and other clinical features are closely related, application of annexin II specific siRNA in gastric cancer HGC-27 cells inhibited annexin II expression, gastric cancer cell migration, MMPs secretion and other capacity are reduced, suggesting that annexin II plays an important role in the process of gastric cancer metastasis. The development and progression of gastric cancer is a complex multi-factor comprehensive results. At present, the etiology and pathogenesis of gastric cancer are more studied, but it is not clear. This study confirmed that annexin II was highly expressed in gastric cancer tissues and had the important role in gastric cancer metastasis. With the deepening of related research, annexin II gene expression, biological function and the role of progress in gastric cancer will be gradually clear, is expected to be used in the diagnosis and treatment of gastric cancer, predict the prognosis of markers and therapeutic targets.

COMMENTS

Background

Gastric cancer is one of the most common malignancies in my country, the first in the digestive tract malignant tumor, ranking the fourth in the world, Because of its symptoms hidden, the disease developed rapidly, when diagnosed in the late, the treatment effect is often poor, a serious threat to human health, but the early treatment of gastric cancer is good, 5 years after radical resection rate of up to 90% or more.

Research frontiers

Annexin II is a calcium-dependent phospholipid-binding protein, mainly in the form of monomer, dimer and tetramer, the study shows: Annexin II are highly expressed in liver cancer, breast cancer, colon cancer, lung cancer and other malignant tumors, and play an important role in the occurence, development and infiltration of tumor.

Innovations and breakthroughs

The authors restropectively analysed the expression of annexin II in 51 cases of gastric carcinoma and 24 cases of adjacent tissues, to investigate the relationship between the positive expression of annexin II and the invasion and metastasis of gastric cancer.

Applications

Annexin II in gastric cancer showed high expression, And the degree of

differentiation of gastric cancer, TNM stage, lymph node metastasis and other clinical features are closely related, suggesting that annexin II plays an important role in the process of gastric cancer metastasis.

Terminology

Annexins is a class of calcium-dependent phospholipid-binding proteins, they are widely present in the cell membrane, in the form of calcium-dependent reversible binding to ceembrane phospholipids, the latter determines Annexins specific biological functions.

Peer-review

This is an interesting study with great promise. AnnexinII was highly expressed in gastric cancer tissues and had the important role in the effect of promoting gastric cancer metastasis.

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ORIGINAL ARTICLE

Retrospective Study

Risk factors for postoperative recurrence after primary bowel resection in patients with Crohn's disease

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Abstract

AIM

To evaluate the risk factors for postoperative recurrence after primary bowel resection in a cohort of Korean Crohn's disease (CD) patients.

METHODS

This study included 260 patients with no history of previous bowel surgery who underwent primary surgery for CD between January 2000 and December 2010 at Asan Medical Center (Seoul, South Korea). The median follow-up period was 101 mo.

RESULTS

During the follow-up period, 66 patients (25.4%) underwent a second operation for disease recurrence.

At 1, 5 and 10 years after the first operation, the cumulative rate of surgical recurrence was 1.1%, 8.3% and 35.9% and clinical recurrence occurred in 1.2%, 23.6% and 68.1%, respectively. In multivariate analysis, undergoing an emergency operation was a significant risk factor for surgical recurrence-free survival (SRFS) [HR = 2.431, 95%CI: 1.394-4.240, *P* = 0.002], as were the presence of perianal disease after the first operation (HR = 1.715, 95%CI: 1.005-2.926, *P* = 0.048) and history of smoking (HR = 1.798, 95%CI: 1.088-2.969, *P* = 0.022). The postoperative use of anti-tumor necrosis factor (TNF) agents reduced SRFS risk (HR = 0.521, 95%CI: 0.300-0.904, *P* = 0.02).

CONCLUSION

History of smoking, postoperative perianal disease and undergoing an emergency operation were independent risk factors for surgical recurrence. Using anti-TNF agents may reduce surgical recurrence.

Key words: Crohn's disease; Recurrence; Risk factor

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Core tip: Our study demonstrated the clinical feature and long-term prognosis of Crohn's disease (CD) in a large cohort of non-Caucasian patients. We investigated risk factors for the postoperative recurrence of CD in a patient population from a single tertiary referral center. History of smoking, postoperative perianal disease and undergoing an emergency operation were found to be independent risk factors for surgical recurrence in CD. Using anti-TNF agents for these patients may reduce surgical recurrence. These findings indicate that affected patients with CD may benefit from close postoperative surveillance and probably from the early administration of anti-TNF agents.

Yang KM, Yu CS, Lee JL, Kim CW, Yoon YS, Park IJ, Lim SB, Park SH, Ye BD, Yang SK, Kim JC. Risk factors for postoperative recurrence after primary bowel resection in patients with Crohn's disease. *World J Gastroenterol* 2017; 23(38): 7016-7024 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7016.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7016

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory gastrointestinal disease with a heterogeneous disease course. Some patients experience low morbidity, whereas others need repeated operations[1]. Conservative medical treatment is regarded as the principal therapeutic approach to treat patients with CD, but the lifetime risk of surgical resection for patients with CD can be 70% to 80%[2,3]. Approximately 10% to 30% of patients who undergo an operation experience clinical recurrence during the first postoperative year, and more than 60% experience during the first postoperative decade. Surgical recurrence rates at 5 and 10 years have been reported as 20% to 25% and 34% to 49%, respectively[4,5]. Thus, to provide optimal patient care, it is important to be able to predict which patients have a higher risk of recurrence.

A range of possible risk factors thought to impact the surgical recurrence rate have been investigated: age at diagnosis, sex, smoking behavior, duration of disease before surgery, family history, behavior of disease (perforating or non-perforating disease), history of medication (use of 5-aminosalicylic acid, corticosteroids, immunomodulators or anti-tumor necrosis factor (anti-TNF) agents), priority of operation (emergency or elective), presence of granulomas in the resected bowel specimen, length of resected bowel, technique of anastomosis used in the surgery and postoperative complications[6,7]. However, such studies on risk factors have largely been inconclusive owing to different patient populations and clinical manifestations.

In this study, we investigated risk factors for the postoperative recurrence of CD, as determined by the need for an additional operation, in a patient population from a single tertiary care center.

MATERIALS AND METHODS

Patients

Patients who underwent intestinal resection for CD at Asan Medical Center (Seoul, Korea) between January 2000 and December 2010 were enrolled in this retrospective study. A total of 401 patients underwent intestinal resection for pathologically confirmed CD during the study period. Exclusion criteria included a history of previous bowel surgery, such as small or large bowel resection or strictureplasty, a concurrent or previous history of other malignancies, and patients who have had follow up for a short period of time (< 12 mo). A total of 260 patients were included in our analysis (Figure 1). The following variables were retrospectively reviewed from the prospectively collected database for these patients: demographics, preoperative disease characteristics, disease phenotype (Montreal classification)[8], any history of perianal disease (*e.g.*, an abscess or fistula), the presence of extraintestinal manifestations, a history of smoking, comorbidity, the operative approach (open or laparoscopy), operative indications (perforating or non-perforating), the rate of stoma formation, the reoperation rate, the duration of follow-up, postoperative complications and regimens of pre- and post-medical treatment.

The study protocol was approved by the institutional review board of Asan Medical Center (registration no: 2016-1139), in accordance with the Declaration of

Helsinki.

Assessment and follow-up

CD distribution was assessed based on pathological and intraoperative findings, radiologic imaging using a small bowel series (SBS), abdomen and pelvis computed tomography (CT), CT enterography, magnetic resonance imaging (MRI) enterography and colonofiberscopy (CFS). Perianal disease included stricture, fistula or an abscess in the perianal area, anal canal or perirectal space at any time during the course of CD. After primary intestinal resection, reassessment was performed by CT enterography, MRI enterography or CFS after 6 to 12 mo. Surgical recurrence was defined as a repeated operation for pathologically confirmed CD on any part of the bowel or a repeated operation for pathologically confirmed anastomotic disease, including instances where the small bowel was diseased at the anastomosis or the stoma site. Clinical recurrence was defined as the recurrence of CD-related symptoms, confirmed by objective signs of radiology or endoscopy findings. As this was a retrospective study the Crohn's disease Activity Index (CDAI) and Rutgeerts' score were not available.

Medical treatment of CD

Our medical treatment policy for treating CD was based on a step-up approach, with more potent therapies added if patients became unresponsive to first-line or less toxic agents, as described in a previous report from our institution[9]. The use of anti-TNF agents in this study was less frequent than in Western studies because of the strict Korean government health insurance reimbursement policy during our study period. During this period, two anti-TNF agents (infliximab and adalimumab) were available in Korea.

Statistical analysis

Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multivariate analyses for survival that included the variables that showed statistical significance in the log-rank test were performed using Cox proportional hazards model with a 95%CI to assess the risk factors associated with surgical recurrence-free survival (SRFS) and clinical recurrence-free survival (CRFS). Statistical significance was defined as $P < 0.05$; all analyses were performed using SPSS software version 21 (IBM Corp., Armonk, NY).

RESULTS

Patient characteristics

The clinicopathological characteristics of the patients are summarised in Tables 1 and 2. The median follow-up time for the 260 patients was 101 mo (range, 12-193 mo). The interval between the first intestinal resection and clinical recurrence was 64 mo (range, 12-148 mo) and the interval between the first intestinal resection and surgical recurrence was 77.5 mo (range, 15-149 mo).

Elective operations were performed for 210 (80.8%) patients; the remaining 50 (19.2%) patients underwent emergency operations because of generalised peritonitis or severe obstruction. Among 260 patients, 122 (46.9%) patients had perianal disease before first surgery. Of these, 42 (34.4%) patients had perianal disease after first operation. Because of the strict Korean government health insurance reimbursement policy, only 27 (10.4%) patients treated with anti-TNF agents before first surgery, and 17 (63%) patients continued anti-TNF agents after first operation. Antibiotics such as ciprofloxacin or metronidazole were administered to 163 patients (62.7%) in case of infectious complications such as intra-abdominal abscess, perianal abscess or fistula before first surgery. All the patients who underwent first surgery used antibiotics for a certain period of time. During the follow-up period, three patients (1.5%) experienced colorectal cancer, one patient experienced lung cancer (0.4%).

Montreal classification of the patients

The majority (215/260, 82.7%) of the patients were diagnosed between the ages of 17 and 40 years, with 9.6% experiencing childhood onset of CD ($\leqslant$ 16 years) and 7.7% diagnosed as older adults (> 40 years). Montreal classifications were distributed as follows: 43.5% exhibited disease in the terminal ileum region (L1), 41.2% in the ileocolon (L3) and 14.6% in the colon region (L2); 67% had penetrating disease (B3, fistula, abscess or perforation), 31% had stricturing disease (B2) and 2% had inflammatory disease (B1, non-stricturing and non-penetrating). Because these patients were treated with surgical intervention, proportion of disease behavior was different from the whole patients with CD in Korea. Disease behavior of whole CD patients at diagnosis was inflammatory (B1) in 1591 patients (77.1%), stricturing (B2) in 174 (10.3%), and penetrating (B3) in 211 (12.6%)[9].

Clinical and surgical recurrence

Clinical recurrence occurred in 1.9%, 24.2% and 68.3% of the patients at 1, 5 and 10 years, respectively, after the first intestinal resection (Figure 2). In multivariate analysis (Table 3), history of smoking, presence of perianal disease after the first operation and undergoing an emergency operation were associated with CRFS.

During the follow-up period, 66 patients (25.4%) underwent a second intestinal resection for disease recurrence. Among these, 13 (20%) patients underwent a third intestinal resection, four patients (31%) a fourth and one patient (25%) a fifth. The cumulative rate of surgical recurrence was 1.1%, 9.2%

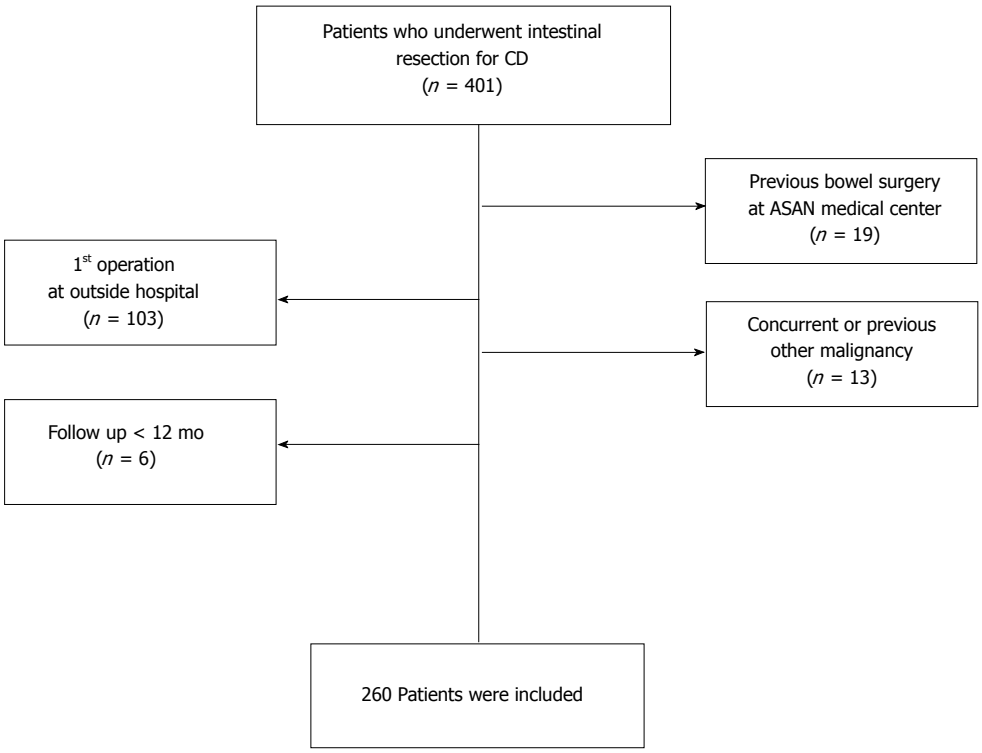


Figure 1 Inclusion criteria.

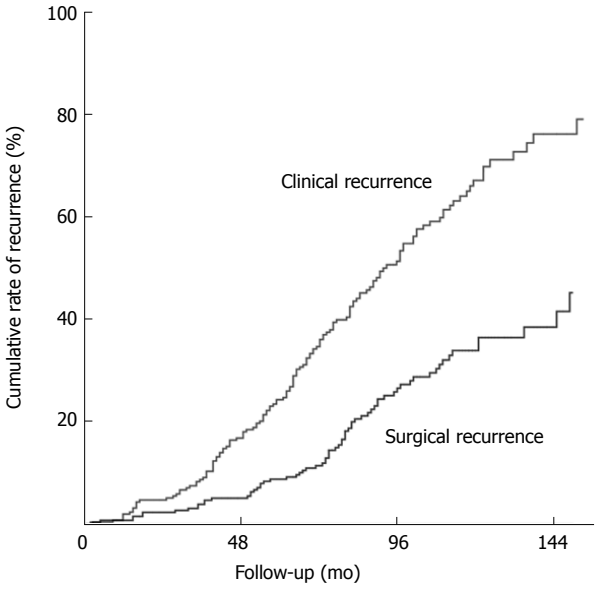


Figure 2 Cumulative rate of clinical and surgical recurrence.

and 36.4% at 1, 5 and 10 years, respectively, after the first operation (Figure 1). In univariate analysis, the presence of perianal disease after the first operation, undergoing an emergency operation and postoperative use of anti-TNF agents were all associated with SRFS.

Multivariate analysis revealed the following risk factors for SRFS (Table 3): undergoing the first resection as an emergency operation (HR = 2.431, 95%CI: 1.394-4.240, P = 0.002), history of smoking (HR: 1.798, 95%CI: 1.088-2.969, P = 0.022) and the presence of perianal disease after the first intestinal resection (HR = 1.715, 95%CI: 1.005-2.926, P = 0.048); in addition, the risk was reduced with the postoperative use of anti-TNF agents (HR = 0.521, 95%CI: 0.300-0.904, P = 0.020). Younger age at diagnosis seemed to be associated with an increased risk of surgical recurrence, but it was not statistically significant (HR = 3.512, 95%CI: 0.944-13.068, P = 0.065). However, no impact on SRFS was found for disease location, presence of postoperative complications, length of resected small bowel, perforating disease or preoperative duration of the disease. Azathioprine and 6-mercaptopurine did not demonstrate a clear clinical effect for the prevention of surgical recurrence after the first intestinal resection.

Discussion and conclusions

The increasing tendency in Korea over the past few decades calls for more attention to be paid to the natural course of CD in these newly developed disease population. Our study demonstrated the clinical feature and long-term prognosis of CD in a large cohort of non-Caucasian patients.

Korean patients with CD differ from Western patients in sex distribution (a predominance of men), disease location (a lower proportion of isolated colonic disease) and the occurrence of perianal fistula (a higher incidence)[10,11]. Although the pathogenesis of CD is largely unknown, differences have been reported in susceptibility genes and phenotypic features of CD between Asian and Western populations. For example, NOD2 or ATG16L1, which are regarded as well-

Table 1 Demographic and clinical characteristics of the patients undergoing intestinal resection *n* (%)

Variables	Number of patients (*n* = 260)
SEX	
Male	187 (71.9)
Female	73 (28.1)
Follow-up period, month	101 (12-193)
Age at diagnosis	23 (12-74)
Age at 1st operation	28 (15-74)
Age at 2nd operation	34 (22-59)
Montreal classification	
Age at diagnosis, yr	
A1 (⩽ 16)	25 (9.6)
A2 (17-40)	215 (82.7)
A3 (> 40)	20 (7.7)
Location	
L1 (Terminal ileum)	113 (43.5)
L2 (Colon)	38 (14.6)
L3 (Ileocolon)	107 (41.2)
L4 (Isolated upper disease)	2 (0.8)
Behavior	
B1 (Inflammatory)	6 (2.3)
B2 (Stricturing)	80 (30.8)
B3 (Penetrating)	174 (66.9)
Family history (Crohn's disease)	7 (2.7)
Presence of extraintestinal manifestation	20 (7.7)
Perianal disease	
at diagnosis	122 (46.9)
after 1st operation	62 (23.8)
History of smoking	70 (26.9)
Current smoker	12 (4.6)
Ex-smoker	58 (22.3)
Non-smoker	190 (73.1)
Preoperative use of medication	
Anti-TNF	27 (10.4)
5-ASA	223 (85.8)
Steroid	147 (56.5)
Immunomodulator[1]	128 (49.2)
Antibiotics[2]	128 (49.2)
Malignancy	163 (62.7)
Colorectal cancer	3 (1.2)
Lung cancer	1 (0.4)

[1]Use of azathioprine/6-mercaptopurine or methotrexate; [2]Ciprofloxacin or metronidazole. EIM: Extraintestinal manifestation; 5-ASA: Aminosalicylates; TNF: Tumor necrosis factor.

Table 2 Operative details and outcomes *n* (%)

Variables	Number of patients (*n* = 266)
Operative approach	
Open	215 (82.7)
Laparoscopy	45 (17.3)
Type of operation	
Elective	210 (80.8)
Emergency	50 (19.2)
Indication for 1st operation	
Perforating	167 (64.2)
Non-perforating	93 (35.8)
OP name	
ICR	85 (32.7)
RHC	72 (27.7)
SB R&A	49 (18.8)
TC/STC	32 (12.3)
TPC/APR	19 (7.3)
Others	3 (1.2)
Type of anastomosis	
Handsewn	5 (1.9)
Stapled	225 (86.5)
Permanent stomy	28 (10.8)
Postoperative use of medication	
Anti-TNF	95 (36.5)
5-ASA	244 (93.8)
Steroid	82 (31.5)
Immunomodulator	220 (84.6)
Presence of complication	58 (22.3)
Wound infection	17 (6.5)
Anastomosis leakage	12 (4.6)
Intra-abdominal abscess	11 (4.2)
Entero-cutaneous fistula	7 (2.7)
Bleeding	4 (1.5)
Ileus	3 (1.2)
Others	4 (1.5)
Time to clinical recurrence, mo	64 ± 29.8
Time to surgical recurrence, mo	77.5 ± 30.8

ICR: Ileocecal resection; RHC: Right hemicolectomy; SB R&A: Small bowel resection and anastomosis; TC: Total colectomy; STC: Subtotal colectomy; TPC: Total proctocolectomy; Others: Segmental resection of colon.

established CD susceptibility genes among Caucasians, are not replicated in Asians, whereas the association of TNFSF15 with CD is stronger in Koreans and Japanese than in Caucasians[12,13].

The surgical recurrence rate was 25% to 45% 10 years after the first intestinal resection[14,15], although postoperative clinical recurrence occurred earlier during the disease course. Our study found that cumulative surgical and clinical recurrence rates were 36.4% and 68.4%, respectively, at 10 years after the first intestinal resection.

Our study demonstrated that patients with CD who had a higher risk of surgical recurrence after primary bowel surgery were those who underwent the first resection as an emergency operation, those with history of smoking and those with postoperative perianal disease. The use of postoperative anti-TNF agents seemed to reduce surgical recurrence.

Korean patients with CD show higher incidence of perianal disease than Western patients[9]. Previous study in our center[11], perianal fistulas occurred in 46.8% of patients. Also, the cumulative frequency of perianal fistula was 54.3% after 15 years. These results are in contrast to those of Western studies in which the cumulative frequency of perianal fistula in patients with CD attending referral centers was reported to be 13%-38%[16]. Furthermore, the presence of perianal disease was one of the predictors of postoperative recurrence, as has been suggested by previous studies in Asian population[17-19]. Gao *et al*[17] also assessed risk factors for surgical recurrence and advocated perianal CD correlated with a higher clinical recurrence rate. Korean CD patients with perianal disease had a higher risk of surgical recurrence[18,20]. The present study showed that patients with CD who developed perianal disease after the first resection had a high risk of surgical and clinical recurrence, indicating that these patients had a poor disease

Table 3 Univariate and multivariate analysis of factors associated with surgical and clinical recurrence free survival after 1st intestinal resection

	Univariate	Multivariate		
	P value	HR	95%CI	*P* value
Clinical recurrence free survival				
Type of operation	0.001[a]			0.002[a]
Elective		1		
Emergency		1.856	1.246-2.763	
Perianal disease after the 1st operation	0.077	1.446	1.005-2.081	0.047[a]
History of smoking	0.001[a]	1.727	1.220-2.444	0.002[a]
Surgical recurrence free survival				
Age at diagnosis, yr	0.097			0.065
A1 (⩽ 16)		3.512	0.944-13.068	
A2 (17-40)		1.547	0.475-5.036	
A1 (> 40)				
Type of operation	0.001[a]			0.002[a]
Elective		1		
Emergency		2.431	1.394-4.240	
Perianal disease after the 1st operation	0.056	1.715	1.005-2.926	0.048[a]
History of smoking	0.047[a]	1.798	1.088-2.969	0.022[a]
Postoperative use of anti-TNF	0.016[a]	0.521	0.300-0.904	0.02[a]

[a]*P* < 0.05; HR: Hazard ratio.

outcome after the primary operation. Similarly, despite a wide variation on reported risk of recurrence, the presence of perianal disease is thus considered to be a risk factor for postoperative recurrence in Caucasian population[3,21,22]. The presence of perianal disease is a sign of an increased inflammatory process and this may also influence intestinal CD.

Most patients with CD can be operated on electively, with only few patients requiring acute surgical intervention. In the present study, an indication for an emergency first intestinal resection was significantly associated with surgical recurrence. Riss *et al*[23] reported that an urgent indication for surgery was found to be significantly associated with the rate of surgical recurrence. Another retrospective study of 116 consecutive patients undergoing their first ileocolectomy at a large Austrian referral centre between 1997 and 2006 demonstrated that urgent index ileocolectomy increased the risk of repeat surgery approximately six-fold[24]. An emergency presentation could be a sign of a significant lack of an immune response to the on-going inflammation, making an emergency operation necessary. Furthermore, preoperative work up and intraoperative evaluation were limited in emergency situation and an emergency operation was usually performed by less experienced surgeons. As a consequence, these patients may be prone to developing early recurrence after the surgical treatment.

Smoking is the most widely recognized environmental risk factor for the initiation and recurrence of Crohn's disease[25]. Sutherland *et al*[26], reported that the 5- and 10-year recurrence rates were significantly higher in smokers (36% and 70%) than in non-smokers (20% and 41%; OR = 2.1, *P* = 0.007). Indeed, smoking is a significant predictive risk factor for all types of recurrences: endoscopic, clinical and surgical[6,27]. Cottone *et al*[25], conducted multivariate analyses to examine predictive factors for three types of recurrence (clinical, endoscopic and surgical recurrences) after primary surgery. They found that smoking was an independent significant factor for clinical, endoscopic and surgical recurrence. In the present study, history of smoking was significant risk factor for clinical and surgical recurrence.

Another important finding was that postoperative exposure to anti-TNF agents significantly reduced the likelihood of surgical recurrence. The introduction of anti-TNF agents has significantly changed the treatment strategy for CD. Recent studies have shown that anti-TNF agents reduced postoperative recurrence[28-30]. In the present study, postoperative use of anti-TNF agents seemed to reduce the risk of reoperation, indicating that medical treatment is one of the important factors affecting surgical recurrence[31]. A randomised controlled trial was able to demonstrate clearly an advantage of anti-TNF agents in maintaining clinical remission in patients with CD[32].

Immunomodulators such as azathioprine or 6-MP has been widely used, and several studies[33,34] showed that immunomodulators could reduce rate of surgery. Similarly, previous study [9] in our center revealed that early use of immunomodulators was independently related to the decreased surgery rate (*P* = 0.01). However, because immunomodulators were generally used after first surgery, it is difficult to determine their impact on decreasing the postoperative recurrence. In present study, 85% of patients used azathioprine or 6-MP after first surgery and use of immunomodulator showed no significant difference in postoperative recurrence. More recently, these drugs are used in combination with anti-TNF drugs to decrease

their immunogenicity and increase anti-TNF drug concentrations.

Antibiotics have not been shown to be effective for induction and maintenance of remission for active CD. The best data for antibiotics are in the short-term treatment of perianal disease and in combination with anti-TNF[35]. Aside from these indications, antibiotics should really be used to treat the inflammatory complications of CD such as enteric fistula or abscess. The main limitations to using these agents long standing are antibiotic resistance and side effect.

Surgical recurrence rates differ immensely between studies. One reason could be that there is crossover with a period of rapidly changing management options, both medical and surgical. Stapled anastomosis has now virtually replaced the hand-sewn technique in the majority of centres performing surgery for CD. Stapled anastomosis following ileocecal resection is an example, as it has been reported to be safe and feasible in patients with CD[36], with a few studies demonstrating a lower incidence of anastomotic recurrence after stapled functional rather than hand-sewn end-to-end anastomosis[37]. The vast majority of patients in the present study (225/230, 97.8%) received a stapled anastomosis. Among them, 210 patients (93.3%) received a functional end-to-end anastomosis with linear stapler, with 15 patients (6.7%) receiving an end-to-end or a side-to-end anastomosis with circular stapler. Only 5 patients (2.2%) received a hand-sewn anastomosis because of severe bowel edema or dilatation.

Several other risk factors for postoperative recurrence have been found over the past decades, such as a family history of CD, perforating type, younger age at diagnosis, the type of anastomotic configuration, sex, disease location, and the presence of postoperative complications[15,38,39], but none of these factors was associated with an increased risk for repeat surgery in the present analysis.

The present study had several limitations. First, the number of patients was relatively small. Owing to this limited study population, we were unable to demonstrate an increased risk of recurrence when there were known risk factors such as a perforating type disease, family history and a presence of complication. As this study was retrospective in design, there was no standardised follow-up protocol of endoscopy or image study for the patients who underwent primary bowel resection.

However, relatively large cohort of patients in Korea was included in this study with near complete follow up, so that these results showed a clinical importance in Asian population.

In conclusion, our present referral center study of long-term course of CD in Korea revealed a several factors that were significantly associated with a higher risk for surgical recurrence: the initial surgery as an emergency, history of smoking and the presence of perianal disease after the first intestinal resection. Anti-TNF agents seemed to reduce surgical recurrence. These findings indicate that affected patients with CD may benefit from close postoperative surveillance and probably from the early administration of anti-TNF agents.

COMMENTS

Background

Majority of Crohn's disease (CD) patients experienced recurrence after their first bowel resection and postoperative recurrence affects the quality of patients' lives. Thus, to provide optimal patient care, it is important to be able to predict which patients have a higher risk of recurrence. They investigated risk factors for the postoperative recurrence of CD in a patient population from a single tertiary referral center in Korea.

Research frontiers

The increasing tendency in Korea over the past few decades calls for more attention to be paid to the natural course of CD in these newly developed disease population. This study demonstrated the clinical feature and long-term prognosis of CD in a large cohort of non-Caucasian patients. In this study, history of smoking, postoperative perianal disease and undergoing an emergency operation were found to be independent risk factors for surgical recurrence in CD. The postoperative use of anti-TNF agents reduced surgical recurrence.

Innovations and breakthroughs

This study evaluated the risk factors which contribute the surgical recurrence and showed that Korean CD patients with postoperative perianal disease had increased risk of surgical recurrence.

Applications

Presence of postoperative perianal disease associated with surgical recurrence and using anti-TNF agents for patients with risk factors may reduce surgical recurrence. Physicians should consider aggressive and early top-down therapy for perianal CD patients.

Terminology

Postoperative perianal disease: Presence of perianal disease after first bowel resection.

Peer-review

This is an interesting study in which the investigators evaluated the risk factors for postoperative recurrence after primary bowel resection in a cohort of Korean CD patients.

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ORIGINAL ARTICLE

Retrospective Study

Trends and outcomes of pancreaticoduodenectomy for periampullary tumors: A 25-year single-center study of 1000 consecutive cases

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Ayman El Nakeeb, Waleed Askar, Ehab Atef, Ehab El Hanafy, Ahmad M Sultan, Tarek Salah, Ahmed shehta, Mohamed El Sorogy, Emad Hamdy, Mohamed El Hemly, Ahmed A El-Geidi, Tharwat Kandil, Mohamed El Shobari, Talaat Abd Allah, Amgad Fouad, Mostafa Abu Zeid, Ahmed Abu El Eneen, Nabil Gad El-Hak, Gamal El Ebidy, Omar Fathy, Ahmed Sultan, Mohamed Abdel Wahab, Gastroenterology surgical center, Mansoura University, Mansoura 35516, Egypt.

Author contributions: El Nakeeb A designed the research; El Nakeeb A, Askar W, Atef E, El Hanafy E, Sultan AM, Salah T, Shehta A, El Sorogy M, Hamdy E, El Hemly M, El-Geidi AA, Kandil T, El Shobari M, Allah TA, Fouad A, Zeid MA, El Eneen AA, El-Hak NG, El Ebidy G, Fathy O, Sultan A and Wahab MA performed the research; El Nakeeb A analyzed the data; El Nakeeb A, Shehta A and El Sorogy M wrote the paper.

Institutional review board statement: This study was approved by the Institutional Review Board of Mansoura University.

Informed consent statement: Informed consent was obtained from all patients to undergo pancreaticoduodenectomy after a careful explanation of the nature of the disease and possible complications.

Conflict-of-interest statement: All authors declare no conflict of interest.

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Abstract

AIM

To evaluate the evolution, trends in surgical approaches and reconstruction techniques, and important lessons learned from performing 1000 consecutive pancreaticoduodenectomies (PDs) for periampullary tumors.

METHODS

This is a retrospective review of the data of all patients who underwent PD for periampullary tumor during the period from January 1993 to April 2017. The data were categorized into three periods, including early period (1993-2002), middle period (2003-2012), and late period (2013-2017).

RESULTS
The frequency showed PD was increasingly performed after the year 2000. With time, elderly, cirrhotic and obese patients, as well as patients with uncinate process carcinoma and borderline tumor were increasingly selected for PD. The median operative time and postoperative hospital stay decreased significantly over the periods. Hospital mortality declined significantly, from 6.6% to 3.1%. Postoperative complications significantly decreased, from 40% to 27.9%. There was significant decrease in postoperative pancreatic fistula in the second 10 years, from 15% to 12.7%. There was a significant improvement in median survival and overall survival among the periods.

CONCLUSION
Surgical results of PD significantly improved, with mortality rate nearly reaching 3%. Pancreatic reconstruction following PD is still debatable. The survival rate was also improved but the rate of recurrence is still high, at 36.9%.

Key words: Pancreaticoduodenectomy; Pancreaticogastrostomy; Pancreaticojejunostomy; Postoperative pancreatic fistula; Periampullary tumor

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Core tip: Pancreaticoduodenectomy (PD) is a complex abdominal procedure. The hospital mortality rate has decreased to less than 5%; however, the rate of postoperative morbidities remains high, from 40% to 50%. Pancreatic reconstruction following PD is still debatable. The long survival rate after PD is clearly improved with time but still poor. Frequency showed PD is increasingly performed. With time, elderly, cirrhotic and obese patients, and patients with uncinate process carcinoma and borderline tumor are increasingly selected for PD. Median operative time and postoperative hospital stay decreased significantly. Hospital mortality declined significantly, from 6.6% to 3.1%. Postoperative complications significantly decreased.

El Nakeeb A, Askar W, Atef E, EI Hanafy E, Sultan AM, Salah T, Shehta A, El Sorogy M, Hamdy E, El Hemly M, El-Geidi AA, Kandil T, El Shobari M, Allah TA, Fouad A, Zeid MA, El Eneen AA, EI-Hak NG, El Ebidy G, Fathy O, Sultan A, Wahab MA. Trends and outcomes of pancreaticoduodenectomy for periampullary tumors: A 25-year single-center study of 1000 consecutive cases. *World J Gastroenterol* 2017; 23(38):7025-7036 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7025.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7025

INTRODUCTION

The first successful localized resection of periampullary tumor was performed by Dr William S Halsted in 1898. For the first time, Allen O Whipple described pancreaticoduodenectomy (PD) in the year 1935, when he modified the procedure that was performed before by Alessendro Codinivillan in Italy and Walter Keusch in Germany[1,2]. In 1963, Whipple performed 37 PDs in his era. From this era till 1980, PD was infrequently performed because the hospital mortality was high (> 25%). After 1990, with development of high-volume centers and improvement of operative technique, surgical equipment, and perioperative care, PD has become a relatively safe and commonly performed procedure[3-5].

PD is one of the most complex abdominal operations that is performed for a heterogeneous group of periampullary lesions, either benign or malignant. PD involves extensive dissection, resection and different reconstruction procedures[3-8]. The rate of postoperative morbidities remains high, from 40% to 50%. However, the hospital mortality rate has decreased to less than 5% in many published series[5-8,9-11].

Many studies were performed to determine the risk factors of post-operative pancreatic fistula and try to present a fistula risk scoring system after PD. These various systems used many factors including pancreatic duct diameter, consistency of pancreas, body mass index (BMI) > 25 kg/m^2, and final pathology[4-6,11-15]. Pancreatic reconstruction following PD is still debatable, even for pancreatic surgeons. Ideally, pancreatic reconstruction after PD should reduce the risk of post-operative pancreatic fistula (POPF) and its severity if developed with preservation of pancreatic functions (exocrine and endocrine functions)[5-8].

The prognosis of pancreatic head adenocarcinoma is one of the most dismal of all cancers. After PD, the 5-year survival is 5% to 20%, representing the worst survival among the periampullary cancers. Numerous prognostic factors have been found to improve survival rate after PD, including lymph node status, free safety margins, tumor size, differentiation, complete excision of mesopancreas, and vascular invasion[11-14].

Many points are still debatable regarding PD, including selection of patients, pancreatic reconstruction, and factors that improve survival rate. Thus, the aim of this study was to evaluate the evolution, trends in surgical approaches and reconstruction techniques, and important lessons learned from performing 1000 consecutive PD for periampullary tumors in the Gastrointestinal Surgery Center of Mansoura University over a period of 25 years.

MATERIALS AND METHODS

Study design

This is a retrospective review of the data of all patients who underwent PD for periampullary tumor in the Gastrointestinal Surgery Center (Mansoura University, Egypt) during the period from January 1993 to April

2017. Patient data were recorded in a prospectively maintained database for all patients undergoing PD since the year 2000; for patients from before 2000, the data were obtained from the archived files. An informed consent for the surgical procedures was obtained from each patient. The Gastrointestinal Surgery Center of Mansoura University is a high-volume center of pancreatic surgery that was established in 1992. The first PD was performed in 1993, and was regularly performed afterwards in our center over a period of 25 years.

Inclusion criteria

This study included 1000 patients who underwent PD for different periampullary tumors (benign and malignant lesions) at our Center in Mansoura University, Egypt during the period from January 1993 to April 2017. Over the 25-year period, 1000 consecutive PD were performed by 20 surgeons. For this study, the data were categorized into three periods, early period (1993-2002), middle period (2003-2012), and late period (2013-2017). This study was approved by the local institutional review board (IRB).

Exclusion criteria

Patients with periampullary lesions who were explored during the same period and failed to complete the PD procedure due to the presence of locally advanced or distant metastatic disease that was not detected in preoperative radiological workup.

Preoperative assessment

Preoperative diagnostic workup included clinical assessment, detailed laboratory investigations, including tumor markers, and radiological investigations (abdominal ultrasound, abdominal triphasic computed tomography (CT), CT angiography, magnetic resonance cholangiopancreatography, chest X-ray and bone survey). Preoperative biliary drainage was performed by endoscopic retrograde cholangiopancreatography in selected patients[16].

Surgical procedures

Over the study period and with accumulating experience, evolution of the surgical approach and techniques occurred.

Dissection technique

In the early period, the anterior approach was utilized in surgical dissection. Afterwards, we shifted to utilize the posterior approach (one of the artery first approaches), aiming to identify vascular invasion at an early stage of the dissection, and to allow more radical excision of the mesopancreas[17]. Standard regional lymphadenectomy was performed, which included resection of nodes within the outlines of the hepatoduodenal ligament, right side of the superior mesenteric vessels, and inferior vena cava. In the early period, diathermy dissection and ligatures were used during the resection stage. Afterwards, a shift to use modern energy devices occurred, such as for Ligasure and Harmonic scalpel.

Approach

In most of our study period, we utilized an open surgical approach through extended right subcostal or inverted J incisions. In the late period, we started to utilize laparoscopic approach, with laparoscopy-assisted approach used in the beginning. This included complete dissection by the laparoscopy followed by reconstruction carried out through a small upper midline or transverse incision. In the last year, we performed a total of 10 laparoscopic PDs. This included completing the whole approach (dissection and reconstruction) by laparoscopy.

Meso-pancreatectomy

A complete removal of all lympho-vascular tissues between the uncinate process and superior mesenteric artery was mandatory in PD. These tissues are the most important site for local recurrence after PD. This concept had evolved in the recent years and became a standard step in the radical resection of periampullary tumors. We adopted this concept in the recent years of our study.

Division of the pancreatic neck

Initially, we divided the pancreatic neck sharply by surgical scalpel and then carried out the hemostasis after division. Recently, we started to divide the pancreatic neck by diathermy and Harmonic scalpel.

Reconstruction

In the beginning of our series, we performed simple loop pancreaticojejunostomy (PJ) for the reconstruction of the pancreatic stump. However, a high rate of pancreatic fistula was noticed. A shift of the reconstruction plan occurred to pancreaticogastrostomy (PG). Short-term outcomes were improved and lower rate of pancreatic fistula was noticed, but the long-term outcomes regarding the digestive and nutritional conditions were not appropriate.

With accumulating experience and refinement of the surgical technique, a re-shift to PJ (simple loop or isolated loop) occurred, which improved long-term outcomes[18,19]. Recently, we adopted a tailored approach for pancreatic stump management. In patients at high risk of pancreatic fistula (presence of two or more risk factors) PG is preferred. In low- and moderate-risk patients (absence of risk factors or presence of one risk factor) PJ is preferred.

Postoperative management

All patients were transferred to the intensive care unit (ICU) postoperatively. Antibiotics and analgesic were

given to all patients. Octreotide analogue was given to some patients postoperatively. Abdominal drains and nasogastric tubes outputs were recorded daily. Patients started oral feeding once bowel sounds restarted and they were able to tolerate nutrition by a fluid diet, then a regular diet.

Abdominal ultrasound was done routinely in all patients postoperatively. Serum amylase and liver function tests were measured on postoperative day (POD) 1 and POD 5. Ultrasound-guided tubal drainage was carried out in patients who had an abdominal collection.

Follow-up occurred at 1 wk, 3 mo and 6 mo postoperatively, and then at 1 year. Patients were also seen at outpatient clinics if symptoms developed between follow-up visits.

Definitions

Complications were defined as adverse events resulting in deviation from the normal postoperative course within 30 d after operation. Severity of complications was assessed using the Clavien classification system, from 1 to 5. Major complications represent those requiring endoscopic, radiologic or surgical intervention, and were defined as class 3 or higher[20].

Postoperative pancreatic fistula was defined by International Study Group of Pancreatic Fistula as any measurable volume of fluid on or after POD 3 with amylase content greater than three times the serum amylase activity, and classified into three grades: A, B or C[21-23].

Delayed gastric emptying (DGE) was defined as output from a nasogastric tube of greater than 500 mL per day that persisted beyond POD 10, the failure to maintain oral intake by POD 14, or reinsertion of a nasogastric tube[21-24].

Outcomes of the study

The aim of this study was to evaluate the milestones, trends in surgical approaches, reconstruction techniques and important lessons learned from performing 1000 consecutive PD for periampullary tumors in the Gastrointestinal Surgery Center of Mansoura University over a period of 25 years. The main outcome of the study was the rates of postoperative morbidity, according to Clavien-Dindo classifications, and of mortality after PD. Special concern was focused on POPF, biliary complications, DGE and the predictive factors of each. In addition, we evaluated the survival outcomes of the PD patients, including recurrence, overall survival (OS) and the different predictive factors of each.

Statistical analysis

Statistical analysis of the data in this study was performed using SPSS software for windows, version 20. For continuous variables, descriptive statistics were calculated and reported as median. Categorical variables were described using frequency distributions. The χ^2 test was used to compare categorical variables and one-way ANOVA for continuous variables. The predictive factors for postoperative complications were evaluated by binary logistic regression method. Survival outcomes were calculated by the Kaplan-Meier method. The predictive factors for survival were evaluated by the Cox regression method. *P* values < 0.05 were considered to be significant.

RESULTS

One thousand patients underwent PD for resection of periampullary tumors from January 1993 to April 2017. Of the 1000 patients who underwent PD, 556 involved pancreatic head mass, 312 were ampullary tumors, 61 were duodenal tumors, 41 were cholangiocarcinoma and 30 were uncinate process mass. The median age of patients was 54-years-old. The data were categorized into three periods. For the first 10 years (1993-2002), a total of 300 patients underwent PD (30 cases/year). In the next 10 years (2003-2012), the total number was 442 patients who underwent PD (44.2 cases/year). In the last 5 years (2013-2017), the total number was 258 patients who underwent PD (51.6 cases/year) (Figure 1).

Preoperative data

Elderly patients were increasingly selected for PD, as the median age was 53 in the first 10 years and 55 in the last 5 years. Obese patients were increasingly selected in the last five years. There were no significant changes for selection of patients for PBD in the period of the study. PBD was indicated for patients with high serum bilirubin (> 10 mg%) with high liver enzymes, renal impairment or associated cholangitis (Table 1).

Intraoperative data

Patients with periampullary tumors and well-compensated chronic liver disease were increasingly selected for PD (Table 2). In the early period, we performed simple loop PJ (21.7%) for the reconstruction of the pancreatic stump, then shifted to PG (78.3%). In the second 10 years, 94.3% of cases underwent PG. In the last 5 years, there was a re-shift to PJ (simple loop or isolated loop) (46.1%). Complete meso-pancreatecomy was achieved in all cases in the last 5 years Operative time was significantly reduced, from 6 h in the first 10 years to 5 h in last 5 years. The median intraoperative blood loss decreased from 500 cc in the first 10 years to 300 cc in last 5 years.

Postoperative data

The overall morbidity of all 1000 patients was 32.3%. The postoperative complications decreased markedly in the recent years, from 40% to 27.9%. There was a significant decrease in POPF in the second 10

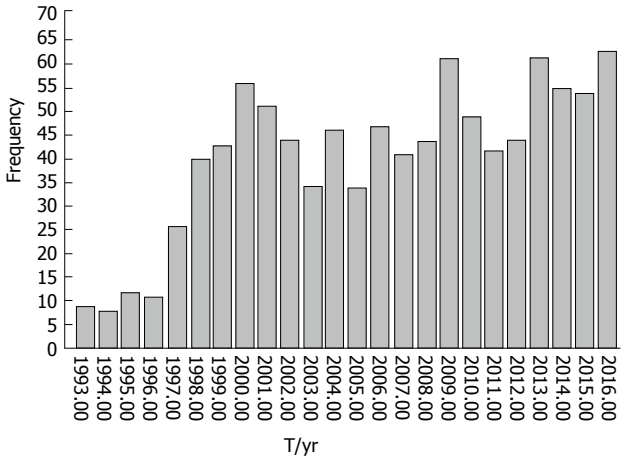


Figure 1 In the first 10 years (1993-2002), the total number of patients who underwent PD was 300 (30 cases/yr), in the next 10 years (2003-2012) 442 patients underwent PD (44.2 cases/yr), and in the last 4 years (2013-2017) 258 cases underwent PD (51.6 cases/yr).

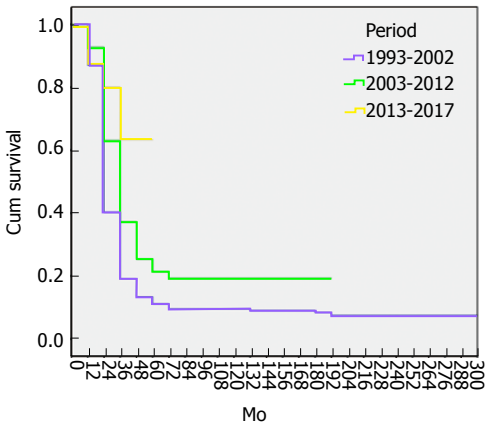


Figure 2 Overall survival curves of patients according to the 3 periods of study. The overall survival rate significantly improved and 5-yr survival rate for the early period was 11%, followed by 21% for the middle period and 64% for the late period (P = 0.0001)

years, from 15% to 12.7% with a decrease in the severity. But the incidence of POPF increased again in last 5 years to 14.7%. DGE was the most common complication (18%). It was secondary to other postoperative complications in 15.2%. Primary DGE presented in 2.8% of cases (Table 3).

The median hospital stay and the day of drain removal were significantly shortest in the late period, decreasing from 9 days to 8 days. The overall hospital mortality of all 1000 patients was 4.3% (43 patients). The hospital mortality declined significantly, from 6.6% to 3.1%. The causes of death were sepsis secondary to POPF in 17 patients, with 6 cases due to cardiac arrest, 6 cases due to liver cell failure, 5 cases due to pulmonary embolism, 3 cases due to pancreatitis, 3 cases due to respiratory failure secondary to severe chest infection, 2 cases due to secondary hemorrhage, and 1 case due to PG–related uncontrolled bleeding.

Seventy patients developed intra-abdominal collection and were managed by ultrasound-guided tubal drainage. Seventy-four patients (7.4%) required re-explorations due to internal hemorrhage (26 patients, with 7/26 due to erosion of gastroduodenal artery), bleeding GJ (17 patients), bleeding PG (15 patients), peritonitis (12 patients) or debridement and drainage (4 patients). Completion spleno-pancreatectomy was needed in 2 cases due to POPF that eroded the gastroduodenal artery and were complicated by secondary internal hemorrhage

The overall recurrence rate in 870 patients who had malignant pathology after PD was 36.9.2%. This rate decreased from 50.4% to 28.7%

Univariate analysis of risk factors for development of POPF found that six variables were significantly associated with POPF (BMI > 25 kg/m^2, liver cirrhosis, soft pancreas, main pancreatic duct < 3 mm, pancreatic duct close to posterior edge < 3 mm, and period of the study). These six risk factors of POPF identified in univariate analysis were further analyzed in multivariate analysis. Soft pancreas, main pancreatic duct < 3 mm pancreatic duct close to posterior edge < 3 mm, BMI > 25 kg/m^2 and period of the study were found to be independent risk factors (Table 4).

Postoperative pathology

There was significant difference among groups regarding site of periampullary tumor, type of pathology, number of dissected lymph nodes, number of infiltrated lymph nodes, lymph node ratio, safety margin, perivascular infiltration and perineural invasion (Table 5).

Survival rate

The 1-, 3- and 5-year OS rates for all cases were 90%, 33% and 19% respectively, with a median survival of 26 mo. There was a significant difference among the groups regarding the median survival and the OS rates at 1-, 3- and 5-years (Tables 3 and 6, Figure 2). The survival analysis in this study revealed that female sex, patients who did not develop major complications, presence of ampullary tumor, type of pathology, negative safety margin, negative lymph nodes, chemoradiotherapy and period of the study were all favorable prognostic variables in univariate and multivariate analyses. The improvement of survival with recent years may be due to complete excision of mesopancreas, greater use of postoperative chemoradiotherapy, improvement of surgical techniques, and strict follow up of most of cases (Table 6).

DISCUSSION

PD is a complex procedure including extensive dissection, resection and multiple reconstruction. Allen O Whipple described PD in the year 1935. From Whipple's era till 1980, PD was performed infrequently

Table 1 Demographic and preoperative data

Variable	Total, *n* = 1000	First 10 yr, 1993-2002	Second 10 yr, 2003-2012	Last 5 yr, 2013-2017	*P*-value
Age in yr	54 (12-88)	53	55	55	0.22
Sex					
Male	614 (61.4)	190 (63.3)	260 (58.8)	164 (63.6)	0.33
Female	386 (38.6)	110 (36.7)	182 (41.2)	94 (36.4)	
DM	145 (14.5)	39 (13)	60 (13.6)	46 (17.8)	0.21
BMI in kg/m^2					
< 25	723 (72.3)	250 (38.3)	326 (73.8)	147 (57)	0.0001
> 25	277 (27.7)	50 (16.7)	116 (26.2)	111 (43)	
Abdominal pain	753 (75.3)	230 (76.7)	329 (74.4)	194 (74.2)	0.12
Jaundice	909 (90.9)	265 (88.3)	406 (91.9)	238 (92.2	0.18
Preoperative biliary drainage	511 (51.1)	163 (54.5)	226 (51.1)	122 (47.3)	0.23
Preoperative serum albumin in mg	4 (3.2-5.2)	4.1	4	3.9	0.23
Preoperative serum bilirubin in mg	4 (0.5-38)	3.1	4.7	4.3	0.0001
Preoperative CEA	6.4 (0.5-394)	8	8	6	0.09
Preoperative CA19-9	27 (0.5-1200)	32	33	34	0.12

Data are presented as *n* (%) or *n* (range), unless otherwise indicated. BMI: Body mass index; CEA: Carcinoembryonic antigen; DM: Diabetes mellitus.

Table 2 Operative data

Variable	Total, *n* = 1000	First 10 yr, 1993-2002	Second 10 yr, 2003-2012	Last 5 yr, 2013-2017	*P*-value
Cirrhosis	129 (12.9)	28 (9.3)	62 (14)	39 (15.1)	0.009
Mass size in cm	3 (0.5-15)	3	3	3	
< 2	418 (41.8)	123 (41)	174 (39.4)	123 (46.9)	0.14
> 2	582 (58.2)	177 (59)	268 (60.6)	137 (53.1)	
Pancreatic texture					
Soft	596 (59.6)	190 (63.3)	263 (59.5)	143 (55.4)	0.23
Firm	404 (40.4)	110 (36.7)	179 (40.5)	115 (44.6)	
Median pancreatic duct diameter in mm	5 (1-15)				0.47
< 3	313 (31.3)	97(32.3)	137 (30.9)	79 (30.6)	
> 3	687 (68.7)	20367.7)	305 (60.1)	179 (69.4)	
Pancreatic duct to posterior border in mm					0.45
< 3	421 (42.1)	128 (42.7)	185 (41.9)	108 (41.9)	
> 3	579 (57.9)	172 (57.3)	257 (58.1)	150 (58.1)	
Pancreatic stump mobilization in cm	2 (1-4)	2	2	2	0.45
CBD diameter in mm	15 (5-30)	15	15	15	0.06
Type of reconstruction					
PG	791 (79.1)	235 (78.3)	417 (94.3)	139 (53.9)	0.0001
Simple PJ	163 (16.3)	65 (21.7)	25 (5.7)	73 (28.3)	
Isolated loop PJ	46 (4.6)	0	0	46 (17.8)	
Duct to mucosa	134 (13.4)	9 (3)	65 (14.7)	60 (23.3)	
Invaginated type with duct to mucosa	644 (64.4)	234 (78)	250 (65.6)	160 (62)	0.0001
Invaginated type without duct to mucosa	221 (22.1)	57 (19)	126 (28.5)	38 (14.7)	
No anastomosis	1 (0.1)	0	1 (0.2)	0	
Standard approach	908 (90.8)	277 (92.3)	388 (87.8)	243 (94.7)	0.004
Posterior approach	92 (9.2)	23 (7.7)	54 (12.2)	15 (5.8)	
Complete meso-pancreatectomy	574 (57.4)	83 (27.7)	233 (52.7)	258 (100)	0.0001
Laparoscopic assisted PD	11 (1.1)	0	0	11 (4.3)	0.0001
Complete laparoscopic PD	10 (1)	0	0	10 (3.9)	
Vascular resection	12 (1.2)	0	4 (0.9)	8 (3.1)	0.003
Primary anastomosis	0	0	3 (0.7)	8 (3.1)	
Gortex	0	0	1 (0.2)	0	
Operative time in h	5 (3.5-10)	6	5	5	0.001
Blood loss in cc	500 (50-4000)	500	400	300	0.001

Data are presented as *n* (%) or *n* (range). PD: Pancreaticoduodenectomy; PG: Pancreaticogastrostomy; PJ: Pancreaticojejunostomy.

because the hospital mortality was high above 25%[2].

Patient selection is still an important factor in decreasing postoperative morbidity and mortality. In our series, the frequency of PD had been increasingly performed after the year 2000. Elderly patients were increasingly selected for PD, as the median age was 53 years in the first 10 years and became 55 years in the last 5 years. In the last 5 years, we accepted patients over the age of 75.

The significant improvement in the surgical

Table 3 Postoperative data

Variable	Total, *n* = 1000	First 10 yr, 1993-2002	Second 10 yr, 2003-2012	Last 5 yr, 2013-2017	*P*-value
Hospital stay in d	8 (5-71)	9	8	8	0.0001
Time to oral intake in d	5 (4-56)	6	5	5	0.33
Total amount of drainage in mL	700 (40-35000)	1200	600	600	0.0001
Drain removal in d	8 (4-71)	8	8	8	0.03
Total postoperative complications	323 (32.3)	120 (40)	131 (29.6)	72 (27.9)	0.02
Dindo grade					
I	114 (11.4)	24	47	43	
II	97 (9.7)	40	45	12	0.11
III	69 (6.9)	36	24	9	
IV and V	43 (4.3)	20	15	8	
Severe complications, ⩾ III					
Minor	211 (21.1)	84	92	55	0.23
Major	109 (10.9)	56	69	17	
Pancreatic fistula	139 (13.9)	45 (15)	56 (12.7)	38 (14.7)	0.01
Grade A	67 (6.7)	14	33	20	0.04
Grade B	48 (4.8)	20	17	11	
Grade C	24 (2.4)	11	6	7	
DGE	180 (18)	76 (25.3)	67 (15.2)	37 (14.3)	0.06
Types of DGE					
Secondary DGE	152 (15.2)	70 (23.3)	54 (12.2)	27 (10.5)	0.03
Primary DGE	28 (2.8)	5 (1.7)	13 (2.9)	10 (3.9)	
Pulmonary complications	46 (4.6)	20 (6.7)	21 (4.8)	5 (1.6)	0.01
Bile leak	73 (7.3)	39 (13)	19 (4.3)	15 (5.8)	0.001
Postoperative bleeding	25 (2.5)	13 (4.3)	7 (1.6)	5 (1.9)	0.49
Pancreatitis	20 (2)	12 (4.3)	7 (1.6)	1 (0.4)	0.004
Bleeding PG	15 (1.5)	8 (2.7)	5 (1.2)	2 (0.8)	0.14
Wound infection	50 (5)	13 (4.6)	25 (5.7)	12 (4.7)	0.77
Re-operation	74 (7.4)	25 (8.3)	33 (7.5)	16 (6.2)	0.21
Recurrence	321 (36.9)	130 (50.4)	125 (32.6)	66 (28.7)	0.0001
Hospital mortality	43 (4.3)	20 (6.6%)	15 (3.4)	8 (3.1)	0.02
Postoperative chemoradiotherapy	275 (27.5)	0	132 (29.9)	143 (55.4)	0.0001
Overall median survival in mo	26 (1-300)	21	30	37	0.0001
1 yr	90%	87%	93%	87%	
3 yr	33%	19%	37%	64%	
5 yr	19%	11%	21%		

Data are presented as *n* (%) or *n* (range), unless otherwise indicated. DGE: Delayed gastric emptying; PG: Pancreaticogastrostomy.

outcome of PD has encouraged surgeons to approach periampullary tumors as aggressively in elderly patients[7,12]. Patients with periampullary tumors and well-compensated chronic liver disease are increasingly selected for PD with accepted surgical outcomes. PD is only recommended in patients with Child A cirrhosis without portal hypertension[13]. PD is associated with an increased risk of postoperative morbidity in obese patients. With time, obesity has lost its status as a limitation for PD and such patients have been increasingly selected[25-27].

Patients with uncinate process carcinoma are increasingly selected for PD as well. However, the loco-regional recurrence rate was found to be common and the OS rate to be lower than other periampullary tumors[28]. The role of postoperative chemoradiotherapy may improve the results. Borderline resectable pancreatic cancer should be included as an indication of PD with advancements in chemoradiotherapy and techniques of vascular resection[29].

The impact of PBD on postoperative outcomes remains controversial. PBD before PD was associated with major postoperative morbidities and stent-related morbidities, including infection, pancreatitis or adhesions. There are no significant changes for selection of patients for PBD in the period of the study. PBD is indicated for patients with high serum bilirubin (> 10 mg%) with high liver enzymes, renal impairment, or associated cholangitis[16,30,31].

In our Center, laparoscopic pancreaticoduodenectomy (LPD) has been introduced as a feasible alternative to open PD since 2013, involving dissection in some cases, then complete LPD was performed at the end of 2013; unfortunately, however, the patients died on POD 7. We restarted again in January 2016 to perform 10 complete LPDs; in all cases, the pancreatic reconstruction was PG. The median operative time was 8 h, so the procedure was performed by two teams (one team for complete dissection and the other one to perform all reconstruction). Only one hospital mortality occurred due to severe pancreatitis, and all cases passed smoothly without any complications. The median hospital stay was 5 days. The most important point to performing a safe LPD is to perform the procedure under skilled hands in selected patients and

Table 4 Univariate and multivariate analyses of risk factors of development of post-operative pancreatic fistula

	Univariate	Multivariate	Exp(B)	95%CI for Exp(B)	
	P-value	*P*-value		Lower	Upper
Age grouping > 60 yr	0.2				
Sex	0.99				
DM	0.58				
BMI >25 kg/m^2	0.0001	0.0001	6.468	4.193	9.977
Preoperative serum bilirubin > 10 mg%	0.62				
Preoperative ERCP	0.52				
Liver cirrhosis	0.05	0.328	0.699	0.341	1.434
Size of the tumor > 2 cm	0.91				
Soft pancreas	0.0001	0.0001	0.218	0.14	0.341
Pancreatic duct diameter > 3 mm	0.0001	0.0001	0.182	0.118	0.279
Pancreatic duct closely related to posterior border of pancreas > 3 mm	0.0001	0.0001	0.372	0.243	0.57
Blood loss > 1000 mL	0.67				
Blood transfusion	0.94				
Type of pancreatic reconstruction	0.62				
Duration of operation	0.75				
Site of the tumor	0.34				
Period of the study	0.001	0.001	0.615	0.461	0.82

BMI: Body mass index; DM: Diabetes mellitus; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 5 Postoperative pathology *n* (%)

Variable	Total, *n* = 1000	First 10 yr, 1993-2002	Second 10 yr, 2003-2012	Last 5 yr, 2013-2017	
Site of the tumor					
Ampullary tumor	312 (31.2)	92 (30.7)	145 (32.8)	75 (29)	
Pancreatic head mass	556 (55.6)	171 (57)	257 (58.1)	128 (49.6)	0.0001
CBD duct tumor	41 (4.1)	16 (5.3)	13 (2.9)	12 (4.7)	
Duodenal tumor	61 (6.1)	20 (6.7)	27 (6.1)	14 (5.4)	
Uncinate process mass	30 (3)	1 (0.3)	0	29 (11.3)	
Pathological diagnosis					
Adenocarcinoma	836 (83.6)	250 (83.3)	357 (80.7)	229 (88.8)	
Undifferentiated carcinoma	20 (2)	1 (1.7)	19 (4.3)	0	
Papillary cystadenocarcinoma	6 (0.6)	4 (1.3)	2 (0.5)	0	0.0001
Lymphoma	4 (0.4)	2 (0.6)	2 (0.5)	0	
Neuroendocrine tumor	28 (2.8)	12 (4)	10 (2.3)	6 (2.3)	
Solid pseudopapillary tumor	20 (2)	5 (1.7)	8 (1.8)	7 (2.7)	
Chronic pancreatitis	24 (2.4)	6 (2)	14 (3.2)	4 (1.6)	
Benign cyst	12 (1.2)	3 (1)	8 (1.8)	1 (0.4)	
Adenoma with dysplasia	41 (4.1)	15 (5)	19 (4.3)	7 (2.7)	
Gastrointestinal stromal tumor	2 (0.2)	0	1 (0.2)	1 (0.4)	
Glomus	1 (0.1)	1 (0.3)	0	0	
Adenosquamous	2 (0.2)	1 (0.3)	1 (0.2)	0	
Pleomorphic adenoma	1 (0.1)	0	1(0.2)	0	
Adenomyoma	3 (0.3)	0	0	3 (1.2)	
Malignant	870 (87)	258 (86)	382 (86.4)	230 (89.1)	0.38
Borderline	48 (4.8)	17(5.7)	18 (4.1)	13 (5.1)	
benign	82 (8.2)	25 (8.3)	42 (9.5)	15 (5.8)	
Number of dissected lymph node	6 (0-40)	5 (0-18)	6 (0-40)	6 (0-40)	0.59
Number of lymph node infiltration	0 (0-14)	0 (0-3)	0 (0-14)	0 (0-14)	0.07
Perineural infiltration	187 (18.7)	62 (20.7)	75 (17)	50 (19.9)	0.09
Perivascular infiltration	134 (134)	40 (13.3)	66 (14.9)	28 (10.9)	0.13
Pancreatic safety margin					
R1	91 (9.1)	40 (13.3)	41 (9.3)	10 (3.8)	0.01
R2	15 (1.5)	7 (2.3)	6 (1.4)	2 (0.7)	

using suitable surgical techniques[32,33].

The ideal and safe pancreatic reconstruction following PD is still debatable. We performed a comparative randomized study between PG and isolated loop PJ which revealed no significant difference between both methods in regards to POPF, but the pancreatic function was preserved with isolated loop PJ[18,19]. Recently, with accumulating experience and refinement of the surgical technique, we adopted a tailored approach for the management of pancreatic stump management. Another prospective randomized study comparing duct to mucosa and invagination PJ

Table 6 Univariate and multivariate analyses of factors affecting the survival

	Median survival	1 yr survival	3 yr survival	5 yr survival	Univariate *P*-value	Multivariate *P*-value	Exp(B)	95%CI for Exp(B)	
								Lower	Upper
> 60 yr	26	91	35	20	0.07				
< 60 yr	24	87	31	18					
Male	24	89	30	16	0.001	0.003	0.77	0.651	0.914
Female	30	91	39	25					
BMI < 25 kg/m^2	27	92	32	19	0.99				
BMI > 25 kg/m^2	25	84	37	20					
Preoperative serum bilirubin < 10 mg%	26	90	34	21	0.6				
Preoperative serum bilirubin > 10 mg%	25	90	33	18					
Preoperative CA19-9 < 37	26	90	35	24	0.17				
Preoperative CA 19-9 > 37	25	89	31	14					
Preoperative CEA < 5	26	90	34	21	0.69				
Preoperative CEA > 5	25	91	33	18					
History of preoperative ERCP	25	88	33	15	0.19				
No history of ERCP	26	92	33	25					
Normal liver	32	90	37	24	0.11				
Liver cirrhosis	25	90	33	19					
Size of the tumor < 2 cm	28	91	37	20	0.16				
Size of the tumor > 2 cm	25	89	31	19					
Type of pancreatic reconstruction									
PG	25	90	31	17	0.11				
Simple loop PJ	32	90	44	28					
Isolated loop PJ	32	80	48	48					
Without POPF	26	90	34	19	0.79				
With POPF	25	89	28	23					
Without major postoperative complications	27	93	35	20	0.03	0.005	0.784	0.661	0.929
With major postoperative complications	24	82	31	18					
Site of the tumor									
Ampullary tumor	33	90	42	22					
Pancreatic head mass	23	81	28	11	0.02	0.3	1.049	0.958	1.148
CBD duct tumor	32	90	30	20					
Duodenal tumor	31	90	42	21					
Uncinate process mass	12	79	18	0					
Type of pathology									
Adenocarcinoma	26	90	27	8					
Neuroendocrine tumor	65	85	71	60					
Solid pseudopapillary tumor	150	100	94	94	0.0001	0.005	0.97	0.951	0.991
Chronic pancreatitis	160	96	96	84					
Benign cyst	130	100	90	70					
Adenoma with dysplasia	85	91	67	48					
Lymph node negative	29	92	38	27	0.0001	0.0001	0.559	0.413	0.757
Lymph node infiltrated	25	87	27	8					
Lymph node ratio									
0	28	91	38	27					
< 0.2	25	89	31	18	0.0001	0.15	0.908	0.797	1.034
0.2-0.4	25	86	28	11					
> 0.4	24	88	21	4					
Without perineural infiltration	25	89	35	21	0.06				
With perineural infiltration	24	91	28	12					
Without perivascular infiltration	29	91	44	24	0.29				
With perivascular infiltration	25	86	35	14					
Pancreatic safety margin									
R0	26	90	35	20					
R1	22	92	27	19	0.0001	0.0001	1.695	1.345	2.136
R2	12	70	19	0					
With postoperative chemoradiotherapy	39	92	53	34	0.0001	0.0001	0.567	0.435	0.741
Without postoperative chemoradiotherapy	24	89	28	15					
Period of the study									
1993-2002	21	87	19	11					
2003-2012	30	93	37	21	0.001	0.0001	0.64	0.555	0.738
2013-2017	37	87	63						

BMI: Body mass index; CBD: Common bile duct; CEA: Carcinoembryonic antigen; ERCP: Endoscopic retrograde cholangiopancreatography; PG: Pancreaticogastrostomy; PJ: Pancreaticojejunostomy; POPF: Postoperative pancreatic fistula.

carried out in our center concluded that invagination PJ is preferred in small pancreatic duct and provides less incidence of postoperative steatorrhea and was associated with less severity of POPF, if developed, than duct to mucosa[19].

In our series, 32.3% of patients undergoing PD had complications. The majority of complications were minor and not life threatening. The postoperative complications decreased markedly in the recent years, from 40% to 27.9%. There was significant decrease in POPF in the second 10 years, from 15% to 12.7% with decrease in severity due to shift of pancreatic reconstruction from PJ to PG. But, the incidence of POPF increased again in last 5 years to 14.7%, due to re-shift again to PJ in order to achieve better long-term outcomes regarding function and morphology of pancreas[18,19]. In this study, the development of major complications had a negative impact on OS.

The median hospital stay was significantly shortest in the last 10 years. In many high-volume centers, there has been significant decrease in postoperative stay after PD as a result of the increase in the frequency of PD, the decrease in incidence of complications, especially of DGE, the decrease in use of pylorus-preserving PD, which is complicated by high incidence of DGE, and the improvements in postoperative care and management of postoperative complications[6,7,34-36].

In this study, the overall hospital mortality was 4.3%. Of the patients who died, 17/43 (39.5%) of the deaths were due to sepsis secondary to POPF. The decrease in hospital mortality following PD over the time is the most prominent achievement in PD. In this study, the hospital mortality rate decreased significantly from 6.6% to 3.1%, which is comparable to the reported mortality rates in other high-volume centers[6-8].

Long-term survival after PD for periampullary tumor adenocarcinoma is still poor. However, the survival after PD has clearly improved with time, due to improvement of surgical techniques, complete excision of mesopancreas, greater use of neoadjuvant and/or adjuvant chemoradiotherapy, and strict follow up of most of cases. There are many high-volume centers in which patients with pancreatic head carcinoma treated by PD have a 5-year survival rate of around 20%[6,7,35,36].

In conclusion, the frequency of PD has increased and become a relatively safe procedure in our Center. With time, elderly, cirrhotic, obese patients, patients with uncinate process carcinoma and borderline tumors have been increasingly selected for PD. Surgical results of PD, including operative time, hospital stay and postoperative complications, have significantly improved, with the mortality rate reaching nearly 3%. Pancreatic reconstruction following PD is still debatable. PG provides better short-term outcomes including POPF, but the long-term outcomes regarding the pancreatic function and nutrition were not appropriate. However, PJ provides better long-term outcomes. The survival rate also improved due to complete meso-pancreatectomy and utilization of adjuvant chemoradiotherapy, but the rate of recurrence is still high at 36.9%. LPD has been introduced as a feasible alternative to open PD.

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ORIGINAL ARTICLE

Prospective Study

Testing for hepatitis B virus alone does not increase vaccine coverage in non-immunized persons

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Author contributions: Bottero J, Boyd A, Carrat F and Lacombe K conceived and designed the study; Bottero J and Rougier H conducted the study and ensured data quality; Gozlan J validated serological and virological results; Boyd A analyzed the data; Boyd A and Bottero J drafted the initial version of the paper; Carrat F, Gozlan J, Rougier H, Girard PM and Lacombe K gave critical revisions to the manuscript. All authors approved the final version of the manuscript.

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Institutional review board statement: The study was approved by the Hôtel-Dieu Hospital Ethics Committee (Paris, France) in accordance with the Helsinki Declaration.

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Abstract

AIM

To determine whether hepatitis B virus (HBV)-testing could serve as a gateway to vaccinate non-immunized individuals in a low-prevalent country.

METHODS

Non-immunized subjects participating in a multi-center, HBV-testing campaign in Paris, France were identified and contacted via telephone 3-9 mo after testing in order to determine vaccination status. Vaccination coverage was evaluated in per-protocol (for all respondents) and intent-to-treat analysis (assuming all non-responders did not vaccinate).

RESULTS

In total, 1215/4924 (24.7%) enrolled subjects with complete HBV serology were identified as non-immunized and eligible for analysis. There were 99/902 successfully contacted subjects who had initiated HBV vaccination after screening: per-protocol, 11.0% (95%CI: 9.0-13.2); intent-to-treat, 8.2% (95%CI: 6.7-9.8). In multivariable analysis, vaccination was more likely to be initiated in individuals originating from moderate or high HBV-endemic countries (P < 0.001), patients with limited healthcare coverage (P = 0.01) and men who have sex with men (P = 0.02). When asked about the reasons for not initiating HBV vaccination, the most frequent response was "will be vaccinated later" (33.4%), followed by "did not want to vaccinate" (29.8%), and "vaccination was not proposed by the physician" (21.5%). Sub-group analysis indicated a stark contrast in vaccination coverage across centers, ranging from 0%-56%.

CONCLUSION

HBV-vaccination after HBV screening was very low in this study, which appeared largely attributed to physician-patient motivation towards vaccination. Increased vaccination coverage might be achieved by emphasizing its need at the organizational level.

Key words: Health service organization; Hepatitis B virus vaccination; Public health; Testing intervention; Vaccine coverage

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Core tip: Testing for hepatitis B virus (HBV) not only serves as a means to identify HBV-infected individuals, but also those who are non-immunized and could further benefit from HBV vaccination. In this mass HBV-screening study within the Paris metropolitan region, vaccine uptake was achieved in 11% of non-immunized patients and was deemed poor. Strategies to increase vaccination after testing need to be considered.

Boyd A, Bottero J, Carrat F, Gozlan J, Rougier H, Girard PM, Lacombe K. Testing for hepatitis B virus alone does not increase vaccine coverage in non-immunized persons. *World J Gastroenterol* 2017; 23(38): 7037-7046 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7037.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7037

INTRODUCTION

Hepatitis B virus (HBV) is one of the most frequent chronic infectious diseases in Western countries[1], with a prevalence of roughly one million in the United States[2] and 14 million in Europe[3]. The effectiveness of HBV vaccination in reducing infection incidence has been demonstrated in several countries with varying HBV prevalence[4-6], making it the single most effective tool for prevention. Nevertheless, coverage rates remain insufficient despite the wide availability of vaccines[3,7] and are particularly low in certain at-risk infection groups[8,9].

In France, the proportion of vaccinated individuals is surprisingly modest compared to other industrialized countries[10], even though vaccination is reimbursed in part by the national healthcare system. Recent epidemiological studies have estimated vaccine coverage at 50%[6], compared to 83% worldwide and higher than 90% in the Western Pacific region[1]. Prevalence of individuals with hepatitis B surface antigen (HBsAg) positive serology has remained exceedingly low over the past decade (0.7%)[11]. Nevertheless, 2500 persons are acutely infected each year, with the majority belonging to high-risk groups[12]. Vaccination campaigns targeted at these risk groups could help curb incident infections.

Accordingly, in current national guidelines, vaccination of non-immunized individuals who fall into these high-risk categories is strongly recommended[13]. Considering that HBV testing is necessary in determining those eligible for vaccination and HBV tests are given frequently in France (at almost 3.4 million tests per year), HBV screening could serve as a wide-reaching gateway to vaccination. Unfortunately, vaccination practices, specifically following HBV-testing, are poorly understood. The objective of this study was then to describe rates of vaccination among non-immunized individuals during a mass-screening program. We also intended to evaluate the reasons for

not initiating HBV vaccination after testing.

MATERIALS AND METHODS

Study design and participants

Participants were selected from the multi-center OPTISCREEN-B study, which took place in the Paris, France metropolitan region from 2011-2012[14,15]. Briefly, the study was conducted in two phases. The first phase was a cross-sectional study aimed at evaluating several rapid test candidates that could detect serological markers commonly used in HBV screening. The second phase was a parallel-group, randomized trial aimed at comparing the use of standard HBV serological tests versus rapid tests (NCT01767597). Participant recruitment occurred at 10 primary healthcare centers, with various objectives related to HBV screening, vaccination, and care - three were sexually transmitted disease (STD) clinics, three were clinics with predominately general practitioners, three focused on immigrant health, and one on incarcerated individuals. The study procedures of both phases have been described in detail[14,15]. The study was approved by the Hôtel-Dieu Hospital Ethics Committee (Paris, France) in accordance with the Helsinki Declaration.

Volunteers were asked to participate during their regular consultations if ⩾ 18 years old and could be available for further contact and medical follow-up at a single university teaching hospital. For both studies, only individuals eligible for HBV screening were invited to participate. Individuals were not included for the following reasons - for both phases: unwilling to participate; for Phase II only: already participated in the OPTISCREEN-B Phase I validation study or were not covered under the national healthcare system. Signed written informed consent was obtained for all eligible participants.

In this sub-study, we restricted our sample population to participants who were identified as non-immunized by complete HBV serological testing [*i.e.* HBsAg, anti-hepatitis B core (HBc) antibody, and anti-hepatitis B surface (HBs) antibody negative serology].

Hepatitis B virus serological testing

During the study visit, roughly 10 mL of blood was drawn and then tested for HBsAg, anti-HBs antibody, and anti-HBc antibody. Serostatus was determined using a commercially-available enzyme-linked immunoassay assay (MONOLISA AgHBs Ultra, anti-HBs plus, anti-hepatitis B core antibody-anti-HBc-plus; Bio-Rad, Hercules, California, United States of Americ). Results were provided 7-14 d after testing and, depending on the study center, were either mailed to the participants or left at the study center for collection.

Hepatitis B virus risk-factor questionnaire

Questionnaires were administered by a trained clinical research assistant during a face-to-face interview with the participant. Questions were asked in lay terms on a variety of sociodemographic characteristics, healthcare coverage, HBV transmission risk-factors, as well as other potential risk-factors. HBV-endemicity of birth country was established according to World Health Organization classification (prevalence of HBsAg-positive individuals): high (8%), intermediate (2%-8%), and low (2%).

Assessing post-test hepatitis B virus vaccination

Non-immunized individuals were identified by serology (HBsAg, anti-HBs antibody and anti-HBc antibody negative) and then contacted via telephone roughly 6-9 and 3-6 mo after testing in Phase I and II, respectively. If a participant could not be contacted after the third attempt, they were considered as having "no reply". Subjects were asked whether or not they initiated immunization against HBV and at which dates their injection(s) were administered. As we conducted follow-up during a telephone interview, physical verification of the participants' vaccination card was not performed. If they indicated no vaccination, they were asked to choose one or several reason(s) from a list for not initiating HBV immunization.

Statistical analysis

The primary outcome in per-protocol analysis was defined as the proportion of non-immunized participants initiating vaccination during follow-up who were able to be contacted. In intent-to-treat analysis, the outcome was defined as the proportion of non-immunized participants initiating vaccination assuming that those who were unable to be contacted did not initiate or complete vaccination. Clopper-Pearson 95% confidence intervals (CI) were calculated for these proportions.

In order to evaluate the determinants of HBV vaccination during follow-up (defined by per-protocol analysis), we used random-effects logistic regression to estimate univariate odds ratios (OR) of various risk-factors and their 95% CIs while accounting for within-center correlation. A multivariable model was then constructed in a backwards-stepwise fashion. A full model containing risk-factors with a P-value ⩽ 0.10 in univariable analysis was initially selected and covariates with a P-value > 0.10, as tested using a likelihood ratio test, were sequentially excluded from the model.

All statistical analysis was performed using STATA (v13.1, College Station, TX, United States of America) and reviewed by two biomedical statisticians. Significance was determined using a P-value < 0.05.

RESULTS

Study participants

The flow of study participants is represented in Figure

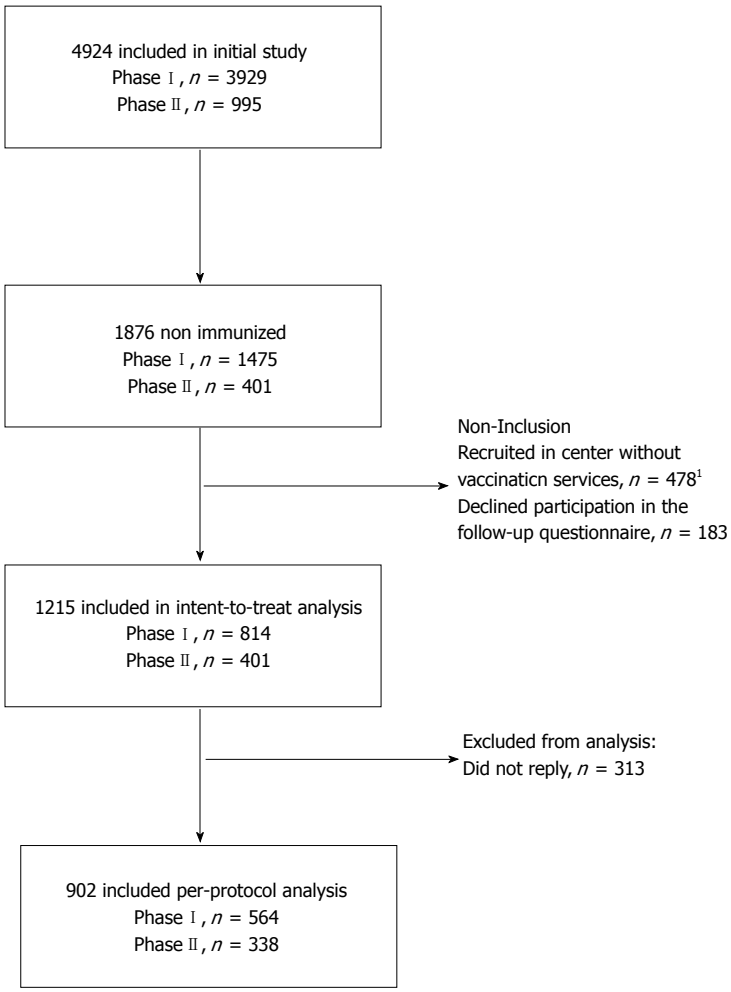


Figure 1 Study flow chart. [1]Only applies to certain participants in Phase Ⅰ.

1. A total of 4924 participants were included in the OPTISCREEN-B study, 1876 (38.1%) of whom were non-immunized and considered for the present sub-study. Of them, 661 were not included because they sought care at a center where, at the time of Phase I, did not offer vaccination services (*n* = 478) or declined participation in the follow-up questionnaire (*n* = 183). In total, 1215 participants were included in analysis.

Description of the study population

Table 1 provides a summary of the study population. The majority of individuals was male, coming from a country of low HBV-prevalence, and covered under the French national healthcare system. Included participants were born in the following geographical regions: West/East Europe, *n* = 710 (58.4%); Mediterranean, *n* = 183 (15.1%); Asia, *n* = 90 (7.4%); Sub-Saharan Africa, *n* = 135 (11.1%); South America, *n* = 43 (3.5%); other, *n* = 54 (4.4%). A total of 1043 (85.8%) of participants were indicated for vaccination according to French recommendations, the most frequent reasons for which were multiple lifetime sexual partners, originating from a moderate/high HBV-endemic country and traveling to high endemic countries. A smaller proportion was men who had sex with men (MSM) or partook in nasal or intravenous drug-use. Characteristics of study participants were compered between enrollment phases in Supplementary Table 1.

Post-test hepatitis B virus vaccination rates

Among the 1215 participants included in analysis, 99 (8.2%) claimed to have initiated HBV vaccination, while the first injection was given a median 31 d (IQR = 12-55) after testing; 803 (66.1%) did not initiate vaccination; and 313 (25.7%) did not respond after the third contact attempt. Participants who did not respond were significantly younger ($P = 0.02$), more likely to come from an intermediate HBV-endemic country ($P < 0.001$), did not have any healthcare coverage ($P < 0.001$), and had a lower number of lifetime sexual partners ($P = 0.001$) (Table 1).

In per-protocol analysis (Table 2), immunization coverage was 11.0% (95%CI 9.0-13.2) during follow-up. This proportion varied substantially across centers (0%-55.6%), with the lowest vaccine coverage observed in STD clinics (7.5%) and centers with mainly general practitioners (6.3%) and the highest

Table 1 Description of the study population *n* (%)

	Total (*n* = 1215)	Follow-up contact		*P* value
		Reply (*n* = 902)	No reply (*n* = 313)	
Male	702 (57.8)	511 (56.7)	191 (61.0)	0.18
Age (yr), mean ± SD	36 ± 15	37 ± 15	34 ± 14	0.02
HBV prevalence of birth country				< 0.001
Low (< 2.0%)	670 (55.1)	527 (58.4)	143 (45.7)	
Intermediate (2.0%-8.0%)	331 (27.2)	222 (24.6)	109 (34.8)	
High (> 8.0%)	214 (17.6)	153 (17.0)	61 (19.5)	
Parents from high endemic region	276 (22.7)	205 (22.7)	71 (22.7)	0.9
Traveled to high endemic region[1]	284 (23.4)	204 (22.6)	80 (25.6)	0.3
Healthcare in high endemic region	177 (14.6)	124 (13.8)	53 (16.9)	0.17
Health insurance plan				< 0.001
Social security/CMU[2]	913 (75.2)	719 (79.7)	194 (62.0)	
CMUc[3]	61 (5.0)	47 (5.2)	14 (4.5)	
AME[4]/Other/ None	241 (19.8)	136 (15.1)	105 (33.6)	
Received transfusion	48 (4.0)	33 (3.7)	15 (4.8)	0.4
Received tattoos	151 (12.4)	100 (11.1)	51 (16.3)	0.02
Received piercing	510 (42.0)	387 (42.9)	123 (39.3)	0.3
Close contact with HBV^+ individual	75 (6.2)	57 (6.4)	18 (5.8)	0.7
Number of life-time sexual partners				0.001
0-1	181 (14.9)	118 (13.1)	63 (20.1)	
2-9	560 (46.1)	409 (45.3)	151 (48.2)	
⩾ 10	474 (39.0)	375 (41.6)	99 (31.6)	
> 1 sexual partner within 12 mo	593 (48.8)	447 (49.6)	146 (46.7)	0.4
Men who have sex with men	93 (8.9)	73 (8.1)	20 (6.4)	0.3
Nasal drug-use	145 (11.9)	108 (12.0)	37 (11.8)	0.9
Intravenous drug-use	5 (0.4)	5 (0.6)	0	0.3
Long-term stay at a medical center	50 (4.1)	38 (4.2)	12 (3.8)	0.8
Previously incarcerated	87 (7.2)	63 (7.0)	24 (7.7)	0.7

Participants with reply and no reply were compared using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables. [1]Period of stay was longer than 3 mo; [2]Couverture médicale universelle, minimum health insurance coverage that is given to persons living in precarious situations (*i.e.* unemployed, poverty, etc.); [3]Couverture médicale universelle complémentaire, additional health insurance coverage that is given to stable residents in France living with limited financial resources; [4]Aide médicale d'état, health insurace generally given to immigrants without proper documentation.

Table 2 Hepatitis B virus vaccination and no reply rates across study centers

	n	No reply *n* (%)	Immunization coverage		
			Vaccinated *n*[3]	Per protocol[1] (%)	ITT[2] (%)
Total	1215	313 (25.8)	99	11	8.2
General practitioner	418	85 (20.3)	21	6.3	5
Policlinique Saint Antoine	165	47 (28.5)	10	8.6	6.1
Travel clinic Saint Antoine	31	5 (16.1)	3	11.5	10
CPAM	222	33 (14.9)	8	4.2	3.6
STD clinics	635	166 (26.1)	35	7.5	5.5
CDAG Saint Antoine	51	10 (19.6)	0	0	0
CDAG Figuier	275	78 (28.4)	22	11.2	8
CDAG Belleville	309	78 (25.2)	13	5.6	4.2
Immigrant-health clinics	140	61 (43.6)	37	46.8	26.4
Croix-Rouge Moulin Joly	9	0 (0)	5	55.6	55.6
CRAMIF	18	0 (0)	9	50	50
Médecin du Monde	113	61 (54.0)	23	44.2	20.4
Prison (UCSA Paris)	22	0 (0)	6	27.3	27.3

[1]Per-protocol end-point defined as the proportion of non-immunized participants initiating vaccination who were contacted; [2]Intent-to-treat (ITT) end-point defined as the proportion of non-immunized participants initiating vaccination assuming that those who were unable to be contacted did not initiate or complete vaccination; [3]Participants initiating HBV vaccination sequence after HBV testing in the OPTISCREEN-B study. CDAG: Centre de dépistage anonyme et gratuit; CPAM: Caisse primaire d'assurance maladie; CRAMIF: Caisse régionale d'assurance maladie d'Ile-de-France; UCSA: Unité de consultations et de soins ambulatoire; STD: transmitted disease.

rates in immigrant-health clinics (46.8%). Assuming those with no reply did not initiate HBV vaccination (intent-to-treat analysis), immunization coverage was 8.2% (95%CI 6.7-9.8), also with high between-center

Table 3 Determinants for initiating hepatitis B virus vaccination sequence

Risk-factor	Univariate		Multivariable[5]	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Female *vs* male	0.61 (0.37-0.99)	0.04		
Age (per yr)	1.00 (0.98-1.02)	0.8		
Parents from high endemic region	1.52 (0.89-2.58)	0.12		
Traveled to high endemic region[1]	1.43 (0.83-2.45)	0.19		
Care in high endemic region	1.45 (0.79-2.67)	0.2		
Transfusion	0.28 (0.03-2.28)	0.2		
Tattoos	1.26 (0.63-2.55)	0.5		
Piercing	1.05 (0.66-1.67)	0.8		
Close contact with HBV^+ individual	1.14 (0.48-2.70)	0.8		
Men who have sex with men	2.05 (0.97-4.34)	0.06	2.53 (1.14-5.60)	0.02
Nasal drug-use	0.37 (0.13-1.07)	0.07	0.41 (0.14-1.21)	0.1
Intravenous drug-use	3.42 (0.41-28.40)	0.3		
Long-term stay at a medical center	1.02 (0.33-3.14)	0.9		
Previously incarcerated	0.71 (0.24-2.06)	0.5		
HBV-prevalence of birth region				
Low (< 2.0%)	1		1	
Intermediate (2.0%-8.0%)	4.39 (2.35-8.19)	< 0.001	3.58 (1.85-6.95)	< 0.001
High (> 8.0%)	4.43 (2.24-8.75)	< 0.001	3.44 (1.65-7.15)	0.001
Health insurance plan				
Social security or CMU[2]	1		1	
CMUc[3]	1.13 (0.35-3.62)	0.8	0.76 (0.23-2.58)	0.7
AME[4], Other, or none	4.54 (2.21-9.30)	< 0.001	2.60 (1.23-5.52)	0.01
Number of life-time sexual partners				
0-1	1			
2-9	0.93 (0.47-1.87)	0.8		
⩾ 10	0.75 (0.35-1.60)	0.5		

Per-protocol analysis on the 902 non-immunized participants who were able to be contacted. [1]Period of stay was longer than 3 mo; [2]Couverture médicale universelle, health insurance coverage that is given to persons living in precarious situations (*i.e.* unemployed, poverty, *etc*; [3]Couverture médicale universelle complémentaire, additional health insurance coverage that is given to stable residents in France living with limited financial resources; [4]Aide médicale d'état, health insurace generally given to immigrants without proper documentation; [5]When constructing the multivariable model, gender was not included due to collinearity with men who have sex with men.

variability (Table 2).

Table 3 describes the per-protocol analysis of risk-factors associated with HBV vaccination. Of note, there was no association between vaccination and study phase (Phase II versus I, OR = 0.66, 95%CI 0.27-1.62, *P* = 0.4). In multivariable analysis, vaccination was more likely to be initiated in MSM (*P* = 0.02), participants born in intermediate/high endemic zones (*P* < 0.001) and those without national healthcare coverage (*P* = 0.006). When replacing gender with MSM in the multivariable model, there was no significant difference between males and females (adjusted - OR = 0.66, 95%CI 0.40-1.10).

Reasons for not initiating vaccination

In the 803 non-vaccinees who were able to be contacted, Table 4 summarizes their reasons for not vaccinating. Roughly one-third stated that they would vaccinate at a later time. Other reasons included no desire to vaccinate (29.8%), vaccination was not suggested by their physician (21.5%), or the participant did not return to obtain their serological results confirming non-immunized status (14.0%). Participants who preferred not to vaccinate were significantly more likely to be male (*P* = 0.002), older (*P* < 0.001), come from a low endemic country (*P* < 0.001), be covered under the French national healthcare system (*P* < 0.001), and have higher number of lifetime sexual partners (*P* = 0.004) (Supplementary Table 2).

DISCUSSION

HBV testing has been proposed as a useful gateway to identify non-immunized individuals and encourage them to initiate vaccination[16], yet data regarding the extent of how successful this strategy would be, if applied, are sparse. Previous studies in France and California have shown that vaccine coverage ranges from 8.6%-11.2% after testing for HBV in the context of STD clinics[17,18]; however, these results were obtained from mostly younger, sexually-active individuals or more specifically, among adults diagnosed with chlamydia, gonorrhoae and/or syphilis who were still susceptible to HBV infection. In the OPTISCREEN-B studies, serving as a testing campaign against HBV infection in a population with diverse at-risk characteristics, we similarly observed a low vaccination rate of 11.0% in per-protocol and 8.2% in intent-to-treat analysis.

The reasons for low vaccine coverage are somewhat difficult to pinpoint. While examining individual characteristics, it was interesting to find that those originating from regions of moderate or high HBV

Table 4 Reasons for not initiating hepatitis B virus vaccination

Reason	*n* (%)
Serology results were never obtained	112 (14.0)
Vaccination was not proposed by physician	173 (21.5)
Vaccination was not indicated (according to physician)	26 (3.2)
Not at risk	14 (1.7)
Vaccination was already done[1]	12 (1.5)
Participant did not want to be vaccinated	239 (29.8)
Not perceived to be at risk	95 (11.8)
Vaccination was already done	21 (2.6)
Not receptive to vaccinations in general	44 (5.5)
Not receptive to HBV vaccination	89 (11.1)
Will be vaccinated later	268 (33.4)

Among the 803 contacted individuals who did not initiate HBV vaccination. More than one response was possible (only applies to 15 participants). [1]No indication for revaccination despite undetectable anti-HBs antibodies.

endemicity and those in difficult socioeconomic situations, based on their healthcare plan, were significantly more likely to initiate HBV vaccination after testing. These data are relieving considering that these two risk-factor groups constitute the largest number of newly identified infections in France[12]. However, it should be mentioned that these groups also had the highest proportions of non-response, particularly individuals without healthcare or from intermediate HBV-endemic countries. MSM were also observed to have a significantly higher vaccination initiation rates, while this key population has shown rather high vaccine uptake in previous intervention studies[19,20]. Unfortunately, two groups with high HBV-infection risk, namely persons having close contact with an infected individual or engaging in nasal or intravenous drug use, were not more likely to become vaccinated.

When addressing the reasons for not vaccinating, approximately one-third responded that they did not want to be vaccinated, mainly because they were not receptive to HBV vaccination or not perceived to be at risk. Vaccine hesitancy has already been illustrated in a study from a French STD clinic, where most non-immunized persons did not trust the safety of HBV vaccines or viewed it as "not well characterized"[21]. We also observed that individuals covered under the French national healthcare plan or those coming from a low HBV-endemic country were more likely not to vaccinate. Their lack of motivation could stem from a lower perception of HBV-transmission risk, which has been previously evidenced in persons with higher socioeconomic status in France[22].

Another one-fifth of non-vaccinated individuals claimed that their physician did not propose vaccination. This result is rather surprising given that professionals have been recommended to initiate vaccination for all non-immunized persons susceptible to transmission since 1995. Few (3.2%) participants stated that they were not indicated for vaccination by their physician, implying that recommendation criteria did not play a major role in the low vaccine coverage observed with this group. One striking feature of our data, however, was that post-testing vaccination rates varied from 0% to 56% across study centers, largely implicating organizational factors in promoting HBV vaccination.

These results support the need for structural reorganization in testing facilities as a means to encourage vaccination. Promising data on interventions at the organization level have been presented in other research, in which increased vaccination rates were achieved after providing focused training to healthcare professionals and individuals seeking testing[23] or when free vaccinations were offered directly after HBV serological results were returned[17]. Other interventions involving financial incentives have also shown substantial effects in at-risk populations, such as intravenous drug-users[24,25]. These strategies would certainly be helpful in counterbalancing vaccine hesitancy, which has been an emerging topic over the past decades[10], notably among health-care providers in general and in France[26-28]. Without these interventions, the proportion of individuals at risk of HBV-infection could rebound and persist as a public health issue[6].

Nevertheless, many of the participants in this analysis came from difficult socioeconomic backgrounds. Past research has underscored the lack of access to preventative health services especially among migrants[29], certain racial/ethnic groups[30], and homeless individuals[31]. Since the majority of vaccination occurs within the context of these services, social inclusion of disadvantaged groups, particularly at primary care centers, would probably be necessary in order to achieve greater vaccine outreach. Although the French healthcare system attempts at addressing care in these groups[32], further improvements could be made with other strategies, such as mobile clinics providing vaccinations[33] or culturally-adapted text message reminders[34]. The services provided at these healthcare centers should also emphasize education on HBV disease and the benefits of vaccination, possibly in combination with care related to other health issues.

There were several noteworthy parallels with the results from this study compared to previous analyses in the OPTISCREEN-B program. The profile of participants accepting vaccination (*i.e.* from regions of moderate to high HBV endemicity, without health care coverage) were also those who more frequently tested for HBV in the past[35], which are perhaps indicators of effective prevention efforts in these at-risk groups. In addition, the between-center variability in the proportion receiving vaccination mirrored that of the proportion of physicians who would propose HBV testing[35]. The similar variation offers evidence that any organizational changes to promoting vaccination would have to include a more comprehensive HBV prevention strategy. Finally, our study group has

observed in a past evaluation that roughly 20%-50% participants newly diagnosed with HBV returned for further consultation at a referent center[15], reflecting the potential for inadequate transition to "appropriate care"[36]. The increased need to foster individuals into appropriate care appears to be shared among non-immunized patients.

Several methodological limitations need to be noted when interpreting the results of our study. First, we used a convenience sample combining participants from two studies. Certain risk-factor groups, such as intravenous drug-users and sex-workers, might not be adequately represented and could have different vaccine coverage than those reported herein. In addition, the lack in numbers may have resulted in inadequate power in order to establish whether they were less likely to complete vaccination. Second, the time-frame of follow-up was relatively short and perhaps some participants received vaccination 6-9 mo or more after their interview. Non-immunized patients did vaccinate a median 30 d, suggesting that few individuals would have initiated vaccination after the end of follow-up and vaccine coverage would have been similar if follow-up were prolonged. Nevertheless, roughly one-third of unvaccinated individuals stated that they would do so in the future. Third, the short time-frame also made it impossible to ascertain whether participants completed HBV vaccination, as the date of last vaccination would have fallen outside the follow-up period. Lastly, the proportion with no reply, which was comparable to other studies[17], could have biased results.

Furthermore, we should highlight that individuals with low levels of anti-HBs antibodies (> 0-10 mIU/mL), who could possibly benefit from booster HBV vaccination, were not included in this analysis. The results from our study might not be generalizable to these individuals.

In conclusion, HBV vaccination after screening was very low in this study, implying that HBV testing alone cannot encourage non-immunized individuals to vaccinate. The fact that large discrepancies in vaccine coverage were observed across centers and that both physician and participant motivation towards vaccination was rather limited provides evidence that involvement at the health structure level is crucial in increasing vaccination coverage. Future interventions and campaigns should work at increasing local awareness of low vaccine coverage rates, especially in groups with poor access to health services, and the need to vaccinate non-immunized individuals as soon as they are identified.

ARTICLE HIGHLIGHTS

Research background

Testing campaigns for hepatitis B virus (HBV) have often focused on identifying infected patients [*i.e.* those with hepatitis B surface antigen (HBsAg)-positive serology] and increasing disease-status awareness. Few evaluations have been performed on how testing campaigns can impact vaccination uptake after receiving non-immunized status.

Research motivation

Since France has a low proportion of vaccinated individuals compared to other Western countries, interventions to increase HBV vaccination rates need to be evaluated in this population. This analysis addressed vaccination initiation among non-immunized participants in a large-scale screening campaign within the Paris metropolitan region. These individuals reflect a wide range of groups at-risk of HBV infection, allowing potential identification of subgroups with low vaccination coverage.

Research objectives

We aimed to describe rates of vaccination among non-immunized individuals during a mass-screening program. We also intended to evaluate the reasons for not initiating HBV vaccination after testing. This allows a first-hand account of why individuals do not vaccinate and helps tailor the needs for future intervention campaigns.

Research methods

Participants were recruited from two large phases of a multi-center, HBV-testing campaign in Paris, France. Non-immunized subjects were identified and contacted via telephone 3-9 mo after testing in order to determine whether they initiated vaccination. We considered vaccination coverage of all respondents (in a per-protocol analysis) and the overall non-immunized study population while assuming no vaccination in non-responders (in an intent-to-treat analysis).

Research results

Overall vaccination uptake was low with 11% of respondents declaring HBV vaccination initiation within 3-6 mo of testing. Few risk-factors for increased vaccination initiation were identified: from moderate or high HBV-endemic countries, with limited healthcare coverage, and men who have sex with men. Compelling differences were observed between centers with vaccination coverage ranging from 0%-56%.

Research conclusions

Given the low vaccine uptake in individuals at potential risk of HBV-infection, there is a major concern in the cascade of care among non-immunized individuals. The contrasting vaccination rates between centers indicate that the challenge to increase vaccination initiation lies within center-specific practices. At the individual level, increasing motivation to vaccinate among physicians and non-immunized persons alike

should be stressed.

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CASE REPORT

Gastric adenocarcinoma of fundic gland type spreading to heterotopic gastric glands

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Author contributions: Manabe S was responsible for the patient, performed the endoscopic examination and treatment, and wrote the initial draft of the manuscript; Mukaisho K contributed to the histopathological diagnosis; all other authors contributed to the retrieval and interpretation of the results and critically reviewed the manuscript; all authors approved the final version of the manuscript.

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Informed consent statement: The patient provided an oral informed consent prior to the conduction of the study.

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Abstract

Herein, we present a case of gastric adenocarcinoma of fundic gland type (GA-FG) spreading to heterotopic gastric glands (HGG) in the submucosa. A 58-year-old man with epigastric pain was referred to our hospital and underwent an esophagogastroduodenoscopy. A Borrmann type II gastric cancer at the antrum and a 10 mm submucosal tumor-like lesion in the lesser curvature of the upper third of the stomach were detected. Histological examination of the biopsy specimens obtained from the submucosal tumor-like lesion suggested a GA-FG. Therefore, endoscopic submucosal dissection was performed as excisional biopsy, and histopathological examination of the resected specimen confirmed a GA-FG and HGG proximal to the GA-FG. Although the GA-FG invaded the submucosal layer slightly, the submucosal lesion of the GA-FG had a poor stromal reaction and was located just above the HGG in the submucosa. Therefore, we

finally diagnosed the lesion as a GA-FG invading the submucosal layer by spreading to HGG.

Key words: Gastric adenocarcinoma of fundic gland type; Heterotopic gastric glands; Endoscopic submucosal dissection; Paracancerous lesion; Pepsinogen-I; H/K-ATPase

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Core tip: A 58-year-old man had a 10mm submucosal tumor-like lesion in the lesser curvature of the upper third of the stomach. Histological examination of the biopsy specimens suggested a gastric adenocarcinoma of fundic gland type (GA-FG), therefore, endoscopic submucosal dissection was performed as excisional biopsy. Histopathological examination of the resected specimen confirmed a GA-FG invading the submucosal layer slightly, and heterotopic gastric glands (HGG) proximal to the GA-FG. A GA-FG spreading to HGG in the submucosa was diagnosed because the submucosal lesion of the GA-FG had a poor stromal reaction and was located just above the HGG.

Manabe S, Mukaisho K, Yasuoka T, Usui F, Matsuyama T, Hirata I, Boku Y, Takahashi S. Gastric adenocarcinoma of fundic gland type spreading to heterotopic gastric glands. *World J Gastroenterol* 2017; 23(38): 7047-7053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7047.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7047

INTRODUCTION

Gastric adenocarcinoma of fundic gland type (GA-FG) is a new histological type of gastric cancer proposed by Ueyama *et al*[1] in 2010. As the concept of GA-FG has spread since then, new findings have been reported[2]. GA-FG tends to invade the submucosal layer while it is still small because it arises from the deep layer of the lamina propria mucosae[1]. On the other hand, heterotopic gastric glands (HGG) are regarded as paracancerous lesions[3,4], and gastric cancers associated with HGG have been reported[5,6]. However, HGG are not considered to contribute to the development of GA-FG because the pathogenesis of GA-FG is different from that of the regular histological type of gastric cancer. We encountered a case of GA-FG suggested to have invaded the submucosal layer by spreading to HGG, which coincidentally existed proximal to the GA-FG. In addition to a GA-FG with a very rare mode of submucosal invasion, this case demonstrated the possibility of GA-FG arising from the gastric mucosa with atrophic change, provided there are remnant gastric fundic glands. Herein, we report this case with a brief review of the literature.

CASE REPORT

A 58-year-old man with epigastric pain was referred to our hospital. Esophagogastroduodenoscopy (EGD) revealed a Borrmann type II gastric cancer at the antrum (Figure 1A) and a 10 mm submucosal tumor-like lesion in the lesser curvature of the upper third of the stomach (Figure 1B and C). Atrophy of the gastric mucosa was classified as O-1 (according to the Kimura and Takemoto classification[7]), although the patient had undergone *Helicobacter pylori (H. pylori)* eradication therapy 4 years ago, and has not taken any proton pump inhibitors since then. Histological examination of the biopsy specimens obtained from the submucosal tumor-like lesion showed that the superficial area retained the normal foveolar epithelium. However, atypical cells mimicking fundic gland cells with mildly enlarged nuclei were observed in the deep layer of the lamina propria mucosae. These findings suggested a GA-FG. Narrow-band imaging with magnifying endoscopy (NBI-ME) performed later showed a regular microvascular pattern and a regular microsurface pattern according to the vessel plus surface classification system[8] with a concomitant depressed area at the center of the submucosal tumor-like lesion expected to be a biopsy scar (Figure 2). Endoscopic ultrasonography (EUS) findings revealed the tumor slightly invading the third layer and a hypoechoic mass located in the third layer near the tumor (Figure 3). Blood test findings revealed carcinoembryonic antigen level of 2.2 ng/mL and carbohydrate antigen 19-9 level of 14.3 U/mL, indicating that the tumor markers were within the normal range. Although gastric wall thickening due to the advanced gastric cancer at the antrum was seen on the abdominal CT scan, nodal or distant metastasis was absent. Based on the above findings, a partial gastrectomy was required for gastric cancer at the antrum. The submucosal tumor-like lesion in the lesser curvature of the upper third of the stomach was suspected to be a GA-FG; hence, we decided to perform endoscopic submucosal dissection (ESD) as excisional biopsy. Since histopathological findings of the ESD-resected specimen may indicate the need for an additional surgery, ESD was performed before surgery for the gastric cancer at the antrum, and en bloc resection was achieved. The ESD-resected specimen was 30 mm in diameter, whilst a 7 mm × 4 mm slightly elevated lesion was identified (Figure 4). Similar to the histological findings of the biopsy specimens, atypical cells mimicking fundic gland cells, mainly chief cells and partially parietal cells, with mildly enlarged nuclei were seen mainly in the deep layer of the lamina propria mucosae. The mucosal surface was covered completely with non-neoplastic foveolar epithelium; thus, the tumor was not exposed to the mucosal surface (Figure 5A).

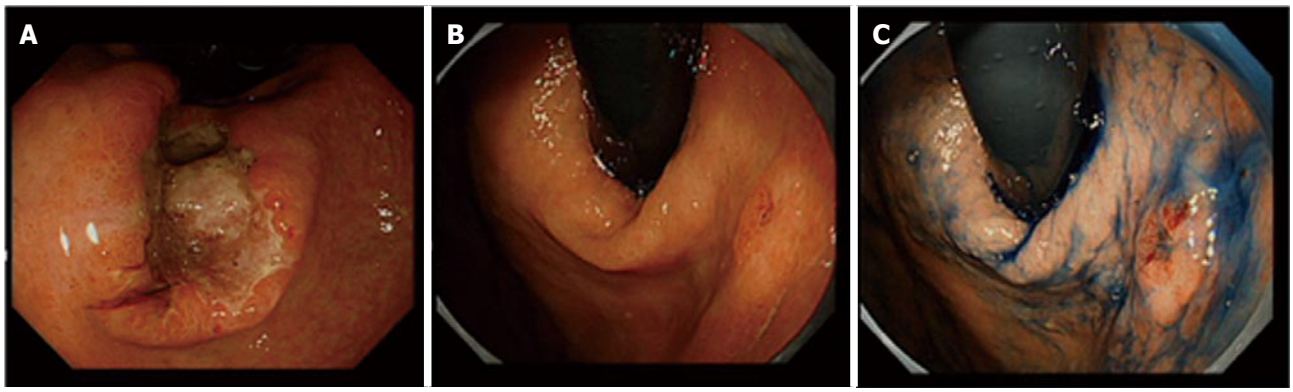


Figure 1 Esophagogastroduodenoscopy findings. A: A Borrmann type II gastric cancer was detected at the antrum; B and C: A 10 mm submucosal tumor-like lesion on the lesser curvature of the upper third of the stomach was detected. Atrophic gastritis O-1 according to Kimura and Takemoto classification was recognized.

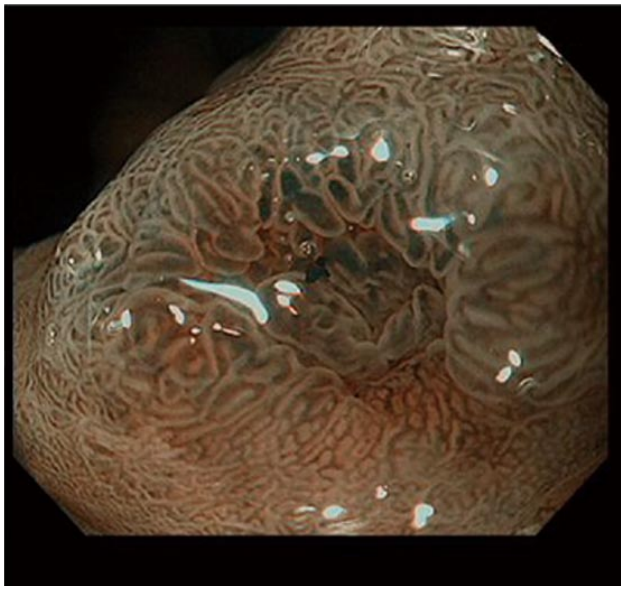


Figure 2 Narrow-band imaging with magnifying endoscopy findings. There were few irregularities in the microvessel architecture and microsurface structure. Therefore, we diagnosed a regular microvascular pattern and a regular microsurface pattern. The depressed area at the center was expected to be a biopsy scar.

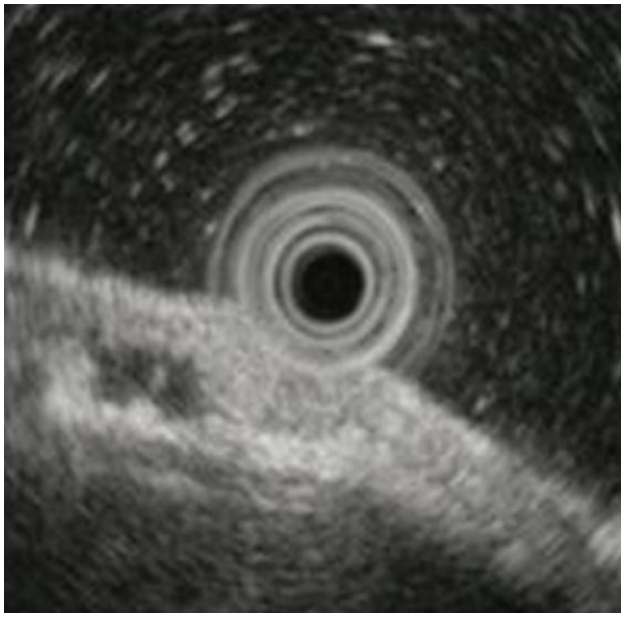


Figure 3 Endoscopic ultrasonography findings. The tumor slightly invaded the third layer, although it was located mainly in the second layer. Moreover, a hypoechoic mass located in the third layer near the tumor was detected.

Upon immunohistochemical examination, the tumor showed positivity for MUC6 and negativity for MUC2 and MUC5AC, indicating a gastric phenotype (Figure 5B-D). Moreover, diffuse positivity for pepsinogen-I and scattered positivity for H/K-ATPase was found, indicating a differentiation dominantly toward chief cells and focally toward parietal cells (Figure 5E and F). Although the lesion was located mainly in the deep layer of the lamina propria mucosae, it had partially invaded the submucosal layer up to 450 μm. Furthermore, HGG were observed in the submucosal layer, proximal to the tumor. The submucosal lesion of the tumor had a poor stromal reaction and was located just above the HGG in the submucosa (Figure 6). Based on these findings, the final diagnosis was: U, Less, 30 × 20 mm, type 0-IIa, 7 × 4 mm, adenocarcinoma of fundic gland type, pT1b1 (450 μm), UL (-), ly (-), v (-), pHM0, pVM0 (according to the Japanese classification of gastric carcinoma[9]). In addition, we have concluded that the GA-FG had invaded the submucosal layer by spreading to the HGG. After obtaining these results, we performed EUS again to examine the presence of other HGG. Thus, several hypoechoic masses considered to be HGG were seen in the entire stomach, and we diagnosed diffuse type HGG. A partial gastrectomy was required for the Borrmann type II gastric cancer at the antrum. Moreover, due to the high risk of cancer arising from the gastric remnant because of the diffuse type HGG, we performed a total gastrectomy upon the patient's request. After the total gastrectomy, the gastric cancer at the antrum was diagnosed as: L, Less, type 2, 40 × 35 mm, por1, pT3 (SS), intermediate type (int), INFa, ly2, v2, pN0, pPM0, pDM0 (according to the Japanese classification of gastric carcinoma[9]), and multiple

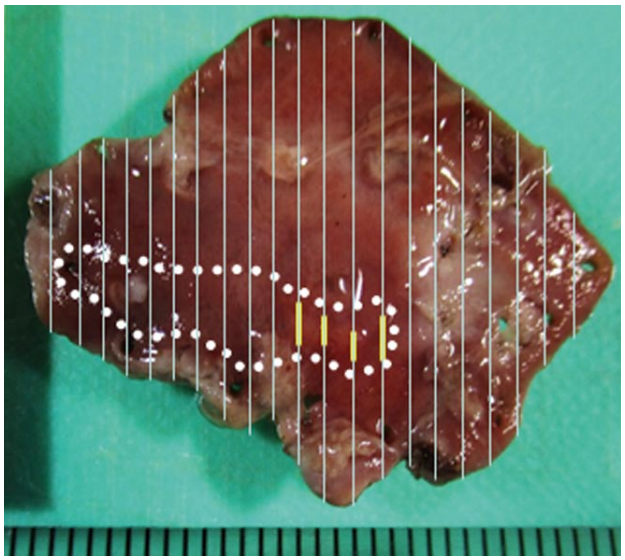


Figure 4 Spread of the gastric adenocarcinoma of fundic gland type and heterotopic gastric glands mapped on the endoscopic submucosal dissection-resected specimen. *Yellow line* spread of the gastric adenocarcinoma of fundic gland type (GA-FG), *White dotted line* spread of heterotopic gastric glands (HGG). The endoscopic submucosal dissection -resected specimen was 30 mm × 20 mm in size. The tumor was slightly elevated and measured 7 mm × 4 mm. The GA-FG and HGG partially overlapped.

HGG in the entire stomach were confirmed. However, we could not find evidence of this cancer related to diffuse type HGG. In addition, considering the risk of multiple gastric cancers associated with diffuse type HGG, the entire stomach was examined histologically. In doing so, we detected an intramucosal cancer in the lesser curvature of the lower gastric body, which was not previously detected by endoscopy before surgery. Although there was no remnant GA-FG near the ESD ulcer scar, a distribution of HGG was seen.

DISCUSSION

GA-FG is a new histological type of gastric cancer[1]. According to Ueyama *et al*[10], endoscopic properties of GA-FG include no intestinal metaplasia or atrophic change in the surrounding mucosa, faded or whitish color, submucosal tumor shape with a soft appearance, and dilated vessels with branching architecture. Although the pathogenesis of GA-FG is unclear, GA-FG tends to arise from the normal gastric mucosa of the fundic gland region without atrophic change or intestinal metaplasia[2]. Therefore, *H. pylori* infection is not considered to contribute to the development of GA-FG, unlike for the regular histological type of gastric cancer.

On the other hand, HGG are gastric glands that are observed in the submucosa and are naturally seen in the lamina propria mucosae[3,4]. Clinically, HGG are regarded as paracancerous lesions and are associated with gastric cancer, particularly, multiple gastric cancers[3,4]. HGG are classified into 4 types based on their number and range of distribution: solitary type with 3 sites or less, localized type with 4-9 sites at the focal area, broad type with 4-9 sites in the broad area, and diffuse type with at least 10 sites which exist in the entire stomach[4]. Of these, the diffuse type is seen in 98% of gastric cancer complications, and the rate of complication with multiple gastric cancers is reported to be 32%[3]. The association between HGG and gastric cancer is unclear. However, one dominant hypothesis is that both HGG and gastric cancer develop because of repeated erosion and regeneration of the mucosa, suggesting that HGG are paracancerous lesions[3,4]. Caution is required when performing EGD for patients with HGG, particularly diffuse type HGG, since there is the possibility that micro-gastric cancers that are difficult to detect endoscopically may be present. Indeed, as in our case, after total gastrectomy, histopathological examination revealed a micro-intramucosal cancer that was not detected endoscopically before surgery. There is no consensus on the treatment of HGG. As mentioned previously, because the rate of complication of gastric cancer is very high, particularly with diffuse type HGG, EGD must be conducted periodically with caution. In our case, in addition to the high risk of gastric cancer arising due to diffuse type HGG, a partial gastrectomy was required for the Borrmann type II gastric cancer at the antrum. Therefore, a total gastrectomy was performed upon the patient's request.

The GA-FG observed in our case presented a submucosal tumor shape, which corresponds to the endoscopic properties reported by Ueyama *et al*[10]. A possible reason the GA-FG presented a submucosal tumor shape is that, in addition to the proliferation of tumor cells in the deep mucosal layer, the HGG were also present in the submucosa. Retrospectively, the hypoechoic mass near the GA-FG that was seen on EUS before ESD was suggested to be HGG. The ESD-resected specimen showed no tumor exposed to the mucosal surface; thus, NBI-ME showed a regular microvascular pattern and a regular microsurface pattern. Since GA-FG arises from the deep layer of the lamina propria mucosa, there is a high rate of submucosal invasion while the tumor diameter is small[1]. In our case also, the GA-FG invaded the submucosal layer up to 450 μm. However, the submucosal lesion of the GA-FG had a poor stromal reaction and was located just above the HGG in the submucosa. Therefore, the GA-FG was considered to have invaded the submucosal layer by spreading to the HGG. There are a few case reports of regular histological type of gastric cancer spreading to HGG[11]. However, to the best of our knowledge, this is the first case of GA-FG spreading to HGG. Currently, in the Japanese classification of gastric carcinoma, there is no definition concerning the depth of tumor invasion that spreads to HGG in the submucosa[9]. However, since similarly, esophageal cancer with intraductal spreading is handled as intramucosal cancer as long as it is

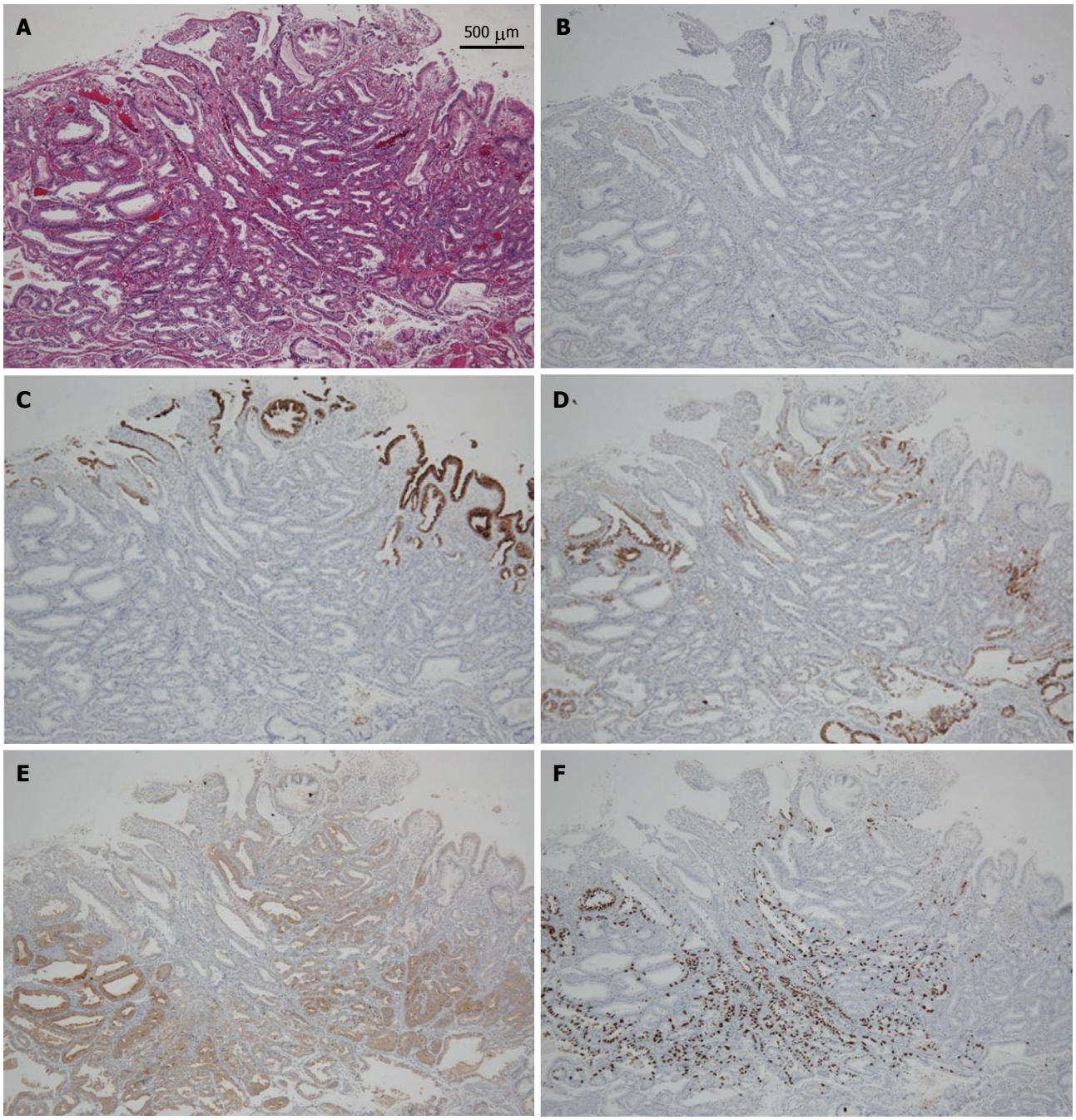


Figure 5 Histopathological and immunohistochemical findings of the endoscopic submucosal dissection-resected specimen. A: H&E staining. There were atypical cells with mildly enlarged nuclei in the deep layer of the lamina propria mucosae. They mimicked fundic gland cells, mainly chief cells and partially parietal cells. The mucosal surface was covered completely with non-neoplastic foveolar epithelium; thus, the tumor was not exposed to the mucosal surface; B: MUC2 staining: almost negative; C: MUC5AC staining: almost negative except for normal superficial foveolar epithelium; D: MUC6 staining: partially positive and indicating a gastric phenotype; E: Pepsinogen staining: diffusely positive in the deep layer of the lamina propria mucosae, corresponding to gastric adenocarcinoma of fundic gland type, and indicating a differentiation toward chief cells; F: H/K-ATPase staining: scattered positive in the deep layer of the lamina propria mucosae, indicating a differentiation toward parietal cells focally.

retained in the esophageal proper glands[12], gastric cancer spreading to HGG should likely be handled as intramucosal cancer[5,6].

As mentioned earlier, atrophic gastritis related to *H. pylori* infection is not considered to be involved in the development of GA-FG. On the other hand, gastric cancer associated with HGG is considered to result from chronic inflammation due to *H. pylori* infection. Therefore, it is thought that there is no correlation between GA-FG and HGG. In our case, the GA-FG and HGG were considered to be coincidentally present in the vicinity. Furthermore, it is thought that HGG resulted from chronic inflammation due to *H. pylori* infection before eradication therapy, and pseudopyloric gland metaplasia in the fundic gland region progressed. The GA-FG may have occurred

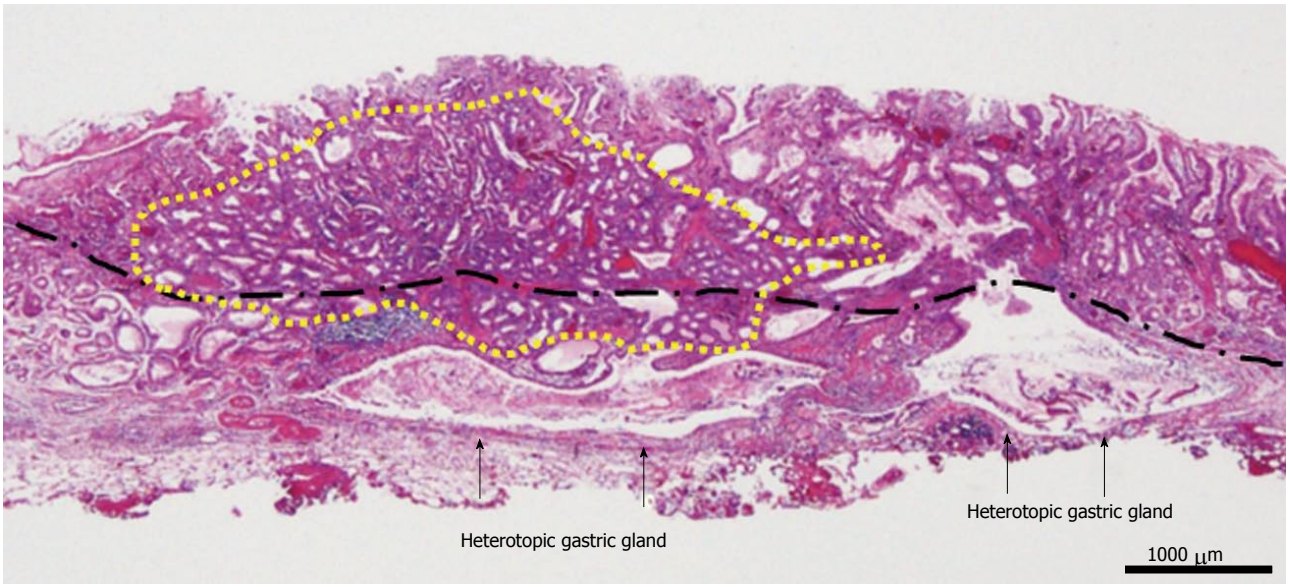


Figure 6 Histopathological findings of the endoscopic submucosal dissection-resected specimen. *Yellow line* the gastric adenocarcinoma of fundic gland type (GA-FG), *Black line* muscularis mucosae. The GA-FG partially invaded the submucosa layer up to 450 μm, although it was located mainly in the deep layer of the lamina propria mucosae. Enlarged ducts of the heterotopic gastric glands were concomitantly observed just under the submucosal lesion of the GA-FG. In addition, the submucosal lesion had a poor stromal reaction.

from the remnant gastric fundic glands despite the progression of pseudopyloric gland metaplasia and spread continuous to the proximally located HGG.

Until recently, GA-FG has been recognized as cancer arising from gastric mucosa without atrophic change[2]. However, cases of GA-FG arising from gastric mucosa with atrophic change have now been reported[13]. Together with these cases, our case suggests that GA-FG could occur even from the gastric mucosa with atrophic change, where HGG could be present, provided the remnant gastric fundic glands exist. This is an important case demonstrating that when performing endoscopy, one needs to keep in mind the possibility of GA-FG presenting on the gastric mucosa with atrophic change, chronic gastritis, or intestinal metaplasia.

COMMENTS

Case characteristics

A 58-year-old man with epigastric pain but no other symptoms.

Clinical diagnosis

A gastric adenocarcinoma of fundic gland type (GA-FG) was suspected based on the histological examination of the biopsy specimens obtained from the submucosal tumor-like lesion that revealed atypical cells mimicking fundic gland cells with mildly enlarged nuclei.

Differential diagnosis

Gastric submucosal tumors, including leiomyoma, leiomyosarcoma, gastrointestinal stromal tumor, and lipoma.

Laboratory diagnosis

Initial laboratory findings were within the normal limits.

Imaging diagnosis

Esophagogastroduodenoscopy revealed a 10 mm submucosal tumor-like lesion in the lesser curvature of the upper third of the stomach, which had a regular microvascular pattern and a regular microsurface pattern based on narrow-band imaging with magnifying endoscopy.

Pathological diagnosis

An endoscopic submucosal dissection (ESD)-resected specimen revealed GA-FG spreading to heterotopic gastric glands (HGG) in the submucosa because the submucosal lesion of the GA-FG had a poor stromal reaction and was located just above the HGG.

Treatment

ESD as excisional biopsy and total gastrectomy.

Related reports

Although there are a few case reports of regular histological type of gastric cancer spreading to HGG, this is the first case of GA-FG spreading to HGG.

Term explanation

GA-FG, which differentiates into chief cells, is a new histological type of gastric cancer proposed by Ueyama *et al* in 2010. HGG, which are observed in the submucosa, are considered to be paracancerous lesions.

Experiences and lessons

We need to keep in mind the possibility of GA-FG presenting on the gastric mucosa with atrophic change, chronic gastritis, or intestinal metaplasia.

Peer-review

Dear Editor, this is well done case report with literature review. Authors described the case extensively and compare it against the literature. Also they provided excellent documentation and provided all necessary explanations.

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CASE REPORT

High-grade myofibroblastic sarcoma in the liver: A case report

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Author contributions: Wen J collected the clinical data, searched the relevant literature, and wrote the paper; Li C, Shen JY ,Wen TF performed the surgical operation; Zhao W performed the pathological diagnosis; Wen TF approved the final manuscript version to be submitted.

Institutional review board statement: This study was approved by West China Hospital Ethics Committee, Chengdu, China.

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Abstract

Only two cases of myofibroblastic sarcoma in the liver have been reported in the literature. Here, we report the case of a male patient with high-grade myofibroblastic sarcoma mimicking echinococcosis in the liver. The 25-year-old male patient complained of right upper quadrant swelling pain for one week and was initially diagnosed with echinococcosis. He was then scheduled for an exploratory laparotomy. During the operation, a huge mass exceeding 16 cm in diameter was found to occupy nearly the entire right trisegment of the liver, with a clear boundary and a round shape, and the mass was resected by right hepatic trisegmentectomy. Immunohistochemical staining revealed that the tumor tissue was positive for desmin, α-smooth muscle actin, CD56, and vimentin and negative for ALK-1, myogenin, calponin, β-catenin, S100, and glypican-3, with a Ki-67 (MIB-1) index of approximately 20%. Based on the histological manifestations and immunohistochemical staining, a diagnosis of myofibroblastic sarcoma was established. The postoperative recovery was uneventful. There was no evidence of recurrence or metastasis through the last follow-up, 6 mo after surgery, despite a lack of postoperative chemotherapy or radiotherapy. To the best of our knowledge, the present case is the first reported case of high-grade myofibroblastic sarcoma in the liver, and it is also the first reported case in a male patient.

Key words: Myofibroblastic sarcoma; Liver; High-grade; Male; Immunohistochemical staining

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Core tip: The development of myofibroblastic sarcoma in the liver is exceedingly rare. We present a case of high-grade myofibroblastic sarcoma in a male patient. The 25-year-old male patient complained of right upper quadrant swelling pain for one week and was diagnosed with high-grade myofibroblastic sarcoma after right hepatic trisegmentectomy based on the histological manifestations and immunohistochemical staining. There was no evidence of recurrence or metastasis through the last follow-up, 6 mo after surgery, despite a lack of postoperative chemotherapy or radiotherapy. The case presented is the first reported case of high-grade myofibroblastic sarcoma in the liver.

Wen J, Zhao W, Li C, Shen JY, Wen TF. High-grade myofibroblastic sarcoma in the liver: A case report. *World J Gastroenterol* 2017; 23(38): 7054-7058 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i38/7054.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i38.7054

INTRODUCTION

Myofibroblastic sarcoma is a rare disease that was initially identified by Mentzel *et al*[1] in 1998, and all its reports are case reports or case series. It is widely accepted that myofibroblastic sarcoma predominantly develops in the head and neck region, followed by the extremities and then the trunk, with the abdominal cavity being rarely involved. The development of myofibroblastic sarcoma in the liver is exceedingly rare, and there have been only two relevant case reports to date[2,3]. Although there is generally a slight male predominance[1,4], both of the case reports described female patients. In the present report, we present a case of high-grade myofibroblastic sarcoma in a male patient. The clinical manifestations, blood test results, imaging findings, histological and immunohistochemical findings, and treatment are discussed here.

CASE REPORT

A 25-year-old male patient was referred to the liver surgery department because of right upper quadrant swelling pain for one week without any concomitant symptoms. His medical history was unremarkable, with no hepatitis or surgical history. No family or genetic history was claimed either, and a general physical examination showed no abnormal signs.

Hematological and blood biochemical tests revealed no abnormalities. Serum tumor markers were within the normal limits: α-fetoprotein (AFP), 4.14 ng/mL; carcinoembryonic antigen (CEA), 1.30 ng/mL; and carbohydrate antigen (CA19-9), 6.06 U/mA. Among all of the hepatitis B markers, only surface antibody was positive.

Abdominal color ultrasound revealed a heteroechoic mass in the right trisegment of the liver, measuring 18 cm × 15 cm × 11 cm, with a clear boundary but with no remarkable blood signal. Abdominal contrast-enhanced color ultrasound revealed a heteroechoic mass showing peripheral hyper-enhancement in the arterial phase and peripheral hypo-enhancement in the portal phase, whereas the central portion showed no enhancement throughout the two phases. Dynamic contrast-enhanced computed tomography (CT) imaging revealed a cyst-like lesion with an obvious capsule located in the right trisegment of the liver; this lesion contained a stripe-like, slightly hyperdense area and patchy hypodense areas that were hyper-enhanced in the arterial phase and hypo-enhanced in the portal phase, whereas the central portion remained unenhanced throughout the arterial and portal phases (Figure 1).

Based on the aforementioned findings, liver echinococcosis was suspected. For treatment, a right hepatic trisegmentectomy was scheduled.

Upon laparotomy, a huge solid mass exceeding 16 cm in diameter with a clear boundary was found to occupy nearly the entire right trisegment of the liver. The remnant liver had a normal appearance, showing no cirrhosis. There was also no ascites or peritoneal dissemination, and enlarged lymph nodes were not found in the peritoneal cavity. Intraoperative ultrasound showed that the right hepatic vein was occluded, whereas the middle hepatic vein was pushed away by the mass. In addition, no disseminated lesions were found to be located in the remnant liver. The resected tumor measured 19 cm × 16 cm × 11 cm and weighed 2400 g. Sections through the tumor disclosed grayish-yellow, solid tissue that showed relatively homogenous internal structures (Figure 1).

Histologically, the tumor cells were spindle shaped and located in a loose myxoid background and contained small-to medium-sized nucleoli with readily identified mitotic figures (Figures 2 and 3). Histological images revealed high mitotic activity, with more than 19 mitoses per 10 high-power fields (HPFs), along with certain small necrotic areas (< 50%). Immunohistochemical staining showed that the tumor tissue was positive for desmin (Figure 4), α-smooth muscle actin (α-SMA), CD56, and vimentin and negative for ALK-1, myogenin, calponin, β-catenin, CD34, CD10, S100, glypican-3, CDK4, PCK, and CD117, with a Ki-67 (MIB-1) index of approximately 20%. For differential diagnosis, the tumor tissue was negative for CD31 (Figure 5), Factor VIII (to deny vascular tumor, such as angiosarcoma or epithelioid hemangioendothelioma), Melan A or HMB45 (to deny angiomyolipoma). Based on the histological

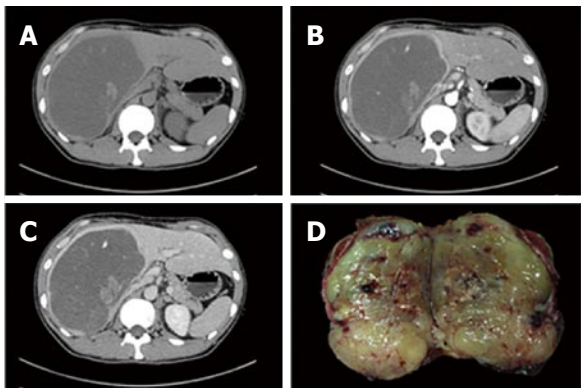


Figure 1 Computed tomography imaging and surgical specimen. Dynamic contrast-enhanced computed tomography (CT) imaging showed that a cyst-like lesion (A) contained a stripe-like, slightly hyperdense area and patchy hypodense areas that were hyper-enhanced in the arterial phase (B) and hypo-enhanced in the portal phase (C), whereas the central portion remained unenhanced throughout the arterial and portal phases. Sections through the tumor disclosed grayish-yellow, solid tissue that showed relatively homogenous internal structures (D).

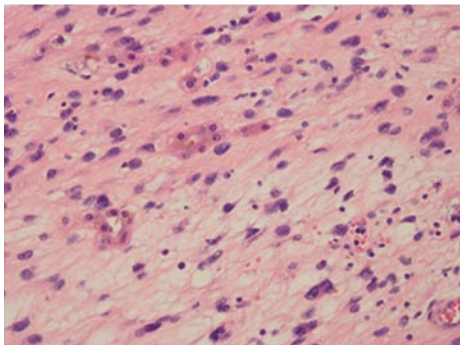


Figure 2 Histological image showing that the tumor cells were spindle shaped and located in a loose myxoid background (hematoxylin-eosin staining; original magnification, × 400).

and immunohistochemical findings, a diagnosis of myofibroblastic sarcoma was established, and this sarcoma was further classified as grade 3 according to the French Federation of Cancer Centers Sarcoma Group (FNCLCC) system[5].

The postoperative recovery was uneventful. The patient refused postoperative chemotherapy and radiotherapy; fortunately, however, there was no evidence of recurrence through the last follow-up, 6 months after surgery, despite the lack of postoperative chemotherapy or radiotherapy.

DISCUSSION

Since Mentzel *et al*[1] first defined a distinctive clinicopathological entity now known as low-grade myofibroblastic sarcoma in 1998, myofibroblastic sarcoma has been increasingly investigated, with most reports consisting of case reports or case series[2,3,6-11].

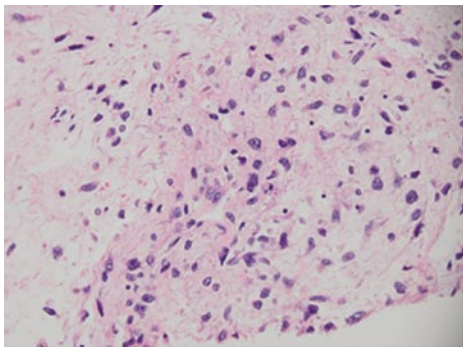


Figure 3 Histological image showing that the tumor cells contained small- to medium-sized nucleoli with readily identified mitotic figures (hematoxylin-eosin staining; original magnification, × 400).

Although the World Health Organization (WHO) included low-grade myofibroblastic sarcoma, when characterized reproducibly, in the WHO Classification of Tumors in 2002[12], the definition of intermediate- and high-grade myofibroblastic sarcoma with less reproducible characteristics has not reached a consensus, even in the latest edition of the WHO Classification of Tumors of Soft Tissue and Bone[4].

Given the rare incidence of myofibroblastic sarcoma, there have been few reports describing its etiology. Although a slight male predominance was suggested in the latest WHO classification[4], Chan *et al*[11] found that the majority of cases involve female patients. To date, the present case is the first reported case of high-grade myofibroblastic sarcoma in the liver and also the first case reported in a male patient, whereas the two existing case reports described female patients[2,3]. Painless swelling or an enlarging mass is the most common complaint[4], with other symptoms depending on the location; for example, obstruction is typical when the lesion develops in the nasal cavity[7]. In the present case, the chief complaint was right upper quadrant swelling pain, which was also present in a report by Yi *et al*[3], whereas Pan *et al*[2] reported no pain but abnormal liver function. In the present case and the report by Yi *et al*[3], the lesion showed a well-defined growth pattern, whereas this type of tumor is commonly reported to display an infiltrative and destructive growth pattern[4,7].

According to the WHO classification, ultrasound examination should be conducted first, but CT or magnetic resonance imaging (MRI) is recommended for further investigation, with equivalent value[4]. Imaging by dynamic contrast-enhanced CT in the report by Yi *et al*[3] was in close accordance with what was found in the present case. The lesion, which was well defined and round shaped and showed hypodensity on plain CT scans, was peripherally enhanced, whereas the central portion remained unenhanced throughout the arterial and portal phases.

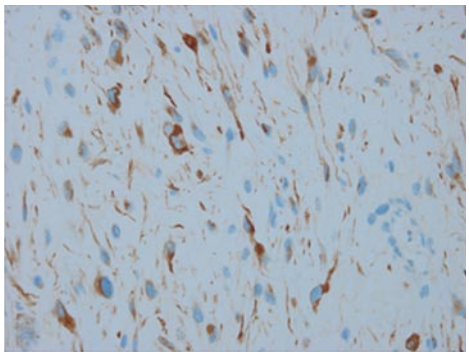


Figure 4 Immunohistochemical image showing that the tumor tissue was positive for desmin (original magnification, × 400).

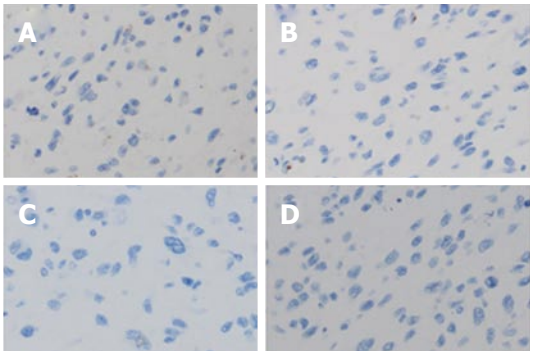


Figure 5 Immunohistochemical staining for differential diagnosis. The tumor tissue was negative for CD31 (A), Factor VIII (B), Melan A (C) and HMB45 (D) (original magnification, × 200).

Although these characteristics have been reported in only two cases, they constitute a "consensus" that could be referred to when myofibroblastic sarcoma in the liver is suspected. Yi *et al*[3] also reported a honeycomb appearance on T2WI images and a progressive enhancement pattern as characteristic manifestations on MRI.

The presence of ultrastructural features, such as fibronexus (microtendons) and stress fibers, is no longer emphasized as important for the diagnosis of myofibroblastic sarcoma[13]. Instead, the diagnosis of myofibroblastic sarcoma is now established by histopathology and immunohistochemical staining[13]. Although there is no constant immunophenotype, myofibroblastic sarcoma stains positively for muscle actin, α-SMA, and/or desmin in general, but it is typically negative for S100 protein, epithelial markers, nuclear β-catenin, and h-caldesmon[1,4,13].

Because high-grade myofibroblastic sarcoma was not included in the latest WHO classification[1,4,13], the two grading systems proposed by the National Cancer Institute (NCI) and the FNCLCC are the most widely used[4,5]. The FNCLCC system, which is precisely defined and more reproducible, with better performance in prognostic prediction, is preferred over the NCI system and other systems[14,15]. The present case, which showed high mitotic activity (more than 19 mitoses/10 HPFs) and certain small necrotic areas (< 50%), was classified as grade 3 according to the FNCLCC system[5]. Although low-grade myofibroblastic sarcomas have a local recurrence rate of 5%-10% when treated by wide-margin excision, they rarely metastasize. In fact, Fisher C reported 13 (33%) recurrences and 3 (8%) metastases in 39 published cases with follow-up information[16]. In contrast, high-grade myofibroblastic sarcoma shows a high rate of metastasis, along with recurrence; Fisher C reported 7 (32%) recurrences and 15 (68%) metastases among 22 ultrastructurally confirmed cases[16].

Surgery is the first choice for treatment, with the role of chemotherapy and radiotherapy being controversial. Generally, a wide margin of at least 1-2 cm is recommended because microscopically positive surgical margins are associated with a high risk of local recurrence, distant metastasis and death[4]. Retroperitoneal sarcomas show a much lower survival rate than extremity soft-tissue sarcomas, and high-grade myofibroblastic sarcomas are more aggressive[4]. However, little is known about myofibroblastic sarcoma, especially high-grade myofibroblastic sarcoma, in the liver. There were no signs of recurrence or metastasis in the reported intermediate-grade case[3] or in the high-grade myofibroblastic sarcoma case described in the present report 6 mo after surgery, despite the fact that both patients refused further treatment. Unfortunately, the patient in the report by Pan *et al*[2] was lost to follow-up 3 mo after discharge from the hospital. Meng *et al*[7] reported that myofibroblastic sarcoma of the nasal cavity and paranasal sinus exhibited diverse histological characteristics and strongly aggressive behavior, indicating that myofibroblastic sarcomas with different anatomic distributions may have diverse histological characteristics, biological behaviors and prognoses. We hypothesize that as long as it is resected with a sufficient margin, irrespective of grading, myofibroblastic sarcoma in the liver probably has an optimistic prognosis, as indicated by the report by Yi *et al*[3] and the present case. However, long-term follow-up and more patient data are needed before we can draw reliable conclusions.

Myofibroblastic sarcoma is a rare disease. The present case is the first reported case of high-grade myofibroblastic sarcoma in the liver, and it is also the first reported case in a male patient. The diagnosis of myofibroblastic sarcoma was established by histopathology and immunohistochemical staining. With a well-defined growth pattern and resection with a sufficient margin, regardless of its grade, the myofibroblastic sarcoma in the liver showed no signs of recurrence or metastasis 6 mo after surgery.

COMMENTS

Case characteristics

A 25-year-old male patient complained of right upper quadrant swelling pain for

1 week without any concomitant symptoms.

Clinical diagnosis
Liver echinococcosis was suspected.

Differential diagnosis
Liver echinococcosis, gastrointestinal stromal tumor, vascular tumor, such as angiosarcoma or epithelioid hemangioendothelioma, angiomyolipoma.

Laboratory diagnosis
All laboratory tests were within normal limits.

Imaging diagnosis
CT imaging found a cyst-like lesion with an obvious capsule located in the right trisegment of the liver.

Pathological diagnosis
The diagnosis of high-grade myofibroblastic sarcoma was established by histopathology and immunohistochemical staining.

Treatment
The patient successfully underwent a right hepatic trisegmentectomy.

Related reports
There have been no case reports on high-grade myofibroblastic sarcoma in the liver. Only two reports were published reporting intermediate-grade myofbroblastic sarcoma or myofibroblastic sarcoma. The diagnosis was established on the basis of morphology and immunohistochemistry features. Surgical resection was the treatment of choice with gratifying results.

Experiences and lessons
The diagnosis of myofibroblastic sarcoma is established by histopathology and immunohistochemical staining. Surgical resection is the treatment of choice with gratifying results.

Peer-review
The authors report a case of myofibroblastic sarcoma of the liver. The manuscript is well-written and the case is pathologically interesting.

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REVIEW

Less common etiologies of exocrine pancreatic insufficiency

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Author contributions: Singh VK, Haupt ME, Geller DE, Hall JA and Quintana Diez PM designed the "search terms" of the literature review, analyzed the data, and summarized the findings; all authors critically reviewed and revised the manuscript, and approved the final version of the article, including the authorship list.

Conflict-of-interest statement: Singh VK is a consultant for Ariel, Kowa, Novo Nordisk, and AbbVie; he has been an advisory board participant for Akcea and Nordmark; Geller DE and Hall JA are employees of AbbVie and may own AbbVie stock and/or options; Haupt ME and Quintana Diez PM are former employees of AbbVie and may own AbbVie stock and/or options.

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Abstract

Exocrine pancreatic insufficiency (EPI), an important cause of maldigestion and malabsorption, results from primary pancreatic diseases or secondarily impaired exocrine pancreatic function. Besides cystic fibrosis and chronic pancreatitis, the most common etiologies of EPI, other causes of EPI include unresectable pancreatic cancer, metabolic diseases (diabetes); impaired hormonal stimulation of exocrine pancreatic secretion by cholecystokinin (CCK); celiac or inflammatory bowel disease (IBD) due to loss of intestinal brush border proteins; and gastrointestinal surgery (asynchrony between motor and secretory functions, impaired enteropancreatic feedback, and inadequate mixing of pancreatic secretions with food). This paper reviews such conditions that have less straightforward associations with EPI and examines the role of pancreatic enzyme replacement therapy (PERT). Relevant literature was identified by database searches. Most patients with inoperable pancreatic cancer develop EPI (66%-92%). EPI occurs in patients with type 1 (26%-57%) or type 2 diabetes (20%-36%) and is typically mild to moderate; by definition, all patients with type 3c (pancreatogenic) diabetes have EPI. EPI occurs in untreated celiac disease (4%-80%), but typically resolves on a gluten-free diet. EPI manifests in patients with IBD (14%-74%) and up to 100% of gastrointestinal surgery patients (47%-100%; dependent on surgical site). With the paucity of published studies on PERT use for these

conditions, recommendations for or against PERT use remain ambiguous. The authors conclude that there is an urgent need to conduct robust clinical studies to understand the validity and nature of associations between EPI and medical conditions beyond those with proven mechanisms, and examine the potential role for PERT.

Key words: Celiac disease; Inflammatory bowel disease; Exocrine pancreatic insufficiency; Malabsorption; Epidemiology; Pancreas; Pancreatic cancer; Secretion/absorption; Surgery

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Core tip: Exocrine pancreatic insufficiency (EPI) results from primary pancreatic diseases or secondarily impaired exocrine pancreatic function. Pancreatic enzyme replacement therapy (PERT) may prevent serious nutritional complications when such patients have symptomatic EPI. However, EPI may be more prevalent in patients with non-pancreatic diseases, diabetes, and pancreatic cancer than has generally been appreciated. Scant published evidence on EPI in these less common etiologies precludes firm recommendations on management. Robust clinical studies are urgently needed to understand the relationships between EPI and medical conditions beyond those with proven mechanisms, and examine the potential role for PERT.

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INTRODUCTION

The pancreas is a dual function organ that possesses both exocrine and endocrine components that are critical for the digestion, absorption, and metabolism of nutrients. Normal digestion requires the exocrine function of the pancreas for macronutrient digestion. This occurs primarily *via* enzymatic hydrolysis by pancreatic enzymes, in particular lipase, amylase, and proteases (trypsin and chymotrypsin)[1]. Exocrine pancreatic insufficiency (EPI) refers to the presence of maldigestion and malabsorption of nutrients and is a consequence of primary loss of functional parenchyma and/or secondarily impaired exocrine pancreatic function and insufficient pancreatic enzyme activity. EPI is sometimes defined simply as an enzyme output less than 10% of that necessary to sustain normal digestion; however, there is no consensus in the literature on the definition of EPI. Furthermore, the clinical presentation of EPI can vary widely depending on the underlying cause, as well as disease stage, diet, and other factors.

EPI is most commonly caused by diseases that destroy the pancreatic parenchyma, such as chronic pancreatitis and cystic fibrosis, as well as pancreatic resection[1,2]. The incidence of EPI in chronic pancreatitis ranges from 30% of patients with mild disease to 85% with severe disease[3]. Approximately 85% of infants with cystic fibrosis have EPI at birth[4]. However, EPI is also observed in other conditions that include unresectable pancreatic cancer; metabolic diseases (diabetes mellitus)[1,2]; impaired hormonal stimulation of exocrine pancreatic secretion by cholecystokinin (CCK); celiac disease or inflammatory bowel disease (IBD) due to loss of intestinal brush border proteins[1,2]; small intestinal bacterial overgrowth[5,6], although not all investigators have found a clear association[7]; impaired coordination between motor and secretory functions (asynchrony); impaired enteropancreatic feedback, and/or inadequate mixing of pancreatic juices with ingested food after gastrointestinal surgery; and other diseases that affect the pancreas, such as hemochromatosis[8,9] (Tables 1 and 2)[1,2].

In this publication, we first briefly review the diagnosis of EPI and management with pancreatic enzyme replacement therapy (PERT). Most knowledge about EPI comes from studies in patients with chronic pancreatitis or cystic fibrosis, and has already been thoroughly explained in previous reviews. Therefore, our article focuses on several other disease states for which the association with EPI is less straightforward, such as inoperable pancreatic cancer, diabetes mellitus, celiac disease, IBD, and gastrointestinal surgery. Finally, we summarize the limited available data on PERT for the treatment of patients with EPI due to these less common etiologies.

LITERATURE SEARCHES

Searches of BIOSIS Previews, Derwent Drug File, Embase, Embase Alert, International Pharmaceutical Abstracts, MEDLINE, and SciSearch were performed to identify eligible literature from the earliest available date to December 5, 2016. The search terms for EPI were "EPI" or "exocrine pancreatic insufficiency" or "maldigestion" or "malabsorption" or "nutritional deficiency" or "steatorrhea" or "(fat* or oil* or elastase) pre/2 (stool* or fece* or fecal)" or "pancreatic near/3 (function or test)" with the terms NOT "chronic pancreatitis" or "cystic fibrosis". For pancreatic cancer, the search included the following terms: "pancreatic cancer" or "pancreatic adenocarcinoma" or "pancreatic tumor". For diabetes mellitus, the search strategy included "diabetes" and "type 1" or "IDDM" or "insulin-dependent" or "type 2" or "NIDDM" or "noninsulin-dependent" or "type 3" or "type 3c" or "type Ⅲ" or "pancreatogenic". For celiac disease, the search included "celiac disease" or "celiac*". For IBD, the search strategy included "inflammatory bowel disease" or "ibd" or "Ulcerative Colitis" or "Crohn*"

Table 1 Causes of exocrine pancreatic insufficiency

Definite association with EPI
Chronic pancreatitis
Pancreatic tumor/cancer
Cystic fibrosis
Pancreatic resection
Pancreatic hemochromatosis
Mechanisms associated with EPI not fully identified
Type 1 and 2 diabetes
Type 3c (pancreatogenic) diabetes
Gastrointestinal diseases
Celiac disease
Inflammatory bowel disease
Crohn's disease
Ulcerative colitis
Gastrointestinal surgery
Aging

EPI: Exocrine pancreatic insufficiency.

or "Crohn Disease". For gastrointestinal surgery, the search strategy included "gastrointestinal surgery" or "digestive system surgical procedures" or [(post or surg*) near/5 ("gastr*" or "bariatric" or "duodenal switch" or "biliopancreatic diversion")] or "bariatric surger*" or "gastrectom*" or "gastric bypass" or "stomach bypass". Reviews, practical guidelines, letters, editorials, and articles were evaluated. The searches returned 582 hits, from which 163 published articles were initially selected. Subsequently, articles were selected based on their clinical relevance, and additional papers were found after a review of the reference lists of these articles. Only a few were designed as prospective controlled studies with clearly defined methodology; this underscores the lack of data to support associations and mechanisms relevant to the conditions explored.

DIAGNOSIS OF EPI

Patients with EPI may exhibit a wide variety of clinical symptoms and nutritional deficiencies (Table 3). Clinical symptoms associated with EPI include steatorrhea (large-volume, foul-smelling stools), diarrhea, weight loss, flatulence, and abdominal pain. EPI may be diagnosed when fecal fat excretion is > 7 g/d on a 100-g fat/d diet. In EPI, fat malabsorption often develops prior to protein and carbohydrate malabsorption because lipase has a higher susceptibility to intraluminal denaturation and proteolytic destruction compared with other enzymes[10,11]. Furthermore, the deficiency in pancreatic lipase cannot be compensated by gastric lipase, the only other lipolytic enzyme in adult humans[12]. Because the exocrine pancreas has a large functional reserve capacity, clinical symptoms may not manifest until exocrine pancreatic function is < 10% of normal[13]. Untreated malabsorption places patients at high risk for developing nutritional deficiencies[14], which can manifest as other health problems, including decreased bone mineral density resulting in osteoporosis or osteomalacia[15-17]; bone metabolism deficiencies and muscle spasms; impaired night vision and decreased immune competence[16,18,19]; coagulation problems[16]; and ataxia and peripheral neuropathy[16]. Additionally, EPI has been associated with high morbidity and mortality secondary to malnutrition-related complications and an increased risk of cardiovascular events[20].

In routine clinical practice, EPI may be difficult to diagnose, particularly in the early stages when patients are less symptomatic. Often patients make dietary modifications to reduce symptoms. Patients may have low serum levels of fat-soluble vitamins, micronutrients, and lipoproteins[21]. Severe symptomatic EPI can be diagnosed by the presence of steatorrhea, diarrhea, flatulence, or weight loss[22], which often manifest when fecal fat excretion is > 7 g/d (Table 4). Early diagnostic studies relied on direct pancreatic function tests (*i.e.*, those involving collection and analysis of secretions directly from the duodenum or pancreatic duct, including the secretin-pancreozymin and Lundh tests[2]), which remain the most sensitive and specific methods for assessing exocrine pancreatic function. Direct tests, however, are limited by their cost, duration, and invasive nature, which involve endoscopic aspiration or tube aspiration of secretions from the duodenum for several hours. During the past 20 years, the use of non-invasive indirect methods has become more common. These tests are more readily performed in multiple settings and are based on the measurement of fecal elastase and fecal fat[2,23,24].

The coefficient of fat absorption is the gold standard for diagnosing fat maldigestion; however, it is poorly accepted by patients and laboratory personnel because it requires a strict diet containing 100 g of fat daily for 5 d and collection of all feces for the last 3 d (classical Van de Kamer test)[2]. In addition, fat excretion > 7 g/d indicates steatorrhea but is not informative about whether this is due to EPI or extrapancreatic causes. Fecal elastase is a pancreatic enzyme that is stable during passage through the gastrointestinal tract; some consider its measurement as the new gold standard for EPI diagnosis[2]. However, the current cutoffs that are used to define EPI might be improved if the cutoff were reduced to 128 μg/g stool[25] or 84 μg/g stool[26]. For better sensitivity, formed stool samples are best, as loose samples may spuriously dilute and lower the elastase levels and give a false positive result[16,24]. Fecal elastase, measured by enzyme-linked immunosorbent assay, has a good sensitivity for moderate EPI (75%) and high sensitivity for severe EPI (95%), and has a higher specificity (79%-96%) compared with the direct tests[27]. It should be noted that decreased fecal elastase values have been reported in patients with conditions not typically associated with EPI, such as HIV infection (23%-54%), advanced renal disease (10%-48%), and irritable bowel syndrome (6%)[28]. It is commonly accepted

Table 2 Factors involved with exocrine pancreatic insufficiency in different medical conditions[1,16,77,78,107,129,131,133,143,144,152-154,164]

Mechanism involved	Pancreatic cancer	Diabetes mellitus	Celiac disease	IBD	GI surgery
Normal pancreas		√	√	√	√
Abnormal pancreas	√	√	√	√	
Low or absent pancreatic enzyme production	√	√	√	√	√
Lack of stimulus for pancreatic enzyme production			√	√	√
Postcibal asynchrony	√	√	√	√	√
Pancreatic or biliary tract abnormalities	√	√		√	
GI malabsorption			√	√	√

EPI: Exocrine pancreatic insufficiency; GI: Gastrointestinal; IBD: Inflammatory bowel disease.

Table 3 Common signs and symptoms of exocrine pancreatic insufficiency[1,14-16,18,19,22]

Sign/symptom	Associated findings
Excessive flatulence	Abdominal bloating or distension, cramps, belching
Steatorrhea	Fatty, bulky stools; increased bowel movements
Malnutrition	Weight loss, anorexia, fatigue
Vitamin D deficiency	Deficient bone mineralization, osteomalacia, osteoporosis
Vitamin K deficiency	Coagulation abnormalities, ecchymoses, bone metabolism deficiencies
Vitamin A deficiency	Night blindness, decreased immune competence
Vitamin E deficiency	Ataxia and peripheral neuropathy
Hypocalcemia	Muscle spasms, osteomalacia, osteoporosis
Hypoalbuminemia	Nail leukonychia

that a fecal elastase-1 level ⩽ 200 μg/g stool indicates EPI, with levels of 100 to 200 μg/g typically indicating mild to moderate impairment and levels < 100 μg/g reflecting severe impairment[29-31]. Fecal elastase testing is considerably more sensitive than the fecal chymotrypsin or PABA test and is the standard clinical marker for moderate to severe EPI[32-34]. However, there is poor correlation between fecal elastase levels and coefficient of fat absorption, making fecal elastase less attractive for clinical research and regulatory purposes[35]. Additionally, because fecal elastase values are unaffected by PERT, enzymes do not need to be stopped before testing; unfortunately, this also means that fecal elastase testing is ineffective for monitoring response to PERT, unlike direct measurement of fat absorption[3].

Although not widely available, other tests for the diagnosis of EPI include the ^{13}C-mixed triglyceride (^{13}C-MTG) breath test and secretin-enhanced diffusion-weighted magnetic resonance cholangiopancreatography imaging (MRCP). In the ^{13}C-MTG test, the patient ingests a small amount of ^{13}C-marked triglycerides which are degraded by lipases in the intestine to ^{13}C-marked fatty acids. The absorbed ^{13}C fatty acids are metabolized by the liver, and $^{13}CO_2$ is exhaled[36]. Lower lipase activity is associated with less $^{13}CO_2$ in the exhaled breath. This test can also be used to assess the effects of PERT[37]. Pancreatic exocrine function can also be assessed by changes in duodenal filling, pancreatic duct caliber, and accumulation of fluid in the pancreatic parenchyma, as monitored by MRCP following stimulation with exogenous secretin[38,39].

PERT

PERT is the backbone of EPI treatment. Patients with abnormal fecal fat excretion, steatorrhea, and/or weight loss are generally considered candidates for PERT[20]. The aims of PERT are to compensate for deficiencies in endogenous enzyme secretion, correct maldigestion and malabsorption, and ameliorate symptoms resulting from a loss of exocrine function. To achieve this, the enzymatic activity delivered into the duodenum in conjunction with gastric emptying must be sufficient to optimize digestion and nutrient absorption[21]. A main goal of PERT is to restore sufficient intestinal lipase levels[11]. Unprotected lipase is irreversibly inactivated in the acidic environment of the stomach (pH ⩽ 4). Consequently, inhibition of gastric acid secretion has been used to prevent lipase inactivation. Modern preparations consist of pancreatic enzymes encapsulated in microspheres or microgranules, with an enteric coating designed to release the enzymes into the pH-neutral environment of the intestinal lumen[40]. A number of porcine lipase preparations are approved for PERT[41], and the reader is referred to publications from national and professional organizations for recommended dosages[16,42-44] Replacement of protease and amylase is also important in EPI, where some of its symptoms relate to deficiency of these two enzymes, as well. Pancrelipase of porcine origin contains the three enzymes (lipase, protease, and amylase) in adequate ratios to treat EPI.

In randomized controlled trials, PERT improved the coefficient of fat absorption, clinical symptoms,

Table 4 Symptoms and tests used in the diagnosis of exocrine pancreatic insufficiency[2,16,23,24]

Clinical symptoms
Steatorrhea
Diarrhea
Flatulence
Weight loss
Laboratory findings
Fecal fat > 7 g/d on a 100-g fat/d diet
Inconvenient; special high-fat diet and prolonged collection of feces
Considered gold standard
An abnormal coefficient of fat absorption is not specific for EPI
Fecal elastase-1 level ⩽ 200 μg/g stool; < 100 μg/g stool = severe EPI
Simple, convenient, and widely available
Measured on a random stool sample
Liquid stools may lead to falsely low results due to dilution
Less accurate in mild stages of disease
Positive qualitative fecal fat (Sudan Ⅲ) staining
Special high-fat diet
Less accurate; semi-quantitative microscopic method
Insensitive for mild disease
Fecal chymotrypsin ⩽ 6 U/g stool
Less sensitive than fecal elastase for mild EPI
Fluorescein dilaurate (pancreolauryl test)
Easy to perform
Not widely available
^{13}C-mixed triglyceride breath test
Well established
Not widely available
Imaging/endoscopy
Pancreatic duct dilatation
Main pancreatic duct calculi
Endosonographic criteria of chronic pancreatitis
Secretin-enhanced diffusion-weighted magnetic resonance cholangiopancreatography imaging
New
Not widely available

EPI: Exocrine pancreatic insufficiency.

and quality of life (QoL) of patients with EPI and significantly slowed gastric emptying[45-48]. Patients with EPI experienced a reduction in stool frequency and fat/water content, as well as abdominal pain and flatulence[47]. PERT is generally well tolerated; treatment-emergent adverse events include headache, infection, abdominal pain, flatulence, diarrhea, and dyspepsia[45-47,49]. However, because only porcine PERT products are currently available, allergic reactions, including anaphylactic shock, could potentially occur. Furthermore, fibrosing colonopathy, a rare but serious complication, has been reported in children[50] and adults[51] with cystic fibrosis receiving high-dose PERT, but there have been no reports in subjects with chronic pancreatitis.

PANCREATIC CANCER AND EPI

Pancreatic cancer ranks fourth among cancer-related deaths in the United States and has a 5-year survival rate of 7.2%[52,53]. This review focuses on inoperable pancreatic cancer, as the relationship between pancreatectomy and EPI is already well-recognized. EPI in patients with pancreatic cancer is related to the loss of pancreatic parenchyma and/or obstruction of the main duct, which impedes the production of pancreatic enzymes or their transportation into the duodenum. The most important predictors for EPI are localization of the tumor to the pancreatic head, ⩾ 90% destruction of normal tissue, degree of ductal obstruction, and surgical loss of pancreatic tissue[1,16,54]. The severity of ductal obstruction is proportional to the length of the obstructed duct; hence, enzyme secretion decreases as the cancer spreads distally, from head to body to tail[1,54,55].

The reported occurrence of malabsorption and exocrine dysfunction varies between 66% and 92% of patients with pancreatic cancer[30,56-59], with 65% to 75% of patients experiencing fat malabsorption and 50% of patients experiencing some degree of protein malabsorption[60,61]. In a prospective study of patients with an inoperable tumor of the pancreatic head region, 66% had EPI at diagnosis and 92% had a fecal elastase level < 200 μg/g by the 6-mo follow-up; 77% of patients were being treated with PERT[59]. In a systematic review, the prevalence of EPI was 25% to 50% in patients with advanced pancreatic cancer who did not undergo resection[62]. Although EPI is usually moderate in severity[61], in a prospective study, Partelli *et al*[31] detected extremely reduced (fecal elastase ⩽ 20 μg/g) in 25%, severely reduced (> 20 to < 100 μg/g) in 14%, and moderately reduced exocrine pancreatic secretion (⩾ 100-200 μg/g) in 11% of patients with advanced pancreatic cancer[31]. Pancreatic function abnormality seems to be higher in patients with tumors located in the pancreatic head versus in the body or tail[31,63]. Furthermore, in a prospective study, significantly more patients with a pancreatic head tumor had extremely reduced exocrine pancreatic secretion (fecal elastase ⩽ 20 μg/g) versus patients with a body or tail tumor; notably, a significant correlation was found between extremely reduced exocrine pancreatic secretion and poor survival.

Several studies have also reported inadequate enzyme secretion (trypsin, lipase, amylase, elastase, and chymotrypsin) in patients with pancreatic cancer compared with healthy controls[55,58,64]. Elastase production may be reduced earlier and to a greater extent compared with the output of other enzymes, for unknown reasons[64]. Additionally, fecal amylase activity was significantly decreased in pancreatic cancer patients compared with healthy controls[65].

PANCREATIC CANCER AND PERT

Approximately 80% to 90% of patients with pancreatic cancer have unresectable or advanced metastatic disease, leaving only palliative treatment options to manage symptoms[66]. Gastrointestinal and diet management problems negatively impact patients' QoL[67]; consequently, early treatment of EPI has been suggested to reduce symptoms[59] and to improve weight gain and fat absorption in patients with

pancreatic cancer[60,66]. The National Comprehensive Cancer Network has advised that PERT be given to patients with pancreatic cancer who show symptoms of EPI[68]. Other organizations have noted that PERT may help maintain weight and promote QoL in patients with pancreatic cancer[40,69].

The recommendations for PERT use in pancreatic cancer patients were made despite a paucity of data to support them. Only two randomized placebo-controlled trials have investigated the use of PERT in pancreatic cancer (Table 5). In a double-blind trial of 21 patients with unresectable cancer of the pancreatic head, patients treated with 50000 units of lipase/meal gained 1.2% in body weight in 8 wk, while those receiving placebo lost 3.7%[66]. Fat absorption also improved by 25% with PERT, whereas it dropped by 25% with placebo. Steatorrhea did not significantly differ between groups; however, there was a trend for lower stool frequency in patients receiving PERT. When patients receiving placebo were switched to open-label PERT, they demonstrated weight stabilization and improvements in steatorrhea-related symptoms. In a double-blind, placebo-controlled study in patients with unresectable pancreatic cancer (43% had severe EPI, defined as fecal elastase-1 < 100 μg/g stool), mean weight loss after 8 wk of PERT (-1.49%) was not significantly different compared with placebo (-2.99%)[70]. However, PERT did improve nutritional status in a subset of patients with unresectable cancer of the pancreatic head region. Additionally, in an uncontrolled study of patients with unresectable pancreatic cancer, patients with moderate to severe fat or protein malabsorption showed improved nutrient absorption with PERT[60].

DIABETES MELLITUS AND EPI

Type 1 diabetes is considered a primary autoimmune process characterized by typically early onset, an eventual absolute lack of insulin, and islet cell antibodies[71]. Type 2 diabetes is a metabolic disorder characterized by hyperglycemia in the context of insulin resistance and a relative lack of insulin[71]. A third type of diabetes, type 3c or pancreatogenic diabetes[71-73], occurs secondary to parenchymal pancreatic disease and is characterized by an absent pancreatic polypeptide response to nutrients and loss of islet cells by inflammatory destruction and fibrosis[74,75]. The relationship between EPI and diabetes is complex due to the close anatomic and physiologic linkages between the exocrine and endocrine pancreas; pathological conditions of the endocrine tissue can cause impairment of exocrine function and vice versa. Furthermore, depending on the particular diagnostic tests that are used, there is the chance of inadvertently classifying type 3c diabetes as type 1 or 2, confounding understanding of their relative prevalence and relationship to EPI.

Type 1 and type 2 diabetes

Marked alterations in the exocrine pancreas are observed in patients with diabetes, including changes in size, morphology, and histology[76]. No studies have examined at what point during the course of diabetes these pancreatic abnormalities develop. Diabetic pancreata are often atrophic and can have prominent fatty involutions and calcification[77,78]. Atrophy is more pronounced in type 1 *vs* type 2 diabetes[79,80]. Moreover, the pancreata of diabetic patients are significantly smaller and have higher lobulation compared with healthy controls[79,80]. In a cadaveric study, the mean weight of pancreata in type 1 diabetic patients weighed about a half of that of controls[81], while magnetic resonance imaging studies in adults with recent-onset diabetes found only a 26% to 31% reduction in pancreatic volume index after adjustment for body weight compared with healthy controls[82,83]. Additionally, pancreatic volume in diabetic patients was significantly lower when elastase and/or chymotrypsin levels were low[77]. Atrophy of the gland and acini, lymphocytic infiltration, moderate to severe fibrosis, and fatty changes were noted on autopsy in the exocrine pancreas of Japanese patients with diabetes[84].

EPI associated with diabetes is typically mild to moderate and not associated with overt steatorrhea. The prevalence of EPI is higher in type 1 diabetes (26%-57%)[85-89] compared with type 2 diabetes (20%-36%)[78,85,86,88], significantly so in a pooled literature analysis of 3662 patients with diabetes (39% *vs* 28%, respectively, using a cutoff of fecal elastase 200 μg/g stool; $P < 0.00001$)[76]. Severe reductions in fecal elastase levels (< 100 μg/g) have been observed in 11% to 30% of patients with type 1 diabetes[85-88,90] and 3% to 20% of patients with type 2 diabetes[85,86,88,91,92]. Notably, in a large screening study of diabetic patients, correlations between exocrine insufficiency and early onset/longer duration of diabetes, insulin use, and lower body mass index (BMI) have been demonstrated[86]. Fecal elastase levels have also been found to correlate with worse glycemic control, less residual β-cell function, and higher BMI[93,94].

Fecal fat excretion inversely correlates with fecal elastase levels in type 1 diabetes; however, excessive fecal fat excretion occurred in 22% of patients with normal fecal elastase levels[87]. In a cohort of diabetics with fecal elastase levels < 100 μg/g, 59% of patients excreted ⩾ 7 g of fat per day[86]. Interestingly, 45% of type 1 diabetics with pathological fat excretion were asymptomatic in one prospective study[89]. Fecal fat excretion did not correlate with the type or duration of diabetes, age at onset, glycemic control, or BMI[89,95]. The prevalence of EPI was 33% using the direct secretin-cerulein test in patients with type 1 diabetes; among patients with an abnormal secretin-cerulein test result and steatorrhea ($n = 8$), 50% had decreased lipase but none had an enzyme secretion level

Table 5 Pancreatic enzyme replacement therapy clinical trials

Study	Study design, duration (when given), and number of patients	Disease	Results	Adverse effects
Bruno *et al*[66]	DBRPC, 8 wk, 24 adults (21 analyzed)	Pancreatic cancer	The mean absolute difference for PERT *vs* placebo in percentage change in body weight was 4.9% (P = 0.02); other outcomes were numerically improved with PERT *vs* placebo [fat absorption coefficient, 12% increase *vs* 8% decrease (P = 0.13); stool frequency, decrease of 1/d *vs* increase of 2/d (P = 0.07)]	No treatment-related AEs
Woo *et al*[70]	DBRPC, 8 wk, 67 adults	Pancreatic cancer	The mean change in body weight at 8 wk was similar with PERT *vs* placebo (-1.49% *vs* -2.99%; P = 0.381), but the mean change in nutritional status was superior with PERT *vs* placebo in the subset of patients with cancer of the pancreatic head (PG-SGA score, -42.65% *vs* 32.93%; P = 0.039)	Three patients died [PERT group, 2/34 (6%); placebo group, 1/33 (3%)] There were no PERT-related serious AEs
Perez *et al*[60]	Open-label, 12 adults	Pancreatic cancer	Most patients with moderate to severe fat (6/7) or protein (3/3) malabsorption improved, but no patients with mild fat or protein (0/8) malabsorption improved	No descriptions regarding TEAEs
Ewald *et al*[49]	DBRPC, 16 wk, 80 adults	Type 1 diabetes	No significant change in HbA_{1c}, fasting glucose, or postprandial glucose; increase in mean vitamin D from baseline to week 16 (PERT, from 54.1 to 59.4 nmol/L; placebo, 60.2 to 62.7 nmol/L)	TEAEs occurred in 33 patients (84.6%) in PERT group and in 35 (85.4%) in PBO group; most frequent AEs were headache, infection, pain, diarrhea, and dyspepsia
Carroccio *et al*[150]	DBRPC, 2 mo, 40 children	Celiac disease	Significant mean ± SD weight gain in first 30 d (1131 ± 461 g with PERT *vs* 732 ± 399 g with placebo; P < 0.006), not significant at 60 d	No undesired side effects were reported
Evans *et al*[141]	Open-label, up to 4 yr, 20 adults	Celiac disease	Significant increase in fecal elastase from median of 90 μg/g to 365 μg/g (P < 0.0001) and improvement in chronic diarrhea with reduction in median stool frequency from 4/d to 1/d ($P \leqslant 0.0001$), but no weight increase (P = 0.3)	No descriptions regarding TEAEs
Leeds *et al*[135]	Open-label, up to 2 yr, 20 adults	Celiac disease	Significant improvement in chronic diarrhea with reduction in median stool frequency from 4/d to 1/d ($P \leqslant 0.0001$), but no weight increase (P = 0.3)	No descriptions regarding TEAEs
Huddy *et al*[181]	Open-label, 10 adults	Esophagectomy	Improvement in diarrhea and steatorrhea (9/10), increased weight (7/10)	Nausea in 1 patient
Armbrecht *et al*[183]	DBRPC crossover trial, 2 wk (plus 1-wk washout), 15 adults	Total gastrectomy	Improved stool consistency (score, 7.6 with PERT *vs* 9.3 with placebo; P < 0.05), but not the number of bowel movements or abdominal symptoms	No descriptions regarding TEAEs
Hillman *et al*[166]	Open-label, 6 mo, 30 adults	Partial gastrectomy	Mean ± SE weight gain of 6.73 ± 0.77 (P < 0.001), mean ± SE decrease in steatorrhea of 49.7% ± 7.7% (P < 0.001)	No descriptions regarding TEAEs
Brägelmann *et al*[184]	DBRPC, 14 d, 52 adults	Total gastrectomy	Improvement of overall well-being (15/23 with PERT *vs* 6/24 with placebo; P = 0.006), but no improvement of specific symptom	No descriptions regarding TEAEs

AE: Adverse event; DBRPC: Double-blind, randomized, placebo-controlled; GIP: Gastric inhibitory polypeptide; GLP-1: Glucagon-like peptide-1; HbA_{1c}: Glycated hemoglobin A_{1c}; PBO: Placebo; PERT: Pancreatic enzyme replacement therapy; PG-SGA: Patient-generated subjective global assessment; TEAE: Treatment-emergent AE.

< 10% of normal, which is typically when steatorrhea manifests[89]. In the same study, the fecal elastase test had low sensitivity (36%-55%) and specificity (59%-77%) to reproduce the secretin-cerulein test results; the authors concluded that low fecal elastase levels do not reliably indicate EPI in type 1 diabetes.

Secretory abnormalities have been noted in diabetics[96-100]. Frier *et al*[96] observed reductions in exogenously stimulated secretion of amylase (66%) and trypsin (54%) in type 1 diabetics, and the degree of dysfunction correlated with disease duration in a small controlled study. Bicarbonate output was also significantly reduced and showed a significant inverse correlation with the daily insulin dosage in patients with a disease duration < 10 years. Furthermore, hyperglucagonemia, which is observed in some type 2 diabetic patients, is associated with a marked inhibition of pancreatic enzyme output, including lipase, amylase, and trypsin[97]. Increased somatostatin, also found in some diabetic patients, dose-dependently inhibits secretion of pancreatic bicarbonate, amylase, and trypsin[98-100].

Several theories have been proposed to explain the reduced exocrine function in diabetes, including

imbalances between stimulatory and inhibitory islet hormones, pancreatic atrophy or fibrosis, autonomic neuropathy, altered release of gastrointestinal regulatory mediators, and autoimmunity[79,80,84,101,102]. Disturbances in acinar-islet interactions with imbalances between stimulatory (insulin) and inhibitory (glucagon, somatostatin) islet hormones are linked to EPI in some diabetic patients[101]. Insulin has a trophic effect on the acinar cells and a stimulatory effect on exocrine enzyme secretion in animal models and cell cultures, suggesting that insulin deficiency may play a role in pancreatic atrophy[101,103]; insulin deficiency in diabetic patients may lead to pancreatic atrophy[79,80,84]. If insulin deficiency were the primary reason for exocrine dysfunction, however, then all patients with type 1 diabetes would be expected to have EPI.

Regulation of pancreatic enzyme elaboration and secretion depends on gastrointestinal hormones and local neuronal signals[101]. Unsurprisingly, therefore, autonomic diabetic neuropathy and secondary gastroparesis can impair enteropancreatic reflexes, such as changes in gut peptides, that may mediate as much as 50% of the postprandial exocrine pancreatic response[75,101,104]. Diabetic microangiopathy can reduce pancreatic perfusion and cause arterial lesions that can lead to pancreatic fibrosis[105,106]. Patients with type 2 diabetes are also at an increased risk for biliary disease, which can diminish secretions from the pancreas[107]. Finally, autoimmune diseases can involve both the exocrine and endocrine glands, as antibodies against islet cells can cross-react with acinar cells[34]. Autoantibodies against exocrine pancreatic antigens were detected in 77% of patients with type 1 diabetes, but were not detected in any patients with type 2 diabetes[108]. In summary, screening for EPI in patients with type 1 or type 2 diabetes is appropriate when symptoms suggest pancreatic insufficiency.

Type 3c diabetes

Pancreatogenic or type 3c diabetes occurs secondary to pancreatic disease, injury, or resection and accounts for 5% to 10% of the Western diabetic population[8,9,109,110]. Despite the prevalence of type 3c diabetes, the American Association of Clinical Endocrinologists and American College of Endocrinology have not formally included it in their guidelines[73]. The etiologies of type 3c diabetes include chronic pancreatitis (76%-79%), pancreatic cancer (8%-9%), hereditary hemochromatosis (7%-8%), cystic fibrosis (4%), and post-pancreatic resection (2%-3%)[8,9]. Furthermore, the prevalence of type 3c diabetes in chronic pancreatitis is correlated with the degree of exocrine dysfunction (with a prevalence of 63%, 32%, and 13% with severe, moderate, and mild dysfunction, respectively)[111]. Per diagnostic criteria, all patients with type 3c diabetes display signs of EPI[112], and this EPI is more severe compared with that of patients with type 1 and type 2 diabetes, as demonstrated by lower stimulated bicarbonate and trypsin output[96] and lower fecal elastase levels[94].

Because of the close anatomical relationship between exocrine and endocrine cells, type 3c diabetes may result from progression of the primary pancreatic exocrine disease that destroys islet cells by pancreatic inflammation or fibrosis[111,113]. Indeed, the impairment of pancreatic endocrine function in chronic pancreatitis proceeds in parallel with the destruction and spread of fibrosis inside islet cells[111,114,115]. Additionally, α- and β-cell responses were reduced in patients with autoimmune pancreatitis[115]. Mechanisms besides simple islet cell destruction may also be involved, as even small adenocarcinomas are associated with type 3c diabetes[116].

DIABETES MELLITUS AND PERT

Despite a paucity of clinical data for PERT use in patients with diabetes, position statements have stated that symptomatic patients with fecal elastase levels <100 μg/g may be treated with PERT[16,117] but should be carefully monitored because of the risk of disturbances in glucose homeostasis[118]. Of course, increased glucose uptake may reduce the risk of hypoglycemia[34]. There is some evidence that PERT can improve glucose metabolism by augmenting the effects of incretins and increasing postprandial insulin secretion[48,119]; however, no significant differences in hemoglobin A1c, fasting glucose, or oral glucose tolerance test results were observed between patients with type 1 diabetes treated with PERT and placebo (Table 5)[49].

In summary, there are different gastrointestinal motility and comorbid conditions in patients with diabetes mellitus that may result in EPI or decreased digestion or absorption of fat and protein. Early EPI is very difficult to diagnose in diabetic patients, where the condition appears and progresses insidiously across years. Although endocrinologists have not formally recognized type 3c diabetes, most of the conditions that lead to type 3c diabetes have a well-known association with EPI and the need for PERT. An interdisciplinary approach is needed to better define the possible association of EPI with diabetes and potential mechanisms, and to separate them from pancreatic processes that may or may not be related to diabetes. Furthermore, guidelines are needed to help clinicians decide when to test diabetic patients for EPI, and when use of PERT is beneficial.

AGING AND EPI

There are few studies on the effects of aging on exocrine pancreas function, and most[120-122] but not all studies[123] have found that EPI appears to increase with age. For example, in a study of older individuals (age 60 to > 79 years) without gastrointestinal

diseases or diabetes, fecal elastase-1 levels correlated negatively with age and were significantly lower in individuals > 70 years of age compared with a control group (age 20-28 years)[122]. Among subjects over 60 years of age, 21.7% had fecal elastase-1 levels below 200 μg/g stool.

CELIAC DISEASE AND EPI

Celiac disease is an inappropriate T-cell–mediated reaction to gluten that causes inflammatory injury to the small intestine; the estimated worldwide prevalence is 1% to 2%[124,125]. Diarrhea is common with celiac disease and is typically attributed to gluten-related indigestion, malabsorption, and fluid secretion. The primary treatment is a gluten-free diet, which usually improves gastrointestinal function, diarrhea, and weight gain. Nonetheless, 17% to 61% of patients with treated celiac disease have persistent diarrhea[126,127]. Diagnostic testing of celiac patients with chronic diarrhea on a gluten-free diet determined that EPI was present in 12% (based on pancreatic test or trial of PERT) to 18% (based on steatorrhea and trial of PERT)[126,128].

Pancreatic dysfunction occurs in some patients with celiac disease but is typically transient and normalizes with a gluten-free diet[129,130]. However, some patients do have substantially impaired exocrine pancreatic function, leading to maldigestion, malabsorption, and malnutrition. Comorbid chronic pancreatitis has been one possible explanation for these severe cases, with several publications reporting a higher incidence of chronic pancreatitis in patients with celiac disease[131,132]. In patients with untreated celiac disease, the prevalence of EPI (measured and defined by the fecal elastase test) ranges from 4% to 80%[133-138]. Subnormal secretion of at least 1 pancreatic enzyme was observed in 22% to 33% of patients with untreated celiac disease[130,139,140]. In a small controlled study, a significant inverse correlation was demonstrated between the severity of intestinal damage and fecal elastase levels in patients with celiac disease[133]. Carroccio *et al*[140]. reported normalization of fecal chymotrypsin in almost all patients with celiac disease on a strict gluten-free diet[140],which speaks to the functional aspect of EPI due to lack of stimulus rather than to structural damage. It has been suggested that impairment of exocrine pancreatic dysfunction is related to mucosal villous atrophy[129,133], and thus can improve when mucosal regeneration occurs with a gluten-free diet and other treatments[137,141].

In a single-center study by Rana *et al*[142], 36 patients with celiac disease serologically and histopathologically diagnosed were studied with fecal elastase, endoscopic ultrasound (EUS), and elastography. At study entry, 10 of the patients (28%) were diagnosed with EPI based on abnormal fecal elastase levels; 9 (90%) of these patients had villous atrophy of the duodenum, and 1 patient had a history of several episodes of acute pancreatitis. The 10 patients were subjected to a gluten-free diet, and after 3 mo 7 patients had a repeat fecal elastase test that had normalized in all cases, except for the patient with prior acute pancreatitis events. Elastography results were normal in all 8 patients who consented to EUS, except for the patient with prior acute pancreatitis events. The authors concluded that EPI, identified based on fecal elastase levels in adult patients with celiac disease, may be unrelated to structural changes in the pancreatic parenchyma and might be reversible by a gluten-free diet in most patients.

The pathophysiological mechanisms of EPI in celiac disease may be multifactorial. A primary mechanism could be a defective postprandial response to intraluminal contents by an atrophic upper intestinal mucosa with altered synthesis, storage, and/or secretion of secretin and CCK, which are potent stimulators of pancreatic secretion. Postprandial plasma CCK levels were significantly lower in patients with untreated celiac disease compared with controls and were significantly correlated with fecal elastase levels[133]. Impaired CCK release leads to reduced pancreatic stimulation and secretion, postcibal asynchrony between gastric emptying and gallbladder contraction, and fat maldigestion[143,144]. Decreased secretin release by the extensively damaged jejunal mucosa has also been reported[145]. General malnutrition is associated with defects in pancreatic secretion[146]; consequently, it is not unexpected that protein malnutrition in celiac disease is associated with a decrease in pancreatic enzyme output, as well as structural changes in the pancreas, including atrophy of acinar cells with fewer secretory granules, pancreatic fibrosis, and a smaller pancreatic head[147,148]. One study, however, reported that EPI in celiac disease may be independent of nutritional status[139]. There is some evidence for malabsorption of amino acids in patients with untreated celiac disease[149], which might contribute to EPI by restricting the substrates for synthesis of digestive enzymes.

CELIAC DISEASE AND PERT

Pancreatic function tests are usually not performed on newly diagnosed patients or patients with uncomplicated celiac disease; these tests should be considered if there is persistent diarrhea or steatorrhea despite a gluten-free diet or if there are signs of overt malnutrition. Patients on a gluten-free diet with low fecal elastase levels should receive PERT[16]. Data from a double-blind randomized trial of children with celiac disease on a gluten-free diet demonstrated that PERT increased body weight versus placebo during the first 30 days after diagnosis (Table 5)[150]. Similarly, PERT reduced chronic diarrhea from 4 to 1 stools/day in 90% of patients with celiac disease in 2 other uncontrolled studies[135,141].

Gastroenterologists specializing in celiac disease have not recognized a definite association between

celiac disease and EPI and are silent on the possible association and the need for treatment with PERT. Further studies are required to demonstrate whether there is any direct association between celiac disease and EPI.

IBD AND EPI

Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing immune-mediated disorders of the gastrointestinal tract, characterized by chronic gastrointestinal inflammation. It is suggested that these disorders result from an aberrant immune response and loss of tolerance to the normal intestinal flora. Patients with IBD are at an increased risk for developing EPI, particularly if they have $\geqslant$ 3 daily bowel movements (BMs), loose stools, and a history of surgery[34,151]. Autopsy studies have found pancreatic lesions in 38% of patients with CD and 53% of patients with UC without prior evidence of pancreatitis[152]. Although still widely used, the fecal elastase test has poor diagnostic accuracy in patients with diarrhea[12]. In a cross-sectional study of 237 unselected patients with IBD, 21% demonstrated exocrine dysfunction as measured by the PABA test, and 19% exhibited abnormally low bicarbonate secretion in response to a secretin test; the frequency of abnormal results was similar in patients with CD and UC[153]. Furthermore, 8.4% of patients had a pancreatic duct abnormality[153].

CD

As a group, patients with CD have significantly decreased lipase, amylase, and trypsin activity compared with controls; these changes are not correlated with disease duration or location or extent of a previous bowel resection[154,155]. Factors related to impaired pancreatic function were disease activity, localization, and extent of bowel involvement[154]. The prevalence of EPI based on low fecal elastase levels varies between 14% and 30% of patients with CD[151,156]. Angelini *et al*[157] determined that 35% of patients with CD have impaired bicarbonate and/or enzyme secretion[157]. Depending on the involvement of the gastrointestinal tract (ileum, colon, or both), abnormal fat excretion varies between 17% and 35% in patients with CD[158].

Possible mechanisms for the development of EPI in CD include pancreatic autoantibodies, duodenal reflux, and reduced secretory hormone secretion. About one third of patients with CD have autoantibodies against pancreatic components[159-161], suggesting that EPI could result from immunologic induction of pathways that impair exocrine function. These antibodies appear specific for CD, as opposed to an individual with UC or without IBD[159,160]. Other possible mechanisms for pancreatitis in patients with CD include duodenal reflux into the pancreatic duct through an inflamed and incompetent ampulla of Vater and fistula formation between the pancreatic duct and the duodenum[162]. These processes could play a role in CD-associated EPI by damaging the pancreatic duct. Indeed, pathological changes in the pancreatic duct that may impede flow have been reported in patients with CD and UC[153]. Finally, scarring or inflammation may reduce intestinal hormone secretion, thus insufficiently stimulating the pancreas[154].

UC

In unselected patients with IBD, 22% of patients with UC had fecal elastase levels $\leqslant$ 200 μg/g, and 9% had severe EPI (fecal elastase $\leqslant$ 100 μg/g)[151]. Additionally, using a secretin-cerulein test, 50% of patients with UC demonstrated bicarbonate and/or enzyme insufficiency, while 74% had an abnormal PABA test[153,157]. By magnetic resonance cholangiopancreatography, 16.5% of patients with UC had a pancreatic duct abnormality, compared with no individuals in the control group[163].

IBD AND PERT

Despite the high prevalence of EPI in patients with IBD, we identified no studies that assessed whether PERT can improve maldigestion or malabsorption in patients with either CD or UC, nor any guidelines for the use of PERT in these populations.

GASTROINTESTINAL SURGERY AND EPI

Upper gastrointestinal surgery can distort the normal anatomy and physiology of digestion, thus disrupting the intricate sequence of events that control normal digestion and absorption. Maldigestion occurs in as many as 80% of patients following such procedures, and EPI may contribute to the pathogenesis[164]. Pancreatectomy results in bulk loss of enzyme-producing cells and is already an indication for PERT, so it will not be discussed here.

Post-gastrectomy diarrhea and/or steatorrhea occur in > 47% of gastrectomy patients, and significant weight loss is common[165-167]. In one study, all patients (n = 15) developed severe EPI 3 mo after total gastrectomy[168]. Steatorrhea was also observed in all patients (n = 30) who underwent a partial gastrectomy[166]. Two additional studies reported pathological fecal fat excretion in 92% and 67% of patients after total gastrectomy[167,169]. Additionally, using the ^{13}C-mixed triglyceride breath test, 82% of patients exhibited fat maldigestion after a Whipple procedure[164]. Finally, using the same diagnostic test, Perez Aisa *et al*[170] recently reported that 38% of patients developed fat malabsorption following partial or total gastrectomy.

EPI and altered pancreatic enzymes and gastrointestinal hormone levels were reported after both total and partial gastrectomies[168,171-173]. Luminal pancreatic enzyme and bile salt concentrations were

markedly reduced after subtotal gastrectomies[173], and significant reductions in the stimulated secretion of pancreatic juice (76%), trypsin (89%), chymotrypsin (91%), and amylase (72%) were observed after total gastrectomy compared with preoperative levels[168]. In another study, total gastrectomy significantly decreased bicarbonate (48%), lipase (39%), and chymotrypsin (24%) output in comparison with non-operated controls[167]. In a third study, only 30% of patients had EPI following subtotal or total gastric resection as measured by the fecal chymotrypsin test[171]. Low levels of gastrin and pancreatic polypeptide and high levels of postprandial plasma CCK have also been reported following total gastrectomy[168].

Gastrectomy disrupts several of the normal digestive processes; different factors may contribute to the postoperative changes, including deficient trituration of nutrients, altered gastric emptying, pancreatic denervation, postcibal asynchrony between gastric emptying and gallbladder contraction, and/or decreased absorptive surface and enzyme contact[16]. When the duodenum is also resected (gastroduodenal resection), a reduction in CCK secretion from the duodenum decreases pancreatic stimulation and contributes to EPI[164]. Likewise, Roux-en-Y gastric bypass surgery to treat obesity disrupts the normal digestive process, and almost a third of patients develop EPI post-operatively[174]. However, since the purpose of the procedure is to effect weight loss, it is unlikely that EPI in this situation would be treated.

The vagus nerve plays an important role in the regulation of exocrine pancreatic secretions, as vago-vagal enteropancreatic reflexes mediate responses in the intestinal phase of exocrine pancreatic secretion[175]. Vagotomies, which reduce gastric acid secretion by severing the vagal nerve supply to the stomach, also cause dysfunction of the exocrine pancreas; during extensive gastric surgery, severing of the vagus nerve (truncal vagotomy) can contribute to postoperative EPI, and a vagotomy by itself is sufficient to cause EPI[176]. In 2 studies, patients had decreased pancreatic juice, lipase, trypsin, and bicarbonate secretion following vagotomy[177,178]. In a similar study, fecal fat excretion was significantly increased after vagotomy and 45% of patients developed steatorrhea[179].

Extensive small bowel resections leading to short bowel syndrome can also reduce endogenous exocrine pancreatic secretion. Short bowel syndrome is characterized by malabsorption, with contributing factors including a reduction in gastrointestinal hormones (particularly CCK), postcibal asynchrony, gastric acid hypersecretion, loss of intestinal regulatory feedback, massive loss of absorptive surface, and rapid transit through the small intestine[1,180]. Additionally, total parenteral nutrition and anti-diarrheal agents used to treat short bowel syndrome are associated with pancreatic and gastric hyposecretion[180]. Some of these mechanisms, though not all, involve the pancreas, suggesting a role for EPI. There is wide variability depending upon the individual and the specific region resected. In patients undergoing ileal resection for CD, fecal fat excretion showed a highly significant correlation to the ileal length resected; for patients with only a 30-cm resection or less, the prevalence of abnormal fat excretion was 37%, whereas 100% of patients who underwent a 90-cm resection or greater displayed abnormal fecal fat excretion[158].

Esophagectomy has also been associated with EPI in one study (n = 63); 10 patients (16%) who underwent an esophagectomy had weight loss and fecal elastase levels < 200 μg/g stool and had symptomatic EPI with diarrhea and/or steatorrhea[181]. Potential mechanisms include decreased gastric reservoir, vagal denervation, and the presence of pyloroplasty that may be part of the procedure and cause dumping syndrome.

GASTROINTESTINAL SURGERY AND PERT

Despite a paucity of evidence regarding PERT use following gastrointestinal surgery, PERT is often recommended for post-surgical patients with steatorrhea, diarrhea, weight loss, or maldigestion-related symptoms[164,182]. In patients with EPI post-esophagectomy, 9 of 10 patients with fecal elastase levels < 200 μg/g stool had symptomatic improvement (no diarrhea or steatorrhea) with PERT and 70% experienced weight gain (Table 5)[181]. PERT may also be appropriate for asymptomatic patients with fat excretion > 15 g/d, as these patients are at high risk for developing nutritional deficiencies[164,182]. It has been suggested that PERT in combination with a high-energy diet over 6 to 8 meals/d may improve nutritional status and symptoms in these patients[11]. Because each patient and surgery is unique and patients have different degrees of EPI, PERT dosing must be tailored to the individual symptoms of a patient.

Data regarding the overall benefits of PERT in total or partial gastrectomy patients are conflicting; while some evidence suggests improved stool consistency[183], weight gain[166], quality of life[184], and reduced steatorrhea and fecal fat excretion[166,185], the same benefits were not observed in all studies. For example, in the double-blind crossover study that showed improvements in stool consistency following a total gastrectomy, there were no beneficial effects of PERT on fecal fat output; however, in the subset of patients with massive steatorrhea, there was a significant reduction in fecal fat excretion following treatment with PERT[183]. The variable trial results

prevent definitive conclusions about the benefits of PERT in fecal fat excretion and steatorrhea following gastric surgery.

CONCLUSION

The prevalence of EPI may be higher in patients with diverse non-pancreatic diseases or pancreatic cancer (Table 2) than has generally been appreciated. EPI should be considered as a possible etiology for any patient with diabetes, celiac disease, IBD, gastrointestinal surgery, or pancreatic cancer who presents with malnutrition, weight loss, and/or abnormal fatty stools (Table 4). In patients with symptomatic EPI, dietary modifications should be implemented and PERT may be initiated and doses should be titrated to achieve the optimal response.

Evidence from clinical research on EPI in less common etiologies is scanty and precludes firm recommendations on management. The lack of studies and evidence-based practices on the association of EPI with the medical conditions discussed herein makes conclusions difficult and needs to be substituted with consensus and clinical practice guidelines derived from future prospective, controlled studies, to confirm or refute these associations. EPI is a serious condition that, once confirmed and regardless of the cause, requires PERT treatment to prevent devastating, sometimes fatal, nutritional complications associated with untreated maldigestion and malabsorption. Further studies are needed to define the association of EPI with these conditions and to support recommendations on the timing of diagnostic testing and initiation of PERT.

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MINIREVIEWS

Radiofrequency ablation for hepatic hemangiomas: A consensus from a Chinese panel of experts

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Abstract

Recent studies have shown that radiofrequency (RF) ablation therapy is a safe, feasible, and effective procedure for hepatic hemangiomas, even huge hepatic hemangiomas. RF ablation has the following advantages in the treatment of hepatic hemangiomas: minimal invasiveness, definite efficacy, high safety, fast recovery, relatively simple operation, and wide applicability. It is necessary to formulate a widely accepted consensus among the experts in China who have extensive expertise and experience in the treatment of hepatic hemangiomas using RF ablation, which is important to standardize the application of RF ablation for the management of hepatic hemangiomas, regarding the selection of patients with suitable indications to receive RF ablation treatment, the technical details of the techniques, therapeutic effect evaluations, management of complications, *etc*. A final consensus by a Chinese panel of experts who have the expertise of using RF ablation to treat hepatic hemangiomas was reached by means of literature review, comprehensive discussion, and draft approval.

Key words: Hepatic hemangiomas; Radiofrequency ablation; Consensus

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Core tip: Recent studies have shown that radiofrequency (RF) ablation therapy is a safe, feasible, and effective procedure for hepatic hemangiomas. It is necessary to formulate a widely accepted consensus among the experts in China who have extensive expertise and experience in the treatment of hepatic hemangiomas using RF ablation, which is important to standardize the application of RF ablation for the management of hepatic hemangiomas.

Gao J, Fan RF, Yang JY, Cui Y, Ji JS, Ma KS, Li XL, Zhang L, Xu CL, Kong XL, Ke S, Ding XM, Wang SH, Yang MM, Song JJ, Zhai B, Nin CM, Guo SG, Xin ZH, Lu J, Dong YH, Zhu HQ, Sun WB. Radiofrequency ablation for hepatic hemangiomas: A consensus from a Chinese panel of experts. *World J Gastroenterol* 2017; 23(39): 7077-7086 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7077.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7077

INTRODUCTION

Hepatic hemangioma is the most common benign tumor of the human liver, with an incidence of 0.4% to 20% in the general population and a prevalence of 0.4% to 7.3% incidentally found in autopsy[1-5]. Most incidentally identified and asymptomatic hepatic hemangiomas do not need medical interventions. However, therapies are warranted for giant hepatic hemangiomas (⩾5 cm) that cause significant symptoms or the peripherally located hemangiomas posing the risk of life-threatening spontaneous rupture and hemorrhage[6-10].

Hepatic hemangiomas have been treated with a wide spectrum of therapies. Traditionally, surgical resection and surgical enucleation are the mostly used treatments of choice[6-10]. Minimally invasive therapies for hepatic hemangioma include transcatheter arterial embolization (TAE)[11-14], radiation therapy[15,16], and radiofrequency (RF) ablation[17-37]. Orthotopic liver transplantation has been performed as the treatment choice in rare circumstances[38]. In recent years, RF ablation has been increasingly used for managing hepatic hemangiomas due to its unique advantages compared with other therapies, such as minimal invasiveness, low cost, low incidence of complications, short duration of hospital stay, and increased patient compliance[17-37]. So far, 501 patients with hemangiomas treated by RF ablation have been reported in the literature[17-37]. A widely accepted consensus or guideline, embracing the selection of patients with suitable indications to receive RF ablation treatment, the application of the techniques, therapeutic effect evaluations, and management of complications, is needed to standardize the management of hepatic hemangiomas using RF ablation.

CONSENSUS DEVELOPMENT

The literature search was performed with an inclusion of published articles during the period from 2003 to 2017. The searched database is Medline. Medical subject headings and free-text words were used for searches, including radiofrequency ablation and hepatic hemangioma. Twenty-one original research papers pertinent to RF ablation treatment for hepatic hemangiomas were included. Of the 21 included papers (501 patients), 13 (465 patients) came from China, including ten in English and three in Chinese[17-37]; two came from South Korea, in which 25 patients with hepatic hemangiomas underwent ultrasound-guided percutaneous RF ablation[21,25]; and Western experience contributed six case reports including 11 patients on the RF ablation for hepatic hemangiomas[18,23,24,26,30,36]. Studies were reviewed and selected for further screening analyses and for subsequent consensus studies.

Base on a comprehensive appraisal of the published original research articles related to RF ablation for hepatic hemangiomas, a Chinese panel of experts was contacted and convened by electronic mails and/or phone. The recruitment of the experts was determined based on the following comprehensive criteria: the number of reported cases using RF ablation for hepatic hemangiomas and the impact of the published articles. All the experts work in well-known, high-volume centers, and their clinical experiences are well documented in scientific papers. They reviewed both the literature and their institutional experience. The draft was completed by the corresponding author and his team and then was circulated to all participants for comments. The revised version was redistributed to the experts for approval or further comments. After more than nine months of electronic mail or, on occasion, face-to-face discussions, the final version of consensus was formulated.

OVERVIEW OF HEPATIC HEMANGIOMAS

Pathology and clinical features

Hepatic hemangioma is usually a solitary tumor mass, although multiple lesions may be present in both hepatic lobes in up to 40% of the patients. Hepatic hemangiomas include cavernous hemangioma, sclerosing hemangioma, hemangioendothelioma, and capillary hemangioma. It consists of blood-filled cavities fed by the hepatic arterial circulation, with walls lined by a single layer of endothelial cells, and manifests as a veritable chaotic entanglement of distorted blood vessels confined to a region as small as a few milliliters and as large as 10 cm, 20 cm, and even 40 cm[1-3].

According to the diameter of hepatic hemangioma, it could be divided into three categories: small (<5 cm), giant (5-9.9 cm), and huge (≥10 cm). Giant hemangiomas are also defined by a diameter larger than 4 cm in the literature[1-5].

Diagnosis

In most situations, hepatic hemangiomas (especially when smaller than 4 cm) do not show any signs and/or symptoms, most likely being discovered incidentally during imaging investigations for other unrelated conditions. However, a few patients may present a wide variety of non-specific clinical symptoms such as pain in the right upper abdomen, decreased appetite, premature satiation sensation, nausea, vomiting, and abdominal discomfort such as sense of fullness and postprandial bloating (early or late). Spontaneous or traumatic rupture is the most severe complication. This has a catastrophic outcome if not promptly managed, and is the reason why correct diagnosis and management are extremely important[1-3].

Hepatic hemangiomas can be specifically diagnosed by ultrasonography (US) or contrast-enhanced US, contrast-enhanced computed tomography (CT), and magnetic resonance imaging (MRI) because of their typical imaging characteristics[4]. On US images, hepatic hemangiomas present as a homogeneous, round or oval lesion with well-defined hyperechogenicity, and likelihood of posterior acoustic enhancement. Other imaging techniques, such as contrast-enhanced CT or MRI, are recommended for confirmation in case of inconclusive ultrasonographic results, or if a giant hemangioma requires treatment[4,5]. The typical hemangioma appears on CT or MRI scans as a hypointense, well-defined lesion, which after contrast injection shows peripheral nodular enhancement with progressive homogeneous centripetal filling.

TRADITIONAL TREATMENT OF HEPATIC HEMANGIOMAS

Surgical procedure used to be the first choice of treatment for hepatic hemangiomas, including hemangioma enucleation, lobectomy (segmentectomy) or partial hepatectomy, and tumor suture or ligation[6-10]. Liver transplantation is used for rare cases which need medical intervention but the tumor is too large to undergo surgical resection, ruptures, or has hemorrhage combined with Kasabach-Merritt syndrome[38]. Surgical resection is rather invasive and associated with relatively high risks of perioperative morbidity (27%), mortality (3%) and long hospitalization[6-10].

Radiation therapy can destroy the endothelial cells and smooth muscle cells of hepatic hemangiomas, consequently leading to thrombosis, necrosis, and fibrosis inside the tumors, which provides partial reduction in hemangioma size and relief of symptoms. Currently, radiation therapy is used rarely in hepatic hemangioma because it is not considered as a

curative therapy. Moveover, it may have the risks of radiation hepatitis, veno-occlusive disease, and hepatic neoplasia[15,16].

TAE has been undertaken as an effective alternative treatment for managing hepatic hemangioma. Before TAE, hepatic arterial angiography is carried out to assess the blood supply to the tumor, tumor size, tumor number, tumor position, and intrahepatic vascular variants. The superselective catheterization of the hepatic arteries leading to the tumor body is followed by embolizing the tumor vessels using an embolization agent mixed with pingyangmycin or bleomycin. With the destruction of endothelial cells lining the blood sinus, thrombosis is formed to block the blood supply to the body of hemangioma. However, TAE is not considered as curative because recurrence is common due to vascular recanalization[34]. Moreover, the treatment may have disastrous complications, such as systemic embolization, biliary damage, and hepatic rupture as the size of tumors increases[11,12]. Several reports have advocated TAE as a temporizing and auxiliary preoperative procedure for huge hepatic hemangioma or spontaneously ruptured hemangiomas to decrease the risk of surgery[11-14,34].

HISTORY OF RF ABLATION FOR HEPATIC HEMANGIOMAS

RF ablation is performed by using RF-induced thermal energy to damage the endothelial lining vascular structures as a result of promoting thrombosis[18], to induce necrotic coagulation[19], as well as to destruct erythrocytes and cause vascular smooth muscle cell disappearance and fibrosis in the ablated zone[19].

In 2003, Cui *et al*[17] reported the first cohort of 12 patients who received the percutaneous RF ablation treatment for 15 hepatic hemangiomas (2.5-9.5 cm) under US guidance. In 2004, Zagoria *et al*[18] reported the successful treatment of a symptomatic hepatic hemangioma (5.0 cm) using percutaneous RF ablation under CT guidance. A few more studies show that percutaneous RF ablation therapy is a safe, minimally invasive, and effective locoregional treatment for selected patients with hepatic hemangiomas[21,23]. In 2006, Fan *et al*[20] reported the use of laparoscopic RF ablation therapy for treating 21 patients with 50 hepatic hemangiomas (5.5 cm ± 2.0 cm) located on the surface of the liver or adjacent to the gallbladder. The results showed that laparoscopic RF ablation therapy is a safe, feasible, and effective treatment option for patients with extrorse hemangiomas. In 2016, a prospective study demonstrated the benefits and disadvantages of laparoscopic RF ablation as compared with surgical resection for managing hepatic hemangioma[35]. Sixty-six patients with symptomatic-enlarging hepatic hemangiomas (4 cm $\leqslant$ diameter < 10 cm) either underwent laparoscopic RF ablation (n = 32) or open resection (n = 34). Laparoscopic RF ablation was associated with significantly shorter operative time (138 min *vs* 201 min, P < 0.001) and less blood loss than open resection. Patients after laparoscopic RF ablation experienced significantly less pain and required less analgesia use, significantly shorter length of hospital stay, and lower hospital cost. The study showed that laparoscopic RF ablation, as a minimal invasive treatment option, is as safe and effective a procedure as open resection for patients with symptomatic-enlarging hepatic hemangiomas smaller than 10 cm.

During the initial period of RF ablation for hepatic hemangiomas, authors always selected and treated hepatic hemangiomas < 10 cm using RF ablation because of the lack of experience. Sporadic cases with huge hepatic hemangiomas were treated by RF ablation. Whether RF ablation should be accepted for treating huge hemangiomas is still in debate because of the requirement of long ablation time. Lengthy ablation time is prone to cause hemolysis, which can lead to the complications of hemoglobinuria, hemolytic jaundice, anemia, or even acute kidney injury (AKI)[25-36]. The incidence of complications post RF ablation is proportionally associated with the size of hemangiomas and the ablation time[28-36]. Park *et al*[25] described the 100% ablation of 10 hepatic hemangiomas larger than 5 cm but less than 10 cm and an ablation in 60% (3/5) of hepatic hemangiomas $\geqslant$ 10 cm. Hence, they drew a conclusion that the best indication for RF ablation was giant hepatic hemangiomas, but huge hepatic hemangiomas were the comparative contraindication. Gao *et al*[28] reported the same technical difficulties when they treated 17 huge hemangiomas $\geqslant$ 10 cm in 16 patients with RF ablation using cluster electrodes. In their study a high rate of complete ablation (82.4%, 14/17) was achieved, but ablation-related complications were seen in all the 16 patients with hemangiomas $\geqslant$ 10 cm, including significant systemic inflammatory responses and acute respiratory distress syndrome (ARDS, grade Ⅳ)[39]. Gao *et al*[31] adopted two approaches to treat 21 large hemangiomas in 21 patients with the expectation of lessening the incidence of complications and achieving a higher success rate: (1) using cool-tip cluster electrodes; and (2) closely monitoring the patient's temperature and hemoglobinuria to warrant a termination of the procedure if the temperature exceeds 39 ℃ or signs of hemoglobinuria appeared. Complete ablation was achieved in 90.5% (19/21) of cases and ablation-related complications reduced to 47.6% (10/21). According to the Dindo-Clavien classification for complications[39], all the complications were grade Ⅰ (including hemoglobinuria in ten cases,

Table 1 Indications and contraindications of radiofrequency ablation for hepatic hemangiomas

Indications
The maximum diameter of hemangiomas > 5 cm
Tumor gaining an enlargement of more than 1 cm within 2 yr
Persistent hemangioma-related abdominal pain or discomfort
Consent to receive the RF ablation
Contraindications
Severe bleeding tendency, platelets < 50 × 10^9/L, international normalized ratio > 1.5, severe platelet function disorders (prothrombin time >18 s and prothrombin activity < 40%)
Malignant tumors
Kasabach-Merritt syndrome
Infection, especially biliary system inflammation
Low immune function
Severe primary organ failure such as the liver, kidney, heart, lung and/or brain

fever in four cases, hemolytic jaundice in three cases, anemia in one case, and elevated serum transaminase in four cases). Eighteen patients required one session and three patients with hemangiomas ⩾ 14.0 cm required two sessions of RF ablation. This study by Gao *et al*[31] showed that RF ablation for huge hepatic hemangiomas is minimally invasive, safe, and effective. Other studies reported the similar results[27,30].

INDICATIONS AND CONTRAINDICATIONS FOR RF ABLATION FOR HEPATIC HEMANGIOMAS

Indications

Indications include: the maximum diameter of hemangiomas > 5 cm; on regular imaging follow-up, tumor gaining an enlargement of more than 1 cm within 2 years; persistent hemangioma-related abdominal pain or discomfort with the definite exclusion of other gastrointestinal diseases which cause the epigastric pain *via* gastroscopy examinations; patients' decline to receive surgical treatment but with the consent to receive the RF ablation (Table 1)[25-34].

Contraindications

Contraindications include: patients with severe bleeding tendency, platelets < 50 × 10^9/L, severe platelet function disorders (prothrombin time > 18 s and prothrombin activity < 40%), international normalized ratio > 1.5, malignant tumors, Kasabach-Merritt syndrome, infection, especially biliary system inflammation, low immune function, and severe primary organ failure such as the liver, kidney, heart, lung and/or brain[28-34]. Anticoagulation and/or antiplatelet drugs should be discontinued at least 5-7 d prior to ablation (Table 1).

ANESTHESIA PROTOCOLS

For percutaneous procedures, general anesthesia is recommended to prevent pain and discomfort during RF procedure. Controlled ventilation would reduce ablation attempts and increase the success rate while RF ablation is performed for patients under general anesthesia[31-35].

PROCEDURES OF RF ABLATION

RF ablation for hepatic hemangiomas can be performed *via* a percutaneous, laparotomy or laparoscopic approach. The diversity of approaches helps extend the scope of treatment indications[25-34].

Hepatic hemangiomas deeply located in liver parenchyma are suited to be treated by percutaneous CT-guided RF ablation. After induction of general anesthesia, patients are placed in a supine position. Grounding is achieved by attaching two pads to the patient's thighs. The skin entry point of the RF electrodes is determined by the guidance of CT imaging. Under the monitoring and guidance of CT, the RF electrodes are percutaneously inserted through the liver to target the tumor. After CT images confirm the acting tip of the RF electrode is located in the tumor center, RF procedure is performed[28].

Subcapsular hepatic hemangioma is suitable to be treated *via* a laparoscopic approach using US guidance[20,33,35]. Under general anesthesia, patients were placed in a supine position. After a pneumoperitoneum (CO_2 at 12 mmHg) is established, a thorough intraperitoneal exploration with a 30° laparoscope through a 10-mm umbilical port is performed. Another 10 mm subxiphoid port is created at the midline of abdomen and an additional 10 mm right or left lateral subcostal port is placed if needed, depending on the location of the hepatic hemangiomas. Under US guidance, the RF electrodes are introduced into the peritoneal cavity through the subcostal abdominal wall under the direct laparoscopic view and deployed into the tumor. The RF procedure is monitored by intraoperative US, which can increase the ability to guide the RF electrode placement and evaluate the efficacy of ablation. After ablation, the ablated lesion became hyperechoic because of outgassing from heated tissues. Laparoscopic biopsy of liver lesions before the ablation is not needed with the consideration of avoiding unnecessary bleeding. For the patients with gallbladder stones or simple hepatic cysts, laparoscopic cholecystectomy (LC) or deroofing of the hepatic cysts may be performed during ablation. LC had to be performed beforehand if the lesions are encroaching on the gallbladder fossa.

Laparotomy is more invasive than percutaneous and laparoscopic approaches. With the increasing

experiences in RF ablation and the improvement of laparoscopic techniques, laparotomy is only used for an alternative approach. In case of unexpected incidence of uncontrollable bleeding during the procedure *via* percutaneous and laparoscopic approaches, laparotomy needs to be performed for controlling the bleeding efficiently and preventing the occurrence of severe complications.

TECHNICAL DETAILS TO BE NOTED

Common strategies of ablation for hepatic hemangiomas

A transhepatic route for RF electrode placement to target the tumor is recommended to prevent bleeding from the electrode-poking site of hemangiomas. RF ablation is initiated at the treatment point close to the margin of the tumor to minimize the risk of bleeding and the heat sink effect. The heat sink effect refers to cooling by adjacent visible (>1 mm diameter) blood vessels when ablated tissues are heated. Overlapped ablation zones are warranted by repositioning the RF electrodes in the tumor mass repeatedly under the guidance and monitoring of CT or US imaging, ensuring a complete ablation of the tumors. An intratumoral ablation is necessary to minimize injuries to normal liver parenchyma and the needle tract ablation can prevent the incidence of needle puncture-induced bleeding[31].

In the laparoscopic approach, the Pringle maneuver can be used to decrease the heat sink effect[28]. Furthermore, the laparoscopic approach also offers a direct vision of the entire RF procedure, which is helpful to identify and manage bleeding from the puncture site or tumor rupture[31-33].

Based on our experience on treating 76 huge hepatic hemangiomas[31-33], we prefer to use internally cooled cluster electrodes to treat the tumors by taking their advantage of achieving an efficient and much concentrated thermal energy in the tumor tissue, which is expected to reduce the incidence of ablation-related complications. Internally cooled cluster electrodes, for example Cool-tip ones, are straight electrodes without an array of prongs, and the tip is internally cooled by continuous infusion of cold saline. Thus, the temperature of the electrode itself is not extremely high, which is helpful to avoid the instant charring at the tissue around the probe and reduce tissue impedance. The efficient heat deposition creates a larger ablated zone within a shorter period of time. Another advantage of internally cooled electrodes is that they induce a sustaining high temperature in the tumor with limited "heat sink effect" caused by blood flow in the adjacent vessels, which can enhance the ablation effectiveness for the tumor tissue abutting the vessel. Moreover, a specially-designed Cool-tip electrode is more visible under the guidance of CT or US imaging, which allows the accurate placement of the electrodes in the tumor without injuring the adjacent organs[31].

Special strategies of ablation for huge hepatic hemangiomas

Larger tumor size is a risk factor for ablation-induced complications. For huge hemangiomas, a few precautions should be taken to lessen the risk of severe complications and enable a successful treatment.

It is unnecessary to achieve a complete ablation of a large tumor using one ablation session if the patient shows the sign of elevating body temperature and hemolysis, considering the severe risk of hemolysis-induced AKI[36]. A repeat RF ablation session can be rescheduled to obtain the expected treatment effect[30].

RF ablation combined with TAE could be an option to achieve a synergistic treatment effect for huge hepatic hemangiomas because the blockade of tumor blood supply by TAE can decrease the lesion size to some extent, facilitating the subsequent RF ablation and minimizing the risk of ablation-related complications. Additionally, the hyper-attenuation of iodized oil deposited in the tumor on CT images can facilitate the targeting placement of the RF electrodes in the tumor and thus increase the success rate of complete ablation[34].

Laparoscopic resection of hemangioma boosted by intratumoral coagulation by RF ablation is a safe, effective option for treating huge hepatic subcapsular hemangiomas with low loss of blood and minimal complications. This technique lies in the advantage that a completely coagulated zone created by sequential RF ablation along the dissection margin warrants the successful removal of the tumor tissue without occluding the hepatic vessels before the tumor dissection. Compared with treating the tumor using RF ablation alone, this technique involves the ablation of the tumor tissue at the resection margin rather than the total hepatic hemangioma, thus shortens the ablation time and avoids the incidence of severe ablation-related complications[37].

COMPLICATIONS

Hemorrhage of hepatic hemangioma

Bleeding at the electrode entry site: Due to the hypervascular nature of hepatic hemangioma and intratumoral high blood pressure, there is high risk of bleeding from the puncture site of the tumor. To prevent bleeding, the electrode needs to be advanced *via* a transhepatic approach to target the tumor and a needle tract ablation needs to be performed while withdrawing the electrode. When the RF electrode

placement is performed *via* laparotomy or laparoscopic approach, RF ablation should be launched from the exterior margin of the tumor from the beginning of a lower RF power[31-34].

Rupture of hepatic hemangioma: Abrupt rupture of hepatic hemangioma could result in massive hemorrhage. For hemangiomas located in the surface of the liver, an improper advancement of the RF electrode directly through the tumor will lose the protection of liver parenchyma. In this context, the RF electrode puncture could induce massive bleeding from the electrode puncture site. On the other hand, when an extra-high RF output power is applied at the beginning of RF ablation, a rapid increase of intratumoral temperature and pressure will induce the burst force in the tumor, which will cause an abrupt rupture of the tumor and life-threatening bleeding. Under the direct view of laparoscopy, hemostasis procedure can be applied to the bleeding site such as applying ablation to stop bleeding. In case the bleeding is not controlled by these measures, a conversion to open surgery is advocated to achieve hemostasis.

Puncture injury to adjacent organs

The accidental injuries to the adjacent organs including the gallbladder, gastrointestinal organs, kidney, diaphragm, lungs, and heart need to avoid. While using multipolar expandable electrodes, the deployment of each prong of the electrodes need to be verified carefully[28,40]. The high visibility of Cool-tip electrodes on CT or US images facilitates the insertion of the electrodes in the tumor without causing accidental injury to adjacent organs[31].

Thermal injury to the pleura and diaphragm

For hepatic hemangiomas situated in the subdiaphragm hepatic dome area, thermal injuries to the diaphragm frequently occur during RF ablation *via* the percutaneous approach[32]. The thermal injury to the diaphragm may manifest as the shoulder pain because the diaphragm and the shoulder skin are innervated by the same phrenic nerves arising from nerve roots C3, C4, and C5[32,41]. Diaphragmatic perforation and herniation were reported as major complications of RF ablation for hepatic tumors abutting the diaphragm in nine cases[41]. RF ablation injuries more frequently occur in cases with hepatic hemangiomas than hepatic cancers due to the high thermal energy and duration needed for large-sized hemangiomas. RF ablation *via* a laparoscopic approach can minimize the risk of diaphragm injury because the pneumoperitoneum can elevate the diaphragm to increases the operation space. Therefore, laparoscopic RF ablation therapy should be used as the first-line treatment for hepatic hemangiomas abutting the diaphragm[32].

Thermal injury to the lung

A patient with two huge hemangiomas in the right lobe (16.0 cm and 11.0 cm), treated with percutaneous RF ablation, developed ARDS immediately after an ablative time of total 250 min[28]. The major complication was resolved by conservative treatment[28]. The pathogenesis of ARDS needs to be investigated, and it is speculated that RF ablation of large quantity of tumor tissues induces significant systemic inflammatory responses[42-45]. Pre-ablation TAE as an adjuvant therapy or multiple ablation sessions is recommended to prevent the risk of ARDS[31,34].

Hemolysis

Hemolysis and anemia: RF ablation poses a risk of predisposing huge hypervascular hepatic hemangiomas to the severe complication of hemolysis. Hemolysis can lead to various degrees of hemoglobinuria, hemolytic jaundice, anemia, or even AKI[28-31,36].

AKI: AKI caused by ablation-induced hemolysis in patients with huge hepatic hemangiomas has been reported[36]. Hemoglobin is released upon erythrocyte destruction and is filtered by the glomerulus into the urinary space. In the urinary space, hemoglobin is degraded and releases heme pigments which can cause tubular injury. Furthermore, volume depletion enhances both vasoconstriction and the formation of obstructing casts, and also is of critical importance for the development of heme-induced AKI[36].

Patients with huge hepatic hemangiomas should be sufficiently hydrated before RF ablation and during the procedure. When any signs or symptoms indicating hemolysis emerge in the course of RF ablation, such as rising body temperature and hemoglobinuria, the RF procedure should be terminated and a repeat RF ablation treatment may need to be rescheduled based on a comprehensive evaluation of the tumor[31].

Other complications

Except for relatively severe RF ablation-related complications, some minor complications may take place, such as liver damage, fever, and skin burn injury at the site where the grounding pad is attached. The ablation-induced self-limiting liver injury can resolve within a short period of time without the need of any medication. The necrotic tissue of tumors can cause unspecific inflammatory reaction and mild hyperthermia. In case that the body temperature is higher than 39 ℃, physical cooling can be used to alleviate the discomfort of hyperthermia. To prevent the burn injury to the skin in patients receiving long time RF ablation, multiple grounding pads can be

Table 2 Ablation-related complications and preventive measures

Complication	Preventive measures
Bleeding at the electrode entry site	The electrode needs to be advanced *via* a transhepatic approach to target the tumor and a needle tract ablation needs to be performed while withdrawing the electrode. Radiofrequency (RF) ablation should be launched from the exterior margin of the tumor from the beginning of a lower RF power.
Rupture of hepatic hemangioma	Under the direct view of laparoscopy, hemostasis procedure can be applied to the bleeding site such as applying ablation to stop the bleeding. If it fails, a conversion to open surgery is advocated to achieve the hemostasis.
Puncture injury to adjacent organs	The high visibility of Cool-tip electrodes on computed tomography or ultrasonography images facilitates the insertion of the electrodes in the tumor without causing accidental injury to adjacent organs.
Thermal injury to the pleura and diaphragm	Laparoscopic RF ablation should be used as the first-line treatment for hepatic hemangiomas abutting the diaphragm.
Thermal injury to the lung	Pre-ablation transcatheter arterial embolization as an adjuvant therapy or multiple ablation sessions is recommended to prevent the risk of acute respiratory distress syndrome.
Hemolysis	The patients should be sufficiently hydrated before RF ablation and during the procedure. When any signs or symptoms indicating the hemolysis emerge in the course of ablation, the RF procedure should be terminated and a repeat RF ablation treatment may need to be rescheduled based on a comprehensive evaluation of the tumor.
Liver damage	The ablation-induced liver injury can resolve spontaneously without the need of any medication.
Fever	Physical cooling can be used to alleviate the discomfort of hyperthermia.
Skin burn injury	Multiple grounding pads can be applied or ice pad can be used to cool the skin with the contact of grounding pad.

applied or ice pad can be used to cool the skin with the contact of grounding pad[31-33]. The ablation-related complications and preventive measures are listed in Table 2.

POST-TREATMENT EVALUATION

Contrast-enhanced CT or MRI can be used to evaluate the therapeutic effect of RF ablation for tumors one month after the treatment. On contrast-enhanced CT or MRI, complete ablation is defined as no nodular or irregular enhancement adjacent to the ablation zone and incomplete ablation is defined as irregular peripheral-enhanced foci in the ablation zone. In cases of complete ablation, subsequent CT or MRI examinations are repeated at a 6-mo interval[31-33]. Repeated RF ablation procedures are not needed unless the residual tumor is growing significantly or posing a risk of spontaneous rupture.

CONCLUSION

RF ablation therapy is a safe, feasible, and effective procedure for hepatic hemangiomas, even for huge hepatic hemangiomas. RF ablation has the following advantages in the treatment of hepatic hemangiomas: minimal invasiveness, definite efficacy, high safety, fast recovery, relatively simple operation, and wide applicability.

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ORIGINAL ARTICLE

Basic Study

Detection of *KRAS* G12D in colorectal cancer stool by droplet digital PCR

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Abstract

AIM

To assess *KRAS* G12D mutation detection by droplet digital PCR (ddPCR) in stool-derived DNA from colorectal cancer (CRC) patients.

METHODS

In this study, tumor tissue and stool samples were collected from 70 patients with stage Ⅰ-Ⅳ CRC diagnosed by preoperative biopsy. *KRAS* mutational status was determined by pyrosequencing analysis of DNA obtained from formalin-fixed paraffin-embedded (FFPE) tumor tissues. The *KRAS* G12D mutation was then analyzed by ddPCR in FFPE tumors and stool-derived DNA from patients with this point mutation. Wild-type (WT) tumors, as determined by pyrosequencing, were included as controls; analysis of FFPE tissue and stool-derived DNA by ddPCR was performed for these patients as well.

RESULTS

Among the total 70 patients included, *KRAS* mutations were detected by pyrosequencing in 32 (45.71%), whereas 38 (54.29%) had WT tumors. The frequency of *KRAS* mutations was higher in left-sided tumors (11 located in the right colon, 15 in the left, and 6 in the rectum). The predominant point mutation was *KRAS* G12D (14.29%, n = 10), which was more frequent in early-stage tumors (I-IIA, n = 7). In agreement with pyrosequencing results, the *KRAS* G12D mutation was detected by ddPCR in FFPE tumor-derived DNA, and only a residual number of mutated copies was found in WT controls. The *KRAS* G12D mutation was also detected in stool-derived DNA in 80% of all fecal samples from CRC patients with this point mutation.

CONCLUSION

ddPCR is a reliable and sensitive method to analyze *KRAS* G12D mutation in stool-derived DNA from CRC patients, especially at early stages. This non-invasive approach is potentially applicable to other relevant biomarkers for CRC management.

Key words: Droplet digital PCR; *KRAS*; Stool; Formalin-fixed paraffin-embedded; Pyrosequencing; Colorectal cancer

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Core tip: The potential of droplet digital PCR (ddPCR) to detect *KRAS* G12D mutation in stool DNA from colorectal cancer (CRC) patients was examined as a proof-of-concept for the applicability of this technology to study DNA biomarkers in stool-derived DNA. It was shown that *KRAS* G12D detection in stool-derived DNA from CRC patients by ddPCR is feasible and provides comparable results to the analysis of formalin-fixed paraffin-embedded tissue by pyrosequencing. These results suggest that analysis of *KRAS* mutations and other molecular biomarkers in stool by ddPCR could represent a complementary non-invasive approach to standard screening tests for CRC.

Olmedillas-López S, Lévano-Linares DC, Aúz Alexandre CL, Vega-Clemente L, León Sánchez E, Villagrasa A, Ruíz-Tovar J, García-Arranz M, García-Olmo D. Detection of *KRAS* G12D in colorectal cancer stool by droplet digital PCR. *World J Gastroenterol* 2017; 23(39): 7087-7097 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7087.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7087

INTRODUCTION

Colorectal cancer (CRC) is the second and third most common cancer in women and men, respectively, with more than one million cases diagnosed each year worldwide[1]. Current therapeutic options have increased overall survival (OS), but have also made clinical decisions more complex, especially in patients with an initial diagnosis of metastatic colorectal cancer (mCRC)[2]. Biological agents targeting the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, used either as monotherapy or in combination with standard chemotherapy, are indicated in mCRC patients with *RAS* (*KRAS* and *NRAS*) or *KRAS* wild-type (WT) tumors, respectively[3,4]. These strategies significantly improve progression-free survival (PFS) and OS in *KRAS* WT mCRC patients depending on the therapeutic regimen applied (chemotherapy and line of treatment)[2,5].

KRAS oncogene mutations, mostly found in codons 12 and 13[6], have been described in approximately 30%-40% of CRC tumors[7-9]. These mutations are associated with absence of response to therapy with biological agents[10,11] and have been correlated with worse prognosis[12,13]. In fact, in Europe and the United States, monoclonal antibody-based therapy has been restricted to patients with WT tumors[14], as when administered in association with standard chemotherapy, this treatment may result in an increased cost and toxicity[10]. Drug resistance can occur months after start of combined therapy, likely due to intratumoral heterogeneity and proliferation of small sub-groups of clonal cells carrying resistance mutations that are difficult to identify by most of the currently applied methods[14,15]. Therefore, highly sensitive and specific methodologies are needed to detect and quantify molecular markers, including *KRAS* mutations, which play a pivotal role in early detection and clinical management of CRC patients.

Droplet digital PCR (ddPCR) is increasingly seen as one of the most powerful techniques to accurately detect a wide variety of genetic alterations in many cancer types. These molecular biomarkers have been analyzed by ddPCR in different body fluids such as blood, urine, cerebrospinal fluid, pleural effusions, ascites and sputum (reviewed in[16]).

Stool-derived DNA is a potential non-invasive alternative source of DNA for tumor genotyping in CRC due to the high rate of exfoliation of tumor cells into the bowel lumen[17]. Digital PCR was first described in 1999 by Vogelstein and Kinzler in a study aimed at identifying *KRAS* in DNA obtained from fecal samples of CRC patients[18]. Based on the isolation of single molecules by limiting dilution of DNA samples and individual amplification by PCR, mutations were detected using fluorescent probes. However, this methodology was found to be quite laborious and difficult to translate into clinical practice[19]. The introduction of new instrumentation involving nanofluidic devices and improved emulsion chemistries has allowed for more widespread use of digital PCR, giving way to the current commercially available platforms[19]; of these, emulsion-based ddPCR has undergone huge growth in cancer research. In fact, a recent study has investigated the application of ddPCR to quantify mRNA biomarkers in stool from patients with CRC as a potential non-invasive screening test[17]. Thus, analysis of DNA obtained from fecal samples in patients with CRC may complement currently used procedures for diagnosis and disease follow-up.

In our experience, ddPCR has shown high sensitivity for detection of mutated *KRAS* alleles in circulating cell-free DNA (cfDNA) in plasma from CRC patients[20]. However, early-stage patients sometimes have undetectable levels of circulating tumor DNA (ctDNA)[20,21]. The aim of this study was to evaluate the feasibility of *KRAS* G12D mutation detection in stool-derived DNA from CRC patients by ddPCR, including early-stage patients.

MATERIALS AND METHODS

Patients

Seventy CRC patients were consecutively included in this study from 2014 to 2015 in the Department of General Surgery at Fundación Jiménez Díaz University Hospital (Madrid, Spain). Inclusion criteria were endoscopic histological diagnosis of CRC and eligibility for primary tumor resection with curative intent. Patients with primary tumors located in the rectum who had received prior neoadjuvant treatment were excluded. All subjects signed an informed consent in accordance with a protocol approved by the Ethics Committee for Clinical Research of this institution (PIC 63/2016_FJD).

Fecal sample collection

All fecal samples were collected during hospitalization before surgery without any bowel preparation (with the exception of patient 17, who was subjected to cathartic preparation due to an oversight). Stool samples were collected in sterile containers and stored at -20 ℃ until analysis.

DNA extraction

A total amount of 200-500 mg of fecal sample was used for DNA extraction. DNA was isolated using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from the LS-174T cell line (kindly provided by the Translational Oncology Division, OncoHealth Institute, IIS-FJD, which had previously purchased this cell line from the American Type Culture Collection, ATCC in Manassas, VA, United States) and DNA from peripheral blood mononuclear cells of a healthy donor were extracted with the QIAamp DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA from formalin-fixed paraffin-embedded (FFPE) tumors was extracted using the Cobas DNA Sample Preparation Kit (Roche Molecular Systems, Inc., Branchburg, NJ, United States) following the manufacturer's instructions. The quantity and purity of the DNA obtained was estimated by NanoDrop (ND-2000 UV-Vis Spectrophotometer; Nanodrop Technologies Inc., Waltham, MA, United States).

Mutation detection in FFPE tumor samples by pyrosequencing

Quantitative analysis of *KRAS* mutations was performed by pyrosequencing in accordance with routine practice at the Department of Pathology at Fundación Jiménez Díaz University Hospital, using the CE-IVD marked Therascreen *KRAS* Pyro kit (Qiagen), according to the manufacturer's protocols. Each PCR product was analyzed by pyrosequencing using the Therascreen *KRAS* Pyro reagents (Qiagen), Streptavidin Sepharose High Performance (GE Healthcare Bio-Science AB, Uppsala, Sweden), and a PyroMark Q24 instrument (Qiagen).

Mutation detection in FFPE tumor tissues and stool samples by ddPCR

ddPCR assays were performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, United States) with the Prime-PCR™ ddPCR™ Mutation Detection Assay Kit (Bio-Rad); amplicon size was 57 bp. DNA from LS-174T, a human colon adenocarcinoma *KRAS* G12D heterozygous cell line, was used as a positive control. *KRAS* WT control DNA was obtained from peripheral blood mononuclear cells of a healthy donor. Background was measured by adding water to the reaction mixture instead of

DNA. The PCR reaction mixture (20 μL) contained 10 μL of ddPCR Supermix (no dUTP) for probes, 1 μL of each primer/probe mix (target and reference, labeled with HEX and FAM fluorophores, respectively), and 2-8 μL of stool-extracted DNA. Different amounts of stool-derived DNA were assayed per sample, as the proportion of human DNA with respect to bacterial DNA was unknown and could vary among patients. A total amount of 100 ng of cell-derived control DNA was added per well. In case of DNA from FFPE tumors, 50 ng per well was used. Thermal cycling consisted of 10 min at 95 ℃, 40 cycles of 94 ℃ for 30 s, and 55 ℃ for 60 s. Results were analyzed using QuantaSoft v.1.7 software (Bio-Rad) and reported as number of copies per 20 μL reaction as well as copies per ng of DNA. Three to four replicates of each stool sample were analyzed. FFPE tumors and controls were assayed in duplicate.

Statistical analysis

A non-parametric Mann-Whitney test for significance was performed using R software.

RESULTS

Patient characteristics

A total of 70 stool and tissue samples from CRC patients were collected and included in the study. All patients were classified according to the distance between the primary tumor and anal margin (cm) reported in the preoperative colonoscopy. Forty-one (58.57%) were male, with a median age of 73 years. Only 5 (7.14%) had a diagnosis of mCRC at baseline.

The patients included in this study were representative of all tumor locations, with 28 (40%) located in the right colon, 34 (48.57%) in the left colon, and 8 (11.43%) in the rectum. Interestingly, 45 (64.29%) patients were diagnosed at an early stage (I-IIA). Clinical and pathological features are summarized in Table 1.

KRAS mutations in FFPE tumor samples analyzed by pyrosequencing

KRAS mutations were found in the tumors of 32 patients (45.71%), including 11 in the right colon, 15 in the left, and 6 in the rectum. Most mutations were located at codon 12 (n = 17, 53.12%) or codon 13 (n = 6, 18.75%). The most prevalent mutation was G12D (n = 10, 14.29%). Two were located in the right colon, 6 in the left colon, and 2 in the rectum. This mutation was found more frequently in early-stage tumors (I-IIA, n = 7). The incidence of the different types of mutations is shown in Table 2.

KRAS G12D detection in FFPE tumor samples by ddPCR

FFPE tumors from patients found to have a *KRAS* G12D mutation by pyrosequencing were analyzed using ddPCR. Five CRC patients with WT *KRAS* exon 2 tumors were selected as controls. One control carried a *KRAS* Q61L mutation (exon 3) that did not interfere with our assays. Results obtained from ddPCR analysis of FFPE tumor DNA from these 15 CRC patients were in agreement with pyrosequencing results. A residual number of *KRAS* G12D copies was found in WT tumors. Due to this level of unspecific background signal, mean copies/ng DNA from control patients plus 2 standard deviation (SD) was considered as a threshold for positivity (0.41 copies/ng DNA). All samples from patients with G12D-positive tumors were above this threshold and showed a significantly higher number of mutant copies/ng DNA than patients with WT *KRAS* tumors (median, 106 and 0.19 copies/ng DNA, respectively; P = 0.001). However, the difference in number of WT *KRAS* copies/ng DNA between both groups was not statistically significant (210.00 copies/ng *vs* 208.40 copies/ng DNA, median; P = 0.699).

KRAS G12D detection in stool samples by ddPCR

Subsequently, we analyzed the presence of the *KRAS* G12D mutation by ddPCR analysis of fecal samples from the 10 patients with mutated tumors by pyrosequencing. Stool DNA from 5 patients with tumors carrying the WT *KRAS* exon 2 were also included as controls. A limited number of *KRAS* G12D-positive events were detected in stool DNA from control WT *KRAS* exon 2 samples. Consequently, mean control value plus 2 SD was established as the positivity threshold. Thus, in our study, stool samples were required to contain more than 1.9 copies/20 μL of reaction to be considered positive for the mutation. According to this threshold, which was equivalent to > 3 positive events per sample, the *KRAS* G12D mutation was detected in 8 of 10 patients. Of these 8 positive samples, 6 were from early-stage tumors. Samples from Patients 46 and 64 were considered negative because they had values less than or equal to the positivity threshold. *KRAS* G12D mutation levels in stool samples are shown in Tables 3 and 4. The median number of copies of *KRAS* G12D/20 μL of reaction as well as copies/ng of stool DNA in control patients differed significantly from those with mutated tumors (P = 0.017). The *KRAS* WT sequence was also detected in stool samples of all CRC patients, though there were no significant differences in the median number of *KRAS* WT copies/ng of stool DNA between both groups (P = 0.129).

In summary, the results of pyrosequencing were in 100% agreement with ddPCR analysis in FFPE tissues, whereas ddPCR detected the *KRAS* mutation in 8 out of 10 stool samples.

DISCUSSION

Despite the advances made in CRC research, the

Table 1 Patient characteristics

		Colorectal cancer patients, *n* = 70		
		Right colon, *n* = 28	Left colon, *n* = 34	Rectum, *n* = 8
Sex	Female	13	13	3
	Male	15	21	5
Age (mean)		76.89	72.26	70.63
pT	T1	3	4	2
	T2	6	9	2
	T3	16	18	3
	T4	3	3	1
pN	N0	20	22	8
	N+	8	12	-
Stage	I	9	10	4
	IIa	9	10	3
	IIb	1	1	1
	IIIa	1	3	-
	IIIb	4	5	-
	IIIc	2	2	-
	IV	2	3	-
KRAS status	Mutant	11	15	6
	Wild-type	17	19	2

disease remains a major cause of death worldwide. Recently, the analysis of *KRAS* oncogene mutations has taken on a major prognostic role in CRC clinical management[22] owing to the fact that the presence of these mutations, which have been described in approximately 30%-40% of cases[7,9,23], could determine the absence of response to anti-EGFR therapies and worse outcome in cases of metastatic disease[10-13]. The most frequent *KRAS* mutations are located at codons 12 and 13; of these, G12D and G13D are particularly relevant, representing around 13%-14% and 6%-7% of all cases, respectively[7,23]. Moreover, worse prognosis has been documented among patients with tumors with the G12D mutation[8,23]. In agreement with previous observations, the incidence of *KRAS* G12D mutation in our study population was 14.29%.

Molecular biomarkers in blood and stool may be used as a complementary screening strategy and prognostic tool for the prediction of clinical outcome in patients with or at high risk for CRC[24]. Not only in CRC, but also in many other human malignancies, the analysis of molecular biomarkers in plasma and other body fluids is attracting increasing interest as a highly valuable, non-invasive predictive tool for monitoring disease progression and response to treatment[16]. In CRC, *KRAS* mutations have been analyzed in blood, both in DNA obtained from circulating tumor cells[25] and in cfDNA[26]. *KRAS*-mutation detection by digital PCR has been described using several commercially available platforms[14,27-32], most of which are focused on detecting mutations in plasma. One of these strategies is ddPCR, which has shown a remarkably high sensitivity when detecting these minority *KRAS* alleles present at low levels in plasma DNA.

In a previous study, using ddPCR, we detected the *KRAS* G12V mutation in plasma cfDNA from 9 of 10 patients whose tumors were also mutated[20]. In this study, we found that metastatic patients had a significantly higher number of mutated copies in circulating cfDNA than M0 patients. The only negative sample was obtained from a T1N0M0 patient. These results are in line with other studies: Bettegowda *et al*[21] also reported that ctDNA in plasma increases with disease stage, and only 47% of early-stage patients with a wide variety of cancers had detectable levels of ctDNA. Similarly, Galanopoulos *et al*[26] recently described that the *KRAS* codon 12 mutation rate in cfDNA is significantly higher in CRC patients compared to healthy subjects, though this methodology seems to have limited potential for predicting the existence of premalignant lesions (neoplastic colonic polyps). Taken together, these findings suggest that at early disease stages, levels of mutated copies in circulating cfDNA may be, in some cases, too low for detection. Thus, alternative non-invasive methods are still needed.

Interestingly, in a very recent study, ddPCR was also used to quantify an mRNA biomarker, *ITGA6*, in stool from patients with CRC[17]. Tumor-derived nucleic acids present in stool samples come from the exfoliation of tumor cells of the intestinal mucosa and are a non-invasive, alternative source of genetic material for *KRAS* oncogene mutation screening in CRC patients[33]. Exfoliation of colonocytes into the large bowel lumen is a continuous, naturally-occurring phenomenon that seems to be exacerbated in tumors[34,35]. Thus, colonocyte shedding from malignant lesions is more frequent than from healthy mucosa[36,37]. Hypothetically, DNA from colorectal tumors should be shed into the bowel fecal content before reaching the bloodstream. This would make testing stool DNA for CRC screening more time-sensitive than plasma or other biological fluids[38].

The proof-of-concept study for stool DNA analysis for CRC detection screened 15 point mutations in several genes, including *KRAS*[39]. Subsequently, several case-control and prospective studies[40-42] led to the 2014 approval by the United States Food and Drug Administration of a fecal DNA analysis system called Cologuard™ (Exact Sciences Corporation, Madison, WI, United States) for CRC detection. This system includes an immunochemical assay for human hemoglobin and molecular biomarkers associated with CRC, such as methylation markers (*BMP3* and *NFRG4* gene promoter regions), *KRAS* mutations, and β-actin. The test is based on amplification and detection by Quantitative Allele-specific Real-time Target and Signal

Table 2 *KRAS* mutational status by pyrosequencing

	KRAS wild-type	*KRAS* mutations											
		Codon 12				Codon 13		Others					
		G12D	G12V	G12R	G12S	G13D	G13R	A146V	A146T	A59T	Q61R	Q61H	Q61L
Right colon	17	2	0	1	0	3	1	1	1	0	1	1	0
Left colon	19	6	5	0	0	1	0	1	1	1	0	0	0
Rectum	2	2	0	0	1	1	0	0	0	0	0	1	1
		10	5	1	1	5	1	2	2	1	1	2	1
Total	38 (54.29%)	32 (45.71%)											

Amplification (QuARTS™) technology. However, this system still has limited application in clinical practice due to its elevated cost. Additionally, the technical difficulties of this test include the need for a large volume of stool sample and the high rate of false-positive results, creating a need for more confirmative colonoscopies and additional costs[38]. Further studies evaluating the cost-effectiveness of this test for large-scale population screening are needed[43].

Fecal DNA analysis for mutation detection has also been reported using several digital PCR systems[18,27,44-48], the first of which were the studies carried out by Vogelstein and Kinzler[18,44], which led to the development of BEAMing (named for 'beads, emulsions, amplification, and magnetics')[27]. Other examples are target-enriched multiplex PCR (Tem-PCR)[47], MDHB (multiplex digital PCR coupled with hydrogel bead-array)[46], and MLPA-DABA (multiplex ligation-dependent probe amplification-digital amplification coupled with hydrogel bead-array)[48]. It is worth mentioning that, to date, none of these systems has been further developed and subjected to clinical validation for stool DNA screening.

In our study, DNA from fecal samples of CRC patients was successfully obtained in all cases. Presence of the *KRAS* G12D mutation was determined by pyrosequencing of FFPE tissue as a reference standard. Results of *KRAS* G12D mutation detection in FFPE tumors using ddPCR were in total agreement with pyrosequencing analysis. This was expected, given the fact that ddPCR has been proven to achieve higher sensitivity than pyrosequencing[49]. Once the *KRAS* G12D mutation had been screened in tumor tissues, DNA from stool samples obtained from the same patients prior to surgery was also analyzed. Thus, we were able to detect the *KRAS* G12D mutation in 8 out of 10 stool samples from patients known to carry this mutation in their tumors using both methods. It is noteworthy that 6 of these 8 samples were from early-stage patients (Ⅰ-ⅡA), highlighting the potential of this approach to identify *KRAS* mutations at the initial stages of the disease.

Absorbance at a wavelength of 230 nm has been reported as an indicator of the level of potential PCR inhibitors in fecal samples[50], and it should be noted that the two negative samples showed peak of absorbance at this same wavelength. Thus, the sensitivity of detection in our assay could have been greatly reduced by the presence of PCR inhibitors in these samples.

The sample from Patient 17 is noteworthy for its remarkably high concentration of both mutated and WT copies. It is worth mentioning that this patient was subjected to cathartic preparation prior to sample collection due to an oversight. The rest of the samples were collected without any bowel preparation. We hypothesize that purging could have increased the exfoliation of tumor cells into the bowel lumen. This unexpected observation raises the question of whether bowel preparation could be advisable prior to sample collection to increase the sensitivity of detection in stool screening of *KRAS* mutations by ddPCR.

To our knowledge, this is the first study to evaluate the feasibility of detection of the *KRAS* G12D mutation in stool DNA from CRC patients using this particular ddPCR platform. Our results are in line with the above-mentioned recently published study by Herring *et al*[17], reporting the detection and accurate quantification of an mRNA biomarker in stool from CRC patients using the same ddPCR system. In light of these results, the analysis of CRC biomarkers in stool using ddPCR merits further study in larger cohorts of patients to evaluate the clinical utility of this approach.

We analyzed only the most prevalent *KRAS* mutation (G12D) in our population, as it was the only one with a sufficient number of samples available for analysis (n = 10). Another reason for choosing G12D as a target was that it has the highest incidence in CRC patients worldwide and is associated with poor clinical outcome[8,23]. For lower-incidence mutations, such as G12V, there were too few samples in our study population to provide conclusive results. The analysis of *KRAS* G12D performed in this study represents a proof-of-concept of the feasibility of this strategy as a first step prior to the screening of other relevant hotspot mutations.

This preliminary study demonstrates the capability of ddPCR to detect *KRAS* mutations in stool-derived DNA, acting as a complementary approach to tissue biopsy for tumor genotyping. These results pave the

Table 3 ***KRAS*** **G12D mutation levels in DNA from stool samples of wild-type control patients**

Patient	Tumor location	Tumor stage	G12D							Wild-type						
			Single			Merged				Single			Merged			
			Positive events	Copies/μL	Copies/20 μL reaction	Positive events	Copies/μL	Copies/20 μL reaction	Copies/ng DNA	Positive events	Copies/μL	Copies/20 μL reaction	Positive events	Copies/μL	Copies/20 μL reaction	Copies/ng DNA
53	Left	I	1	0.1	2	3	0.07	1.40	0.011	94	9	180	469	10.50	210.00	1.615
			2	0.17	3.4					12	10.8	216				
			0	0	0					116	9.9	198				
			0	0	0					136	12.1	242				
56	Left	II A	1	0.08	1.6	1	0.02	0.40	0.003	17	1.4	28	76	1.56	31.20	0.240
			0	0	0					25	2.1	42				
			0	0	0					16	1.3	26				
			0	0	0					18	1.4	28				
63	Right	II A	1	0.08	1.6	1	0.02	0.42	0.003	258	20.4	408	957	20.30	406.00	3.123
			0	0	0					222	22.1	442				
			0	0	0					217	19	380				
			0	0	0					260	19.9	398				
71	Right	III B	1	0.08	1.6	3	0.06	1.20	0.009	89	6.7	134	377	7.20	144.00	1.108
			2	0.15	3					104	7.9	158				
			0	0	0					93	6.9	138				
			0	0	0					91	7.3	146				
96	Left	IV	1	0.09	1.8	3	0.06	1.20	0.009	32	3	60	133	2.84	56.80	0.437
			1	0.08	1.6					33	2.6	52				
			1	0.09	1.8					34	3	60				
			0	0	0					34	2.9	58				

way for the ddPCR analysis of other molecular biomarkers of CRC in stool, including other *KRAS*, *NRAS* and *BRAF* mutations. A multiplex assay simultaneously covering all *KRAS* mutations relevant for anti-EGFR therapy decision-making would maximize the benefits and optimize the cost-effectiveness of this approach. Further studies involving larger cohorts of patients and samples collected at different time points throughout the progression of the disease should be performed in order to confirm the prognostic value and economic viability of this tool before implementation in clinical practice.

This study is the first to describe the detection of *KRAS* G12D mutation in stool-derived DNA from CRC patients using a commercially available ddPCR platform, including individuals at early stages of the disease. We hypothesized that ddPCR could be a reliable and sensitive method to analyze *KRAS* mutations in stool-derived DNA providing reproducible and accurate results. Our findings suggest this approach, which is fast, simple and affordable, could be adaptable to the detection of other clinically relevant molecular biomarkers for CRC management. These advantages with respect to other previously described stool-based strategies, together with instrumentation and protocols easily adoptable by any lab, make our approach more feasible for implementation into routine clinical practice. In light of our results, it could be proposed that biomarker analysis by ddPCR of stool samples may complement current CRC screening methods; stool-derived nucleic acid testing by ddPCR offers an alternative tool to tissue genotyping and blood-based biomarker quantification, being less invasive than the former and, probably, more time-sensitive than

Table 4 ***KRAS*** **G12D mutation levels in DNA from stool samples of patients with** ***KRAS*** **G12D mutated tumors**

			G12D							Wild-type						
			Single			Merged				single			Merged			
Patient	Tumor location	Tumor stage	Positive events	Copies/μL	Copies/20 μL reaction	Positive events	Copies/μL	Copies/20 μL reaction	Copies/ng DNA	Positive events	Copies/μL	Copies/20 μL reaction	Positive events	Copies/μL	Copies/20 μL reaction	Copies/ng DNA
12	Rectum	Ⅰ	4	0.32	6.4	14	0.37	7.40	0.051	155	12.6	252	493	13.10	262.00	1.819
			5	0.39	7.8					158	12.5	250				
			5	0.39	7.8					180	14.1	282				
17	Left	Ⅰ	1147	95	1900	4849	97.20	1944.00	41.362	4684	446	8920	19664	456.00	9120.00	194.043
			1243	98	1960					4996	458	9160				
			1269	99	1980					5094	460	9200				
			1190	97	1940					4890	461	9220				
29	Left	ⅢB	5	0.41	8.2	11	0.30	6.00	0.024	94	7.7	154	257	7.10	142.00	0.577
			3	0.24	4.8					90	7.4	148				
			3	0.25	5					73	6.2	124				
30	Right	Ⅰ	6	0.57	11.4	18	0.54	10.80	0.017	238	22.8	456	757	23.10	462.00	0.728
			7	0.64	12.8					268	24.8	496				
			5	0.43	8.6					251	21.8	436				
43	Right	ⅢC	6	0.59	11.8	19	0.59	11.80	0.035	418	42	840	1393	44.10	882.00	2.602
			8	0.7	14					492	45.5	910				
			5	0.46	9.2					483	44.8	896				
46[1]	Left	ⅡC	1	0.09	1.8	3	0.09	1.80	0.014	81	7	140	265	7.80	156.00	1.238
			1	0.09	1.8					97	8.3	166				
			1	0.09	1.8					87	8.2	164				
51	Rectum	Ⅰ	13	1	20	47	0.90	18.00	0.295	1292	103	2060	5110	102.60	2052.00	33.639
			12	0.9	18					1291	102	2040				
			9	0.68	13.6					1297	102	2040				
			13	1	20					1230	103	2060				
64[1]	Left	Ⅰ	0	0	0	0	0.00	0.00	0.000	141	12	240	653	12.20	244.00	1.877
			0	0	0					163	12.1	242				
			0	0	0					154	11.4	228				
			0	0	0					195	13.3	266				
70	Left	Ⅰ	11	1.1	22	45	1.37	27.40	0.036	2515	282	5640	8516	294.00	5880.00	7.656
			24	1.9	38					3313	305	6100				
			10	1	20					2688	291	5820				
75	Left	ⅡA	0	0	0	11	0.33	6.60	0.032	304	27.2	544	964	29.00	580.00	2.788
			2	0.18	3.6					326	29.8	596				
			9	0.8	16					334	30.1	602				

[1]Patients 46 and 64 were considered negative because they had values less than or equal to the positivity threshold.

the later, especially at early stages, as tumor DNA will reasonably reach the fecal content more quickly than the bloodstream, at least during the initial phases of cancer development.

ARTICLE HIGHLIGHTS

Research background

Clinical management of colorectal cancer (CRC) requires analysis of molecular biomarkers, such as *KRAS* or *NRAS* mutations, which are associated with the emergence of resistance to therapy with biological agents. Tumor genotyping is usually performed using DNA from tissue biopsies, and, in recent years, from blood as well. However, at early disease stages, levels of mutated copies in circulating cell-free DNA may be, in some cases, too low for detection. Thus, extremely sensitive and non-invasive alternative methods are still needed to improve detection and achieve accurate quantification of these biomarkers.

Research motivation

Stool is an alternative and non-invasive source of genetic material for tumor genotyping in CRC. To date, several strategies based on analysis of molecular markers in fecal samples have been proposed, though their application in clinical practice remains limited due to their elevated cost and reduced sensitivity at early stages of disease.

Research objectives

The aim of this study was to assess the potential of droplet digital PCR (ddPCR) to detect the *KRAS* G12D mutation in stool-derived DNA from CRC patients as a proof-of-concept for the applicability of this technology as a non-invasive method of studying clinically relevant DNA biomarkers in stool.

Research methods

KRAS mutations were determined by pyrosequencing in DNA obtained from formalin-fixed paraffin-embedded (FFPE) tumor tissues. Then, *KRAS* G12D mutation was analyzed by ddPCR in FFPE tumors and stool-derived DNA in samples obtained from patients carrying this point mutation.

Research results

The *KRAS* G12D mutation was detected by ddPCR in FFPE tumor-derived DNA and in stool-derived DNA in 80% of all fecal samples from CRC patients with this mutation.

Research conclusions

This is the first study to describe the detection of the *KRAS* G12D mutation in stool-derived DNA from CRC patients using a commercially available ddPCR platform, including in individuals with early stages of the disease. ddPCR served as a reliable tool for detecting this clinically relevant mutation in stool-derived DNA from CRC patients. Several stool-based strategies involving digital PCR have been investigated to analyze relevant mutations for CRC management. However, none of these approaches has been further developed and subjected to clinical validation for stool DNA screening to date.

The advantages of ddPCR technology, together with instrumentation and protocols easily adoptable by any lab, support a potential translation of this approach to clinical scenarios. Our results show that *KRAS* G12D detection in stool-derived DNA from CRC patients by ddPCR is feasible and suggests this technology might be useful for the analysis of other molecular markers in stool. The authors hypothesized that ddPCR could be a reliable and sensitive method of analyzing *KRAS* mutations in stool-derived DNA, providing reproducible and accurate results.

This study proposed a new strategy based on detecting *KRAS* mutations in stool-derived DNA using a commercially available ddPCR platform. ddPCR is an emulsion-based amplification technology with fluorescently labelled probes. *KRAS* G12D mutation detection in stool-derived DNA by ddPCR is a fast, simple, and affordable approach which could be adapted to detect other clinically relevant molecular biomarkers for CRC management. This technique is more feasible for implementation into routine clinical practice than other previously described stool-based strategies.

ddPCR provided sensitive, accurate, and reproducible results for detection of the *KRAS* G12D mutation in stool-derived DNA from CRC patients, especially at early stages of the disease. In light of our results, it could be proposed that biomarker analysis by ddPCR in stool samples may complement current CRC screening methods; stool-derived nucleic acid testing by ddPCR offers an alternative to tissue genotyping and blood-based biomarker quantification, is a less invasive tool than the former and is likely more time-sensitive than the latter, especially at early stages, as tumor DNA will reasonably reach the fecal content more quickly than the bloodstream, at least during the initial phases of cancer development.

Research perspectives

KRAS mutations are analyzable by ddPCR in stool-derived DNA from CRC patients, including early-stage patients. This observation merits further studies aimed at evaluating and improving the efficiency of this approach prior to its clinical application. These results pave the way for ddPCR analysis of other molecular biomarkers of CRC in stool. Further studies involving larger cohorts of patients and samples collected at different time points throughout the progression of the disease should be performed in order to confirm the prognostic value and economic viability of this tool before implementation in clinical practice. A multiplex assay simultaneously covering all *KRAS* mutations relevant for anti-EGFR-therapy decision-making would maximize the benefits and optimize the cost-effectiveness of this approach. This strategy should be further investigated as a complementary screening test for early detection of CRC.

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ORIGINAL ARTICLE

Basic Study

Optimal timing for the oral administration of Da-Cheng-Qi decoction based on the pharmacokinetic and pharmacodynamic targeting of the pancreas in rats with acute pancreatitis

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Abstract

AIM

To identify the optimal oral dosing time of Da-Cheng-Qi decoction (DCQD) in rats with acute pancreatitis (AP) based on the pharmacokinetic and pharmacodynamic parameters.

METHODS

First, 24 male Sprague-Dawley rats were divided into a sham-operated group [NG(a)] and three model groups [4hG(a), 12hG(a) and 24hG(a)]. The NG(a) and model groups were administered DCQD (10 g/kg.BW) intragastrically at 4 h, 4 h, 12 h and 24 h, respectively, after AP models induced by 3% sodium taurocholate. Plasma samples were collected from the tails at 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after a single dosing with DCQD. Plasma and pancreatic tissue concentrations of the major components of DCQD were determined by high-performance liquid chromatography tandem mass spectroscopy. The pharmacokinetic parameters and serum amylase were detected and compared. Second, rats were divided into a sham-operated group [NG(b)] and three treatment groups [4hG(b), 12hG(b) and 24hG(b)] with three corresponding control groups [MG(b)s]. Blood and pancreatic tissues were collected 24 h after a single dosing with DCQD. Serum amylase, inflammatory cytokines and pathological scores of pancreatic tissues were detected and compared.

RESULTS

The concentrations of emodin, naringin, honokiol, naringenin, aloe-emodin, chrysophanol and rhe-ochrysidin in the 12hG(a) group were higher than those in the 4hG(a) group in the pancreatic tissues (P < 0.05). The area under the plasma concentration-time curve from time 0 to the time of the last measurable concentration values ($AUC_{0\rightarrow t}$) for rhein, chrysophanol, magnolol and naringin in the 12hG(a) group were larger than those in the 4hG(a) or 24hG(a) groups. The 12hG(a) group had a higher C_{max} than the other two model groups. The IL-10 levels in the 12hG(b) and 24hG(b) groups were higher than in the MG(b)s (96.55 ± 7.84 *vs* 77.46 ± 7.42, 251.22 ± 16.15 *vs* 99.72 ± 4.7 respectively, P < 0.05), while in the 24hG(b) group, the IL-10 level was higher than in the other two treatment groups (251.22 ± 16.15 *vs* 154.41 ± 12.09/96.55 ± 7.84, P < 0.05). The IL-6 levels displayed a decrease in the 4hG(b) and 12hG(b) groups compared to the MG(b)s (89.99 ± 4.61 *vs* 147.91 ± 4.36, 90.82 ± 5.34 *vs* 171.44 ± 13.43, P < 0.05).

CONCLUSION

Late-time dosing may have higher concentrations of the most major components of DCQD, with better pharmacokinetics and pharmacodynamics of anti-inflammation than early-time dosing, which showed the late time to be the optimal dosing time of DCQD for AP.

Key words: Da-Cheng-Qi decoction; Acute pancreatitis; Pharmacokinetics; Oral dosing time; Pharmacodynamics

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Core tip: Our study group had raised the hypothesis of tissue pharmacology of an herbal recipe, which assumed the effect of an herb formula is related to its target tissue distribution or concentration of effective components in target tissues. This study was then designed to screen the optimal oral dosing time of Da-Cheng-Qi decoction (DCQD) in rats with acute pancreatitis based on the pharmacokinetics of the main absorbed components and the pharmacodynamics of DCQD targeting of the pancreas.

Zhang YM, Zhu L, Zhao XL, Chen H, Kang HX, Zhao JL, Wan MH, Li J, Zhu L, Tang WF. Optimal timing for the oral administration of Da-Cheng-Qi decoction based on the pharmacokinetic and pharmacodynamic targeting of the pancreas in rats with acute pancreatitis. *World J Gastroenterol* 2017; 23(39): 7098-7109 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7098.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7098

INTRODUCTION

Da-Cheng-Qi decoction (DCQD) was first described in Shang-Han-Lun, a classical work of Traditional Chinese Medicine (TCM). DCQD consists of four Chinese herbs: dahuang (*Rheum palmatum* L.), mangxiao (*Mirabilite*, $Na_2SO_4 \cdot 10H_2O$), houpu (*Magnolia officinalis* Rehd. et Wils.), and zhishi (*Citrus aurantium* L.). It has been widely used as a purgative to treat diseases with constipation and to clear away internal heat[1], such as in acute pancreatitis (AP), which has a consensus in the relative treatment guidelines in China. The efficacy of DCQD, administered orally or by coloclyster to patients with AP, is obvious. According to clinical observation, it can reduce intra-abdominal hypertension[2] and decrease the risk of developing acute respiratory distress syndrome in severe AP (SAP) patients with systemic inflammatory response syndrome, and shorten their length of hospitalization[3].

In animal experiments, it can ameliorate acute pancreatic, intestinal, lung and liver injury complicated with SAP[4,5]. It can also reduce the generation of reactive oxygen species in AR42J cells and regulate the apoptosis/necrosis switch, to ameliorate the pancreatic inflammation and pathological damage[6]. Other therapeutic activities of DCQD for AP, such as its antioxidant, anti-inflammatory and anti-ulcer properties[7] are important. Its numerous roles indicate the wide applicability of DCQD in AP.

However, early oral dosing with DCQD is contradictory to the conventional therapy that considers fasting and water deprivation are necessary for pancreatic rest in the early stage of AP. Conventionally, any stimulation of the exocrine function of the pancreas by fluid or solid nutrients would promote the release of proteolytic enzymes and affect the disease course negatively[8]. Early oral dosing with DCQD may increase gastric contents, worsen stomachache and abdominal distension, and even aggravate the disease severity. However, it is not clear whether early oral administration of DCQD increases pancreatic secretion and worsens the disease severity. One clinical observation revealed that the best time for gastrointestinal unblocking by herbs was within 48 h after the onset of AP[9]. At the same time, releasing excessive turbidity should be done sooner rather than later to prevent intestinal function failure and disease deterioration[9]. Some studies have indicated that AP patients must pass feces within 24 h after the onset of AP, as one method to support intestinal function to control systemic inflammatory response syndrome and protect organ functions[9]. It is still unclear if the optimal oral dosing time of DCQD should be earlier or later. Therefore, it is important to find the optimal dosing time of DCQD at which DCQD will not worsen the disease severity.

The necrosis of pancreatic acinar cells would worsen the disease, and the induction of apoptosis would decrease the disease severity[6]. Thus, based on the effect of DCQD on regulation of the apoptosis/necrosis switch of pancreatic acinar cells to ameliorate pancreatic inflammation and pathological damage[6], the study aimed to identify the optimal oral dosing time of DCQD in rats with AP according to the pharmacokinetics of the absorbed components and the pharmacodynamics of DCQD targeting of the pancreas.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (n = 66) aged 90 ± 5 d, with body weights of 280-300 g, were purchased from Chengdu Dashuo Bio-Technique Co. Ltd. (Chengdu, China). The animals were maintained as previously described[10]. Before AP induction, rats were fasted for 12 h. This study was performed according to the Guide for the Care and Use of Laboratory Animals of Sichuan University (Chengdu, China) and the Animal Ethics Committee Guidelines of the Animal Facility of the West China Hospital (Chengdu, China).

Preparation of drugs

Sodium taurocholate was supplied by Sigma (St. Louis, MO, United States). Spray dried particles of dahuang, mangxiao, houpu, and zhishi were purchased from Chengdu Green Herbal Pharmaceutical Co. Ltd. (Chengdu, China). Chloral hydrate (10%), paraformaldehyde (4%) and methanol were obtained from Tedia Co. Ltd. (Nos. 509221 and 609144; Fairfield, OH, United States,). Glacial acetic acid (No. 20030911) and ethyl acetate (No. 20070116) were purchased from Chengdu Kelon Chemical Reagent Factory (Chengdu, China). Reference standards of the 10 components of DCQD were purchased from the same companies. According to the proportion of crude drugs (dahuang 12 g, houpu 24 g, zhishi 12 g, and mangxiao 9 g), the granules of the four drugs were stirred with ultra-pure water by magnetic stirrers with a speed setting of grade 5 for 1 h in a water bath at a temperature of 37 ℃ for 30 min. According to the Method of Pharmacology, the least dosage of DCQD is 0.6 g/100 g.BW for rats. In this study, the dosage was 1 g/100 g.BW (10 g/kg.BW) with the concentration of 1 g/mL.

Equipment and conditions

The magnetic stirrer (C-MAG, MS 7) was provided by the IKA Company (Breisgau, Germany). The analytical balance (BSA-224S-CW) was provided by the Sartorius Company (Goettingen, Germany). The micro-infusion pump was obtained from KD Scientific (Holliston, MA, United States). Conventional operation instruments, fixation-machines for rats, and the 1.5 mL and 10 mL centrifuge tubes were purchased from Shimadzu (Kyoto, Japan). The high-performance liquid chromatography tandem mass spectroscopy (HPLC-MS/MS) system consisted of a SIL-HTc autosampler (Shimadzu), a LC-10ADvp pump (Shimadzu), and an API3000 triple-quadrupole LC-MS system (Applied Biosystems, Foster City, CA, United States). This system was controlled by Analyst 1.4. Software (Chinese Pharmacological Society, Beijing, China). The chromatographic column was an Ultimate XB-C_{18} (5 μm, 50 mm × 4.6 mm). The mobile phase consisted of methanol-water (92:8, v/v) delivered at a flow rate of 0.3 mL/min. The column was maintained at ambient temperature, and the injection volume was 80 μL. The Anke centrifuge TGL-16B was supplied by Shanghai An-Ting Science Technology Instrument Factory (Shanghai, China). All aqueous solutions and buffers were prepared with ultra-pure water from a Millipore Rios™-16 water purifier (Millipore, Billerica, MA, United States). Standard stock solutions were prepared

by dissolving the reference standards (100 μg/mL for emodin, aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 20 μg/mL for rhein and rheochrysidin) and internal standard (40 μg/mL for ibuprofen) in methanol[1]. Stock solutions were stored at -20 ℃. Working standard solutions were prepared freshly by diluting stock solutions in sodium hydroxide solution (0.1 mol/L). Internal standard working solution (200 ng/mL) was prepared by diluting the stock solution in methanol-water (1:1, v/v)[1].

Induction of AP and dosing of DCQD

First part: Rats were randomly allocated into the following four groups (n = 6 each): a sham-operated group [NG(a)] and three model groups [4hG(a), 12hG(a) and 24hG(a)]. AP models were induced by retrograde perfusion of 3% sodium taurocholate (1 mL/kg.BW) into the biliopancreatic duct[11] at a rate of 6 mL/h with a micro-infusion pump after anesthetization with 10% chloral hydrate (3 mL/kg.BW) injected into the abdominal cavity. The NG(a) group was established by the same procedure, but with use of saline instead of sodium taurocholate. The dosing time of DCQD for the NG(a) and model groups were 4 h, 4 h, 12 h and 24 h after operation.

Second part: Forty-two rats were divided into a sham-operated group [NG(b)], and 4h-, 12h- and 24h-dosing treatment groups [4hG(b), 12hG(b) and 24hG(b), respectively] with three corresponding control groups [MG(b)s]. Only the three treatment groups were administered the same dosage of DCQD at 4 h, 12 h and 24 h, respectively, after AP induction. The three corresponding control groups were given saline instead of DCQD at the same time. Rats were euthanized at 24 h after drug dosing.

Collection and measurement of samples

First part: Plasma samples (0.5 mL) were collected from the tails at 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after a single dosing of DCQD. Serum samples for amylase (AMS) and pancreatic tissues for the tissue concentrations of the absorbed components of DCQD were collected 24 h after drug dosing. A total of 0.05 mL of internal standard working solution and 0.1 mL of hydrochloric acid buffer solution were added into 0.2 mL of the plasma or tissue homogenate samples, followed by 3.0 mL of ethyl acetate. Then, the mixtures were extracted by vortex mixing for 7 min and centrifuged at 3000 rpm for 7 min at a low temperature. After that, 2.4 mL supernatants were evaporated at 45 ℃, followed by incubation with 0.1 mL of double-solvents (methanol-water: 92:8, v/v). Thereafter, 20 μL of supernatant was injected automatically into the HPLC-MS/MS system for analysis. The 10 major components of DCQD (aloe-emodin, rhein, emodin, chrysophanol, honokiol, rheochrysophanol, magnolol, hesperidin, naringenin and naringin) were detected. The mean contents of the components were detected three times, as in our previous study[12]. The detected DCQD samples were stored in the Public Experiment Platform at West China Hospital (Chengdu, China).

Second part: Serum for AMS and inflammatory cytokines measurement and pancreatic tissues for pathological scoring were collected 24 h after drug dosing. Pancreatic samples were fixed in 10% neutral formalin for paraffin sections and stained with hematoxylin and eosin (HE). All the histopathology specimens were scored in a blinded fashion by two independent pathologists using a scoring system for the extent and severity of tissue injury (0-4, edema, neutrophil infiltration, necrosis and hemorrhage, respectively), as previously described[4,13]. The IL-10 and IL-6 levels were detected by enzyme-linked immunosorbent assay kits. The following formula was used to calculate the value of AMS: AMS (U/dL) = (blankOD - measurementOD)/blankOD × 0.4 × 0.5/10 × 30 min/7.5 min × 100/0.1 × 10

Statistical analysis

The pharmacokinetic parameters were processed by pharmacokinetic statistical software DAS2.0.1 programmed by the Chinese Pharmacological Society. The data were obtained by statistical moment calculation. The following pharmacokinetic parameters were calculated: the maximum plasma concentration (C_{max}), the time to reach maximum concentration (T_{max}), the mean residence time ($MRT_{0\rightarrow t}$), the elimination half-life ($T_{1/2}$) and the area under the plasma concentration-time curve from time 0 to the time of the last measurable concentration ($AUC_{0\rightarrow t}$). The data were processed with statistical software PEMS3.1. All values were expressed as the mean ± SD. A one-way repeated-measure ANOVA, followed by multiple pairwise comparisons using the Student-Newman-Keuls procedure, was used to detect differences among the groups. $P < 0.05$ was considered a statistically significant difference.

RESULTS

Ten major components of DCQD distributed in pancreatic tissues

In this study, all 10 major components of DCQD in pancreatic tissues could be detected by HPLC-MS/MS. The concentrations of naringin, hesperidin, naringenin, aloe-emodin and chrysophanol in pancreatic tissues were relatively high. Compared to the NG(a) group, the concentrations of emodin,

Table 1 Concentrations of the 10 components of DCQD distributed in pancreatic tissues

	NG(a)	4hG(a)	12hG(a)	24hG(a)
Rhein	12.65 ± 3.7	179.15 ± 77.6[a]	199.89 ± 34.8	50.08 ± 17.6[e]
Emodin	18.68 ± 8.7	1.33 ± 0.8[a]	34.46 ± 10.3[c]	39.75 ± 12.4
Naringin	415.30 ± 17.8	19.47 ± 1.4[a]	32.10 ± 2.7[c]	269.16 ± 12.9[e]
Honokiol	2.38 ± 1.2	1.98 ± 0.1	19.79 ± 2.2[c]	4.26 ± 1.0
Magnolol	1.34 ± 0.9	0.97 ± 0.2	0.97 ± 0.2	7.51 ± 0.8[e]
Hesperidin	162.01 ± 34.3	34.94 ± 12.6[a]	24.31 ± 10.3	113.26 ± 13.8[e]
Naringenin	847.98 ± 76.9	858.58 ± 19.6	1077.06 ± 26.8[c]	262.30 ± 100.6[e]
Aloe-emodin	439.05 ± 179.8	89.53 ± 14.8[a]	502.74 ± 70.7[c]	501.45 ± 143.4
Chrysophanol	60.99 ± 16.4	5.16 ± 2.1[a]	197.29 ± 17.9[c]	113.43 ± 23.7[e]
Rheochrysidin	6.79 ± 1.2	0.55 ± 0.2[a]	17.92 ± 1.9[c]	11.67 ± 0.9[e]

Rats were orally administered with DCQD (10 mL/kg.BW). Pancreatic tissues were collected 24 h after a single dosage of DCQD. The concentrations of the 10 components of DCQD were determined by HPLC-MS/MS. The results are presented as the mean ± SD of μg/mg (n = 6). NG(a): the sham-operated group with the dosing time at 4 h after operation. 4hG(a), 12hG(a) and 24hG(a): rats were dosed orally with DCQD at 4 h, 12 h and 24 h, respectively, after AP induction. 4hG(a) *vs* NG(a): [a]P < 0.05, 12hG(a) *vs* 4hG(a): [c]P < 0.05, 24hG(a) *vs* 12hG(a): [e]P < 0.05. AP: Acute pancreatitis; DCQD: Da-Cheng-Qi decoction; HPLC-MS/MS: High-performance liquid chromatography tandem mass spectroscopy.

naringin, hesperidin, aloe-emodin, chrysophanol and rheochrysidin were lower in the 4hG(a) group (P < 0.05), while the rhein concentration was higher (P < 0.05). The concentrations of emodin, naringin, honokiol, naringenin, aloe-emodin, chrysophanol and rheochrysidin in the 12hG(a) group were higher than in the 4hG(a) group (P < 0.05). The concentrations of rhein, naringenin, chrysophanol and rheochrysidin in the 24hG(a) group were lower than in the 12hG(a) group (P < 0.05), while the concentrations of naringin, magnolol and hesperidin in the 24hG(a) group were higher (P < 0.05) (Table 1).

Eight major components of DCQD detected in plasma

Only eight of the ten components were successfully fitted to the concentration-time curves according to the testing data (Figure 1). The T_{max} values were all at 40 min (0.67 h) after a single dosage of DCQD in the NG(a) group. The T_{max} of six components (aloe-emodin, rhein, emodin, chrysophanol, naringenin and naringin) in the 4hG(a) group, all components in the 12hG(a) group and that of five components (aloe-emodin, rhein, rheochrysidin, magnolol and naringin) in the 24hG(a) were delayed (Figure 1).

In the NG(a) group, the C_{max} values of the components were as follows: aloe-emodin, 3218.33 ng/mL; rhein, 8638.42 ng/mL; emodin, 510.97 ng/mL; naringin, 204.56 ng/mL; chrysophanol, 5419.89 ng/mL; rheochrysidin, 146.75 ng/mL; naringenin, 419.94 ng/mL; and magnolol, 19.58 ng/mL. Compared to the NG(a) group, the C_{max} values of aloe-emodin, rhein, emodin, chrysophanol and magnolol were reduced in the 4hG(a) group (Figure 1). Compared to the 4hG(a) or 12hG(a) groups, the C_{max} values of aloe-emodin, rhein, emodin, naringenin and magnolol were higher in the 24hG(a) group, and they were as follows: 5885.13 ng/mL, 8245.18 ng/mL, 88.65 ng/mL, 606.41 ng/mL and 11.018 ng/mL, respectively. Among the three model groups, the C_{max} values of chrysophanol and naringin were highest in the 12hG(a) group, and for rheochrysidin the C_{max} was in the 4hG(a) group; the concentrations were 4054.73 ng/mL, 519.53 ng/mL and 557.06 ng/mL, respectively (Figure 1).

The pharmacokinetic parameters were analyzed by statistical moment calculation, and those in model groups were different from those in the NG(a) group (Table 2). Compared to the NG(a) group, the $AUC_{0\rightarrow t}$ values of aloe-emodin, chrysophanol, emodin and magnolol were smaller in the 4hG(a) group (P < 0.05), while those of rheochrysidin, naringin and naringenin were larger (P < 0.05). The $AUC_{0\rightarrow t}$ of aloe-emodin in the 24hG(a) group was larger than in the 4hG(a) or 12hG(a) groups, and those of rhein, chrysophanol, magnolol and naringin in the 12hG(a) group were larger than in the 4hG(a) or 24hG(a) groups. The $T_{1/2}$ values of rhein, emodin, aloe-emodin, rheochrysidin, naringin and magnolol in the 24hG(a) group were longer than in the 4hG(a) or 12hG(a) groups.

DCQD elevated the IL-10 levels and lowered the IL-6 levels in serum

In serum, the IL-10 and IL-6 levels in the three corresponding control groups were all higher than in the NG(b) group (IL-10: 152.8 ± 18.58/77.46 ± 7.42/99.72 ± 4.7 *vs* 48 ± 12, P < 0.05; IL-6: 147.91 ± 4.36/171.44 ± 13.43/98.48 ± 2.7 *vs* 68 ± 20, P < 0.05). Compared to the corresponding control groups, the IL-10 levels in the 12hG(b) and 24hG(b) groups were increased (96.55 ± 7.84 *vs* 77.46 ± 7.42, 251.22 ± 16.15 *vs* 99.72 ± 4.7, P < 0.05), and the IL-10 level in the 24hG(b) group was higher than in the 4h(b) and 12hG(b) groups (251.22 ± 16.15 *vs* 154.41 ± 12.09/96.55 ± 7.84, P < 0.05) (Figure 2A). The IL-6 levels displayed a decrease in the 4hG(b) and 12hG(b) groups compared to their corresponding control groups (89.99 ± 4.61 *vs* 147.4.36, 90.82 ± 5.34 *vs* 171.44 ±

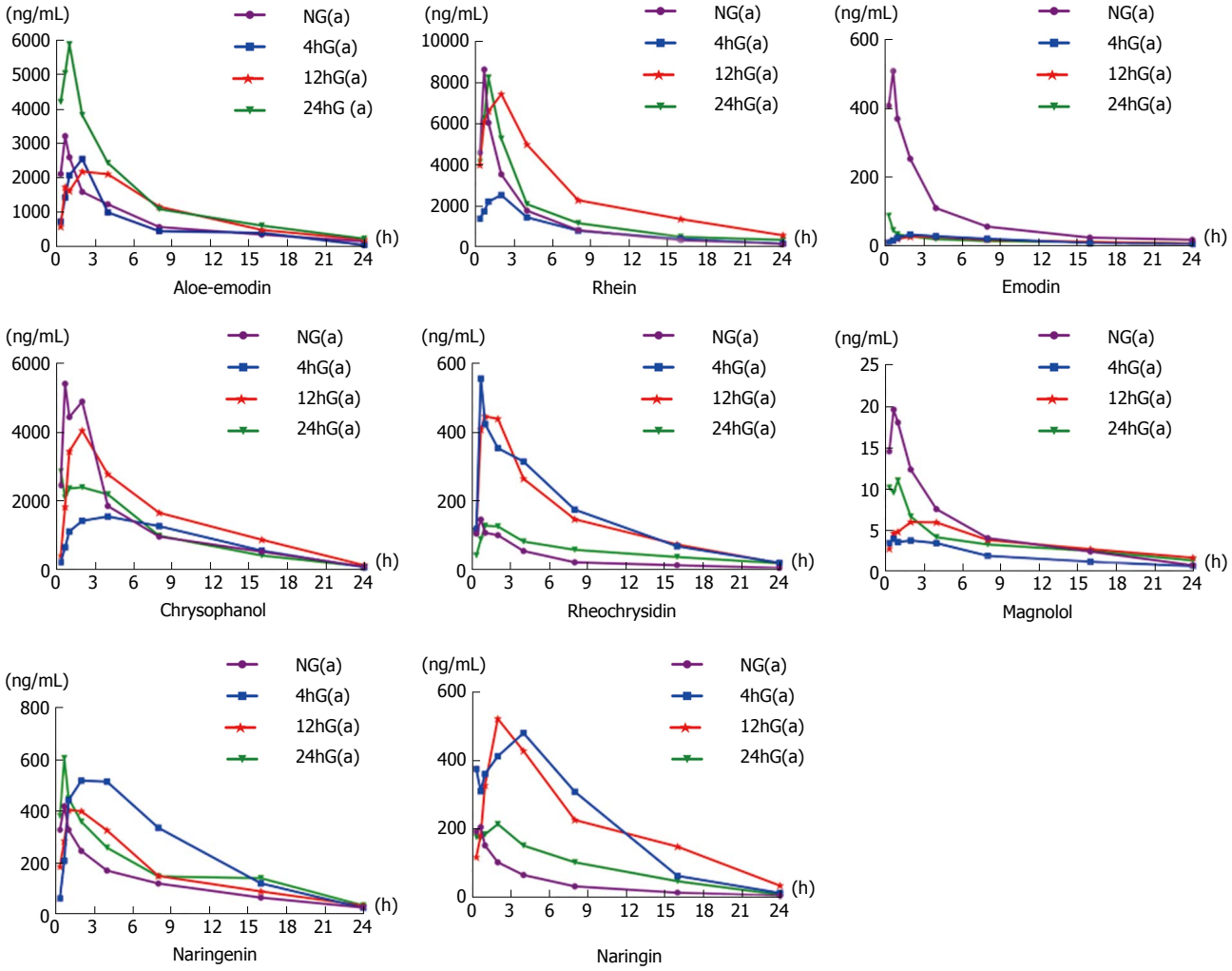


Figure 1 Concentration-time curves of the eight components of Da-Cheng-Qi decoction in plasma of the rats with acute pancreatitis. Rats (n = 6 per group) were orally dosed with DCQD (10 mL/kg.BW). Plasma samples were collected from the tails at 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after a single dosage of DCQD. Plasma concentrations of the components of DCQD were determined by HPLC-MS/MS. The results are presented as the mean ± SD. NG(a): the sham-operated group with the dosing time 4 h after operation; 4hG(a), 12hG(a) and 24hG(a): the dosing times were 4 h, 12 h and 24 h, respectively, after AP induction. AP: Acute pancreatitis; DCQD: Da-Cheng-Qi decoction; HPLC-MS/MS: High-performance liquid chromatography tandem mass spectroscopy.

13.43, $P < 0.05$) (Figure 2B).

Pathological damages in the pancreatic tissues and serum AMS levels

In the NG(b) group, the pancreatic tissues were edematous with a few neutrophils but without obvious hemorrhage and necrotic acinar tissues. In the MG(b) groups, edema, hemorrhage and neutrophils with some necrotic acinar tissues were obvious. In the 12hG(b) group, the pathological damages were improved, while those in the 4hG(b) or 24hG(b) groups had no obvious improvement (Figure 3A). Compared to the NG(b) group, the pathological scores in the MG(b) groups were increased (1.79 ± 0.3/2.2 ± 0.2/2.67 ± 0.67 *vs* 0.7 ± 0.26, $P < 0.05$). Those in the 12hG(b) group were evidently reduced compared to its control group (1.3 ± 0.4 *vs* 2.2 ± 0.2, $P < 0.05$) or the 4hG(b) and 24hG(b) groups (1.3 ± 0.4 *vs 2.63* ± 0.4/2.5 ± 0.3, $P < 0.05$) (Figure 3B).

The AMS levels in the 4hG(a), 12hG(a) and 24hG(a) groups were all higher than in the NG(a) group (724.17 ± 42.8/673.67 ± 26.64/659.65 ± 41.38 *vs* 273.67 ± 93.23, $P < 0.05$) (Figure 3C). Compared to the NG(b) group, that in the MG(b) groups were higher (718.65 ± 51.04/711.68 ± 55.37/666.4 ± 73.4 *vs* 389 ± 98, $P < 0.05$). The AP model was successful. That in the 12hG(b) group was reduced compared to its control group (649 ± 131.69 *vs* 711.68 ± 55.37, $P < 0.05$) (Figure 3D).

DISCUSSION

According to the present study, AP reduced the concentrations of the major components of DCQD to the target pancreas, and the oral administration time also played an important role; their absorption

Table 2 Pharmacokinetic parameters of the 8 detected components of Da-Cheng-Qi decoction in plasma

Parameter	NG(a)	4hG(a)	12hG(a)	24hG(a)
Aloe-emodin				
T1/2 (h)	7.5 ± 3.4	4.8 ± 1.9	7.3 ± 2.7	7.5 ± 4.3
AUC0→t (μg/mL•h)	16582.3 ± 523.7	15180.5 ± 245.3[a]	23266.6 ± 2848.4[b]	32517.8 ± 3109.6[dc]
MRT0→t (h)	7.0 ± 1.8	6.5 ± 1.6	7.7 ± 0.9	6.2 ± 1.3
Tmax (h)	1.22 ± 1.38	1.45 ± 0.62	2.28 ± 1.44	0.67 ± 0.30[dc]
Cmax (μg/mL)	4080.9 ± 2491.6	2890.5 ± 955.0	2948.2 ± 997.6	7706.6 ± 2366.7[dc]
Rhein				
T1/2 (h)	4.7 ± 1.5	6.3 ± 1.8	6.2 ± 1.2	16.6 ± 2.1[dc]
AUC0→t (μg/mL•h)	27164.9 ± 1686.9	19164.9 ± 1680.3	60705.9 ± 2870.4[b]	35470.0 ± 1910.1[dc]
MRT0→t (h)	4.7 ± 1.0	6.3 ± 1.5[a]	7.4 ± 1.3	5.9 ± 3.2
Tmax (h)	0.7 ± 0.3	1.4 ± 0.9[a]	1.3 ± 0.7	1.3 ± 0.4
Cmax (μg/mL)	11033.4 ± 3248.9	3037.9 ± 1040.5[a]	9439.4 ± 3191.6[b]	9938.6 ± 3349.6[d]
Chrysophanol				
T1/2 (h)	5.2 ± 1.7	6.8 ± 3.6	4.5 ± 0.8	6.2 ± 3.8
AUC0→t (μg/mL•h)	28925.5 ± 7837.2	20214.5 ± 1460.6[a]	34977.3 ± 1927.7[b]	23102.4 ± 1614.8[c]
MRT0→t (h)	5.7 ± 0.8	7.2 ± 1.7[a]	7.3 ± 1.0	6.2 ± 1.2
Tmax (h)	1.2 ± 0.6	3.5 ± 2.5[a]	1.7 ± 0.5	1.3 ± 0.5[d]
Cmax (μg/mL)	7708.1 ± 2234.3	2296.1 ± 7643.8[a]	4521.8 ± 1127.1[b]	4772.6 ± 1078.4[d]
Emodin				
T1/2 (h)	8.2 ± 6.6	14.7 ± 11.7	11.7 ± 6.0	20.8 ± 19.6
AUC0→t (μg/mL•h)	1891.5 ± 1692.8	395.3 ± 159.0[a]	395.4 ± 82.4	391.2 ± 135.8
MRT0→t (h)	5.2 ± 1.9	8.4 ± 1.7[a]	9.4 ± 0.8	8.6 ± 1.9
Tmax (h)	0.6 ± 0.3	3.8 ± 3.3[a]	3.2 ± 2.6	0.7 ± 0.7[dc]
Cmax (μg/mL)	546.2 ± 496.8	37.9 ± 18.4[a]	34.1 ± 9.6	91.8 ± 37.4
Rheochrysidin				
T1/2 (h)	6.4 ± 0.9	4.6 ± 1.9	5.4 ± 2.1	8.3 ± 2.3[dc]
AUC0→t (μg/mL•h)	740.2 ± 623.3	3680.6 ± 1903.0[a]	3489.2 ± 1354.1	1301.8 ± 420.9[dc]
MRT0→t (h)	5.7 ± 1.4	6.4 ± 0.9	6.3 ± 1.5	8.2 ± 1.1[dc]
Tmax (h)	0.9 ± 0.6	2.1 ± 1.6	1.7 ± 1.3	1.5 ± 0.6
Cmax (μg/mL)	184.7 ± 92.4	713.8 ± 201.6[a]	642.3 ± 211.5	155.5 ± 56.4[dc]
Magnolol				
T1/2 (h)	8.5 ± 4.9	9.4 ± 2.8	12.2 ± 3.3	22.8 ± 7.6[dc]
AUC0→t (μg/mL•h)	111.4 ± 37.2	44.4 ± 14.3[a]	83.9 ± 27.2[b]	81.5 ± 25.0[d]
MRT0→t (h)	7.2 ± 0.9	7.9 ± 1.2	9.4 ± 1.1	8.9 ± 1.2
Tmax (h)	0.7 ± 0.3	1.3 ± 0.5Δ	2.8 ± 1.4	0.6 ± 0.3[dc]
Cmax (μg/mL)	24.2 ± 8.6	5.8 ± 2.3Δ	7.7 ± 3.6	15.3 ± 13.1[dc]
Naringin				
T1/2 (h)	5.8 ± 1.2	4.9 ± 1.8	5.3 ± 1.4	5.5 ± 1.4
AUC0→t (μg/mL•h)	918.1 ± 106.8	4920.6 ± 310.7[a]	5054.4 ± 435.1	2044.1 ± 26.9[dc]
MRT0→t (h)	5.9 ± 1.1	6.6 ± 0.8	7.9 ± 0.6	6.9 ± 1.0
Tmax (h)	0.7 ± 0.2	1.4 ± 1.4	2.5 ± 1.2	1.8 ± 1.3
Cmax (μg/mL)	229.3 ± 195.6	724.4 ± 584.7	595.1 ± 338.9	322.9 ± 148.4
Naringenin				
T1/2 (h)	7.2 ± 1.7	4.9 ± 1.1a	9.3 ± 3.6[b]	7.7 ± 1.3[d]
AUC0→t (μg/mL•h)	2705.2 ± 164.6	5795.1 ± 291.4[a]	3758.1 ± 250.1[b]	4099.2 ± 148.8[dc]
MRT0→t (h)	6.4 ± 1.7	7.3 ± 0.4	7.7 ± 1.2	7.9 ± 1.8
Tmax (h)	0.8 ± 0.6	2.3 ± 1.4	1.6 ± 1.3	0.6 ± 0.3[d]
Cmax (μg/mL)	552.9 ± 226.7	720.4 ± 165.9	543.3 ± 110.4	624.9 ± 143.7

Rats were orally dosed with DCQD (10 mL/kg.BW). Plasma samples were collected from the tails at 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after a single dosage of DCQD. The samples were assessed by HPLC-MS/MS. The pharmacokinetic parameters were processed by pharmacokinetic statistic software DAS2.0.1 and the data were obtained by statistical moment calculation. The results are presented as the mean ± SD. NG(a): the sham-operated group with the dosing time at 4 h after operation. 4hG(a), 12hG(a) and 24hG(a): rats were dosed orally with DCQD at 4 h, 12 h and 24 h, respectively, after AP induction. Compared with NG(a): [a]$P < 0.05$, 12hG(a) *vs* 4hG(a): [b]$P < 0.05$, 24hG(a) *vs* 12hG(a): [c]$P < 0.05$, 24hG(a) *vs* 4hG(a): [d]$P < 0.05$. AP: Acute pancreatitis; DCQD: Da-Cheng-Qi decoction; HPLC-MS/MS: High-performance liquid chromatography tandem mass spectroscopy.

may be better if the oral administration time of DCQD is approximately 12 h after the onset of AP (Table 1). AP delayed the T_{max} and reduced the C_{max} of the components of DCQD in the circulation of rats (Figure 1). The components of DCQD displayed a higher C_{max} and a longer $T_{1/2}$ when the oral administration time of DCQD was approximately 24 h after the onset of AP (Figure 1 and Table 2). The $AUC_{0\rightarrow t}$ was larger when that time was approximately 12 h after the onset of AP (Table 2). DCQD increased the IL-10 levels and lowered the IL-6 levels (Figure 2A and B), and the later administration of the DCQD dose corresponded to higher IL-10 levels (Figure 2A). Therefore, administering the DCQD dose too early may not be

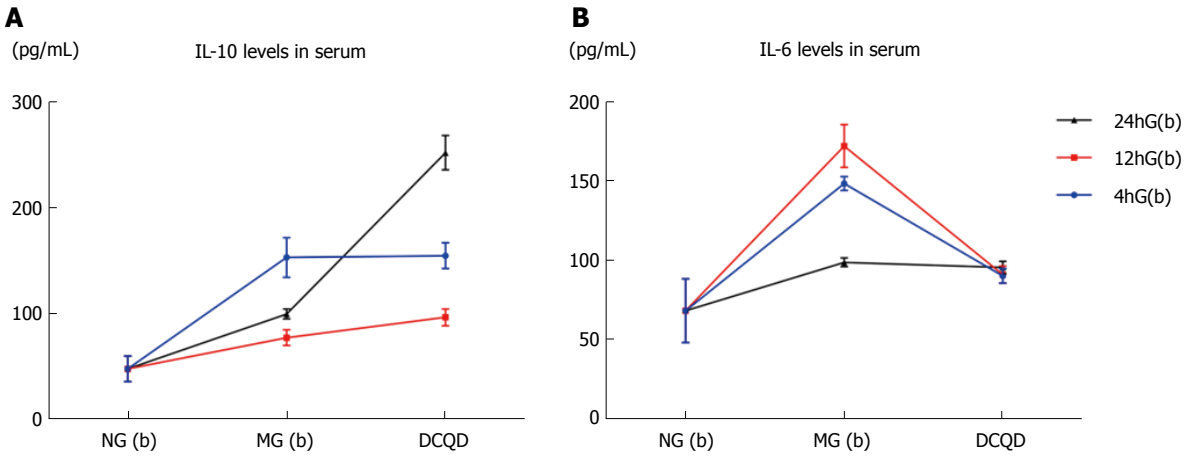


Figure 2 Da-Cheng-Qi decoction elevated IL-10 levels and lowered IL-6 levels in serum of the rats with acute pancreatitis. Rats (*n* = 6 per group) in the three treatment groups were orally dosed with DCQD (10 mL/kg.BW) 4 h, 12 h and 24 h after AP induction. Serum samples were collected at 24 h after a single dosage of DCQD. The results are presented as the mean ± SD. NG(b): the sham-operated group; MG(b): the model group or the control group; 4hG(b), 12hG(b) and 24hG(b): rats were orally dosed with DCQD 4 h, 12 h and 24 h, respectively, after AP induction. AP: Acute pancreatitis; DCQD: Da-Cheng-Qi decoction.

appropriate for AP, and administration should at least be 12 h after the onset of AP.

Pathological circumstances may affect the absorption of the components of DCQD and affect the pharmacokinetics in AP. Pancreatic ischemia, reduced pancreatic blood flow and increased capillary permeability are usually common in AP[14]. The systemic hemodynamic disturbances lead to ischemia of the intestine[15]. In addition, one retrospective analysis (197 patients) showed that 65% of patients with AP had acute gastrointestinal mucosal lesions detected by upper gastrointestinal endoscopy[16]. As well, 59% of patients with AP showed gut barrier dysfunction with increased intestinal permeability in a meta-analysis of 18 studies[17]. Both propulsion and contractility were reduced in necrotizing pancreatitis of rats[18]. It was reported that permeability of the ileum was significantly increased at 6 h, the blood endotoxin level was elevated and bacterial translocation occurred 18 h after induction of SAP-induced by injection of 3% sodium deoxycholate[19].

As we know, oral medicines, including herbs, are generally absorbed by the gastrointestinal mucosa. The process of drug absorption into the blood circulation from the plasma membrane barrier proceeds as follows: drug molecule encounters the gastrointestinal mucous layer, brush border, epithelial cell membrane, intracellular fluid, basal lamina, lamina propria, externa of vessels, cytoplasm of vessels, intima of vessels and then the blood. Therefore, these aforementioned factors may play an important role in the delay of the T_{max} of the components of DCQD in reaching the circulation and establishing their concentrations in pancreatic tissues.

Another factor may be the physicochemical properties of these components. Chrysophanol belongs to the Class Ⅱ poorly water soluble drugs in the Biopharmaceutics Classification System (BCS), with low solubility but high permeability[20,21]. Magnolol, a small-molecule neolignan[22], has an extensive first-pass metabolism and low absorption[23]. Naringin is moderately soluble in water, and it is broken into its aglycon naringenin in the intestine by the gut microflora and then absorbed from the gut[24]. These different characteristics may influence the absorption of these components. Furthermore, the pharmacokinetics of phytochemicals have substantial variation[25] and circulating concentrations of phytochemicals, which could vary widely among individuals, even in the context of controlled feeding studies[26]. In brief, the internal environments of rats and the characteristics of the components of DCQD may be the major factors affecting the absorption of these components.

It is well known that the avoidance of gastric and intestinal secretion has been the cornerstone of management of patients with AP for nearly a century[27]. To espouse the "pancreatic rest" concept, fasting and water deprivation have become the fundamental treating rules. However, the benefits of oral dosing with DCQD in the early stage of AP have been demonstrated, especially in gastrointestinal internal environments. This approach could improve intestinal propulsive function, relieve abdominal distension and abdominal pressure[28], and protect the intestinal immune barrier, with amelioration of the levels of high mobility group box-1 protein (HMGB1) RNA and cyclooxygenase 2 (COX-2) RNA expression[29]. One meta-analysis showed that purgative therapy could shorten the time of first defecation and the hospitalization time[30]. Thus, the advantages of DCQD are obvious. Future studies should be done to determine whether the oral dose of DCQD could increase gastrointestinal and pancreatic secretion.

The finding of DCQD regulating the balance of pro-inflammation and anti-inflammation was consistent with our previous studies[4]. However, the current

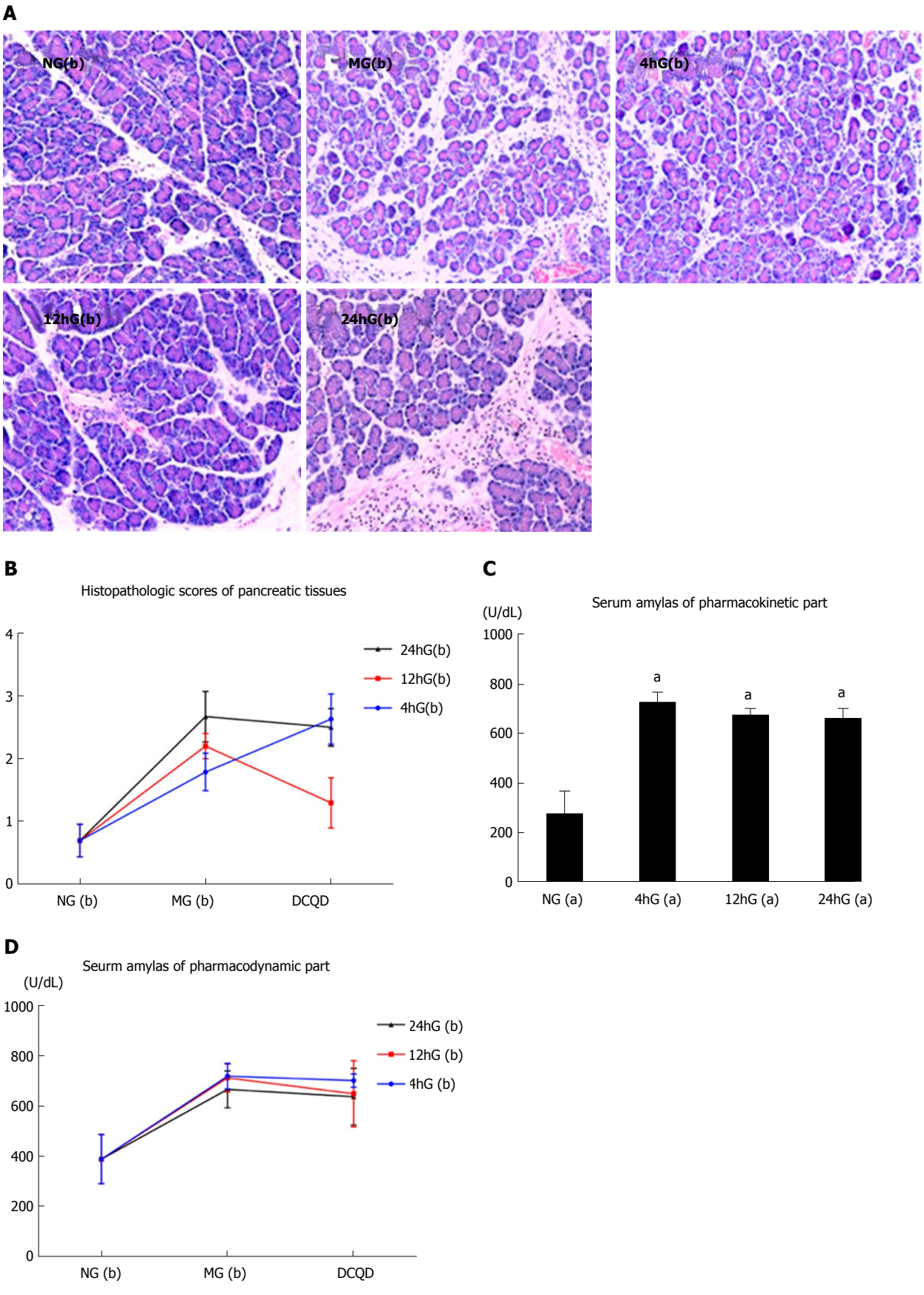


Figure 3 Pathological damages in the pancreatic tissues and changes in the serum amylase concentration. Rats (n = 6 per group) were orally administered DCQD (10 mL/kg.BW) 4 h, 12 h and 24 h after AP induction. Pancreatic tissues were collected for staining with hematoxylin-eosin (A, × 200) 24 h after a single dosage of DCQD; B: Pathological scores of the pancreatic tissues. Serum amylase was detected by enzyme-linked immunosorbent assay (C and D). The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* NG(a). NG(a/b): the sham-operated group; MG(b): the model group or the control group; 4hG(a/b) 12hG(a/b), and 24hG(a/b): rats were orally dosed with DCQD 4 h, 12 h and 24 h, respectively, after AP induction. AP: Acute pancreatitis; DCQD: Da-Cheng-Qi decoction.

study showed that its oral dosing time might affect the inflammatory cytokines and pharmacokinetics of the effective components of DCQD targeting of pancreatic tissues and plasma. The chronomedicine of TCM, with thousands of years of history and in which the midnight-noon ebb-flow theory is typical, is similar to modern chronobiology. Theoretically, the function of Chinese medicine will be most effective at driving out pathogenic factors when the function of some meridian is at its peak[31]. For example, erythrocyte C3b receptor rosette and erythrocyte immune complex rosette can reach peak value when the kidney meridian has its most active function[32].

Along with the improvement of modern technology, the research on the relationship between the dosing time of Chinese medicine and plasma concentration or curative effect has been performed. Nishioka *et al*[33] demonstrated that the dosing time of Sho-Saiko-To could affect the plasma concentrations of the effective components (glycyrrhizin and baicalein). Additionally, the pharmacokinetic processes of emodin and aloe-emodin of DCQD presented a circadian rhythm phenomenon[34]. According to our study, the oral dosing time also affects the drug tissue concentrations. Therefore, the oral dosing time of DCQD is closely related to its pharmacokinetics and pharmacodynamics.

In this study, the pathological damages of pancreatic tissues had been improved only in the 12hG(b) group, and was not improved in the 4hG(b) or 24hG(b) groups. However, this finding was not consistent with those of a previous study showing that a similar dose of DCQD 2 h after AP induction confers some protection against pancreatic tissue damage. Although the weight of rats showed no difference, this finding may be related to the different experimenters, which may have resulted in large differences among the various groups. We should ensure the consistency of experimenters. In clinical practice, orally dosing or coloclysis of DCQD are performed immediately when patients with AP are admitted to hospital. Whether these approaches could increase secretion of the gastrointestinal tract remains unclear. Recently, little research on the optimal administration time of Chinese herbs has been reported, and there are no definite opinions on this topic in the Chinese guidelines, although the guidelines are generally used in clinical practice in China. Therefore, our conjecture needs further clinical studies to be confirmed.

In conclusion, AP and the oral administration time of DCQD could affect the pharmacokinetics of the absorbed components of DCQD in the pancreatic tissues and plasma of rats. Late-time dosing may result in higher concentrations of the major components of DCQD with better pharmacokinetics and pharmacodynamics of anti-inflammation than seen with early-time dosing, thereby showing the late time to be the optimal dosing time of DCQD for AP.

ARTICLE HIGHLIGHTS

Research background

Oral administration with Da-Cheng-Qi decoction (DCQD) is the conventional therapy at the early phase of acute pancreatitis (AP) patients in China. But oral dosing with DCQD is contrary to the idea of pancreatic rest at the early stage of AP, which may inhibit the absorption of the components of DCQD, influence its pharmacokinetics or pharmacodynamics and even worsen the disease severity.

Research motivation

The necrosis of pancreatic acinar cells would worsen the disease and the induction of apoptosis would relieve the disease severity. In clinical practice, oral dosing or coloclysis of DCQD are performed immediately when patients with AP are admitted to hospital. Whether these approaches could increase secretion of gastrointestinal tract remain unclear. What's more, little research on the optimal administration time of Chinese herbs has been reported, and there are no definite opinions on this topic in the Chinese guidelines. Thus, based on effect of DCQD regulating the apoptosis/necrosis switch of pancreatic acinar cells to ameliorate the pancreatic inflammation and pathological damage, the study aimed to screen the optional oral dosing time of DCQD in rats with AP according to the pharmacokinetics of the absorbed components and the pharmacodynamics of DCQD targeting of pancreas.

Research objectives

This objective was to screen the optional oral dosing time of DCQD in rats with AP based on the pharmacokinetic and pharmacodynamic parameters. We hoped to find an optimal dosing time of DCQD without increasing the severity of AP.

Research methods

This animal experiment was divided into pharmacokinetic and pharmacodynamic parts. AP models were induced by 3% sodium taurocholate. Rats were dosed at three different times. Plasma samples were collected from the tails at nine different times. The main components' concentrations of plasma and pancreatic tissues were detected by high-performance liquid chromatography tandem mass spectroscopy, confirmed as a specific, sensitive, accurate and reproducible method. The pharmacokinetic parameters [the maximum plasma concentration (C_{max}), the time to reach maximum concentration (T_{max}), the mean residence time ($MRT_{0\rightarrow t}$), the elimination half-life ($T_{1/2}$) and the area under the plasma concentration-time curve from time 0 to the time of the last measurable concentration ($AUC_{0\rightarrow t}$)] were processed by pharmacokinetic statistical software DAS2.0.1 programmed by the Chinese Pharmacological Society. The IL-10, IL-6 and amylase concentrations in serum and pathological scores of pancreatic tissues were calculated.

Research results

According to the present study, AP reduced the concentrations of the major components of DCQD to the target pancreas, and the oral administration time also played an important role. AP delayed the T_{max} and reduced the C_{max} of the components of DCQD in the circulation of rats. The $AUC_{0\rightarrow t}$ was larger when that time was approximately 12 h after the onset of AP. DCQD increased the IL-10 levels and lowered the IL-6 levels, and the later administration of the DCQD dose corresponded to higher IL-10 levels. Therefore, administering the DCQD dose too early may not be appropriate for AP. However, our results need further clinical studies to be confirmed.

Research conclusions

Late-time dosing may result in higher concentrations of the major components of DCQD with better pharmacokinetics and pharmacodynamics of anti-inflammation than seen with early-time dosing, thereby showing the late time to

be the optimal dosing time of DCQD for AP.

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ORIGINAL ARTICLE

Retrospective Study

Short- and long-term results of endoscopic ultrasound-guided transmural drainage for pancreatic pseudocysts and walled-off necrosis

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Author contributions: Watanabe Y and Mikata R designed research; Watanabe Y, Mikata R, Yasui S, Ohyama H, Sugiyama H, Sakai Y and Tsuyuguchi T acquired the data; Watanabe Y and Mikata R analyzed and interpreted data; Watanabe Y drafted the manuscript; Mikata R and Kato N made critical revisions related to important intellectual content of the manuscript; all authors have read and approved the final version to be published.

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Informed consent statement: In this retrospective study, written informed consent was not provided by the participants, but the documents explaining how the data included in this study would be used were displayed on the bulletin board in Chiba University Hospital.

Conflict-of-interest statement: The authors have no conflicts to disclose. All authors disclosed no financial relationships relevant to this publication.

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Abstract

AIM

To evaluate the short- and long-term results of endoscopic ultrasound-guided transmural drainage (EUS-GTD) for pancreatic fluid collection (PFC) and identify the predictive factors of treatment outcome for walled-off necrosis (WON) managed by EUS-GTD alone.

METHODS

We investigated 103 consecutive patients with PFC who

underwent EUS-GTD between September 1999 and August 2015. Patients were divided into four groups as follows: WON (n = 40), pancreatic pseudocyst (PPC; n = 11), chronic pseudocyst (n = 33), and others (n = 19). We evaluated the short- and long-term outcomes of the treatment. In cases of WON, multiple logistic regression analyses were performed to identify the predictor variables associated with the treatment success. In addition, PFC recurrence was examined in patients followed up for more than 6 mo and internal stent removal after successful EUS-GTD was confirmed.

RESULTS

In this study, the total technical success rate was 96.1%. The treatment success rate of WON, PPC, chronic pseudocyst, and others was 57.5%, 90.9%, 91.0%, and 89.5%, respectively. Contrast-enhanced computed tomography using the multivariate logistic regression analysis revealed that the treatment success rate of WON was significantly lower in patients with more than 50% pancreatic parenchymal necrosis (OR = 17.0; 95%CI: 1.9-150.7; P = 0.011) and in patients with more than 150 mm of PFC (OR = 27.9; 95%CI: 3.4-227.7; P = 0.002).The recurrence of PFC in the long term was 13.3% (median observation time, 38.8 mo). Mean amylase level in the cavity was significantly higher in the recurrence group than in the no recurrence group (P = 0.02).

CONCLUSION

The reduction of WON by EUS-GTD alone was associated with the proportion of necrotic tissue and extent of the cavity. The amylase level in the cavity may be a predictive factor for recurrence of PFC.

Key words: Endoscopic ultrasound-guided transmural drainage; Pancreatic fluid collection; Revised Atlanta Classification; Walled-off necrosis

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Core tip: It remains unclear that which patients with walled-off necrosis (WON) can be resolved by endoscopic ultrasound-guided transmural drainage (EUS-GTD) alone and which ones should be treated by endoscopic necrosectomy or other additional treatment. In addition, some pancreatic fluid collections (PFCs) develop recurrent fluid collection, and it is also unclear which types of PFCs show recurrence. In this study, we demonstrated that PFC size and proportion of pancreatic parenchymal necrosis were related to the resolution of WON treated by EUS-GTD alone. Regarding long-term follow-up patients, mean amylase level in the cavity was associated with PFC recurrence, suggesting a prolonged stent placement in patients with predicted recurrence.

Watanabe Y, Mikata R, Yasui S, Ohyama H, Sugiyama H, Sakai Y, Tsuyuguchi T, Kato N. Short- and long-term results of endoscopic ultrasound-guided transmural drainage for pancreatic pseudocysts and walled-off necrosis. *World J Gastroenterol* 2017; 23(39): 7110-7118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7110.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7110

INTRODUCTION

Pancreatic fluid collection (PFC) is a local complication after pancreatitis. Although most PFCs spontaneously improve, some PFCs remain and become infectious, thereby needing therapeutic intervention. In 2012, the revision of the Atlanta classification categorized PFC into the following four types: acute peripancreatic fluid collection, acute necrotic collection, pancreatic pseudocyst (PPC), and walled-off necrosis (WON). According to this revised classification, the development of PPC is considered to be extremely rare in acute pancreatitis, and most PFCs over 4 wk are classified as WON[1]. This suggests that many PFCs after acute pancreatitis that were treated as PPC because of slight debris in the cavity should be addressed as WON, according to the revised classification.

Endoscopic ultrasound-guided transmural drainage (EUS-GTD) is now widely accepted as a minimally invasive method for managing PFC with minimal complications[2]. According to previous reports, most patients with PPC achieved treatment success by EUS-GTD alone[3-6]. Conversely, the treatment success rate of WON by EUS-GTD alone was relatively lower than that of PPC[4,7]. Endoscopic necrosectomy (EN) was performed in patients with no clinical improvement by EUS-GTD. In a recent review of 10 series of EN, the overall treatment success rate was 76%, with 5% procedure-related mortality and 27% morbidity[8]. Although EN is less invasive than surgical necrosectomy, serious complications associated with EN have been reported lately. More recently, novel, fully covered biflanged metal stents have been reported to be effective and feasible for the treatment of WON[9-12]. However, the criteria for WON that should be treated by EN or/and with these metal stents remain unclear. Therefore, it is essential to clarify the cases that can be resolved by EUS-GTD alone and those that should be treated with EN and/or metallic stents in addition to EUS-GTD. To the best of our knowledge, to date, no report has investigated the predictive factors of treatment outcome managed by EUS-GTD alone using the definition of WON in the 2012 Atlanta classification.

Despite initial treatment success, some PFCs develop recurrent fluid collection owing to disconnected pancreatic duct syndrome (DPDS)[13,14]. Although long-term PFC recurrence is unknown, recommendations for permanent stent placement have been reported; however, stent migration or obstruction should be considered[13].

In this study, we classified PFC into the following four groups according to the 2012 Atlanta classification: WON; PPC; chronic pseudocyst; and others, including trauma, pancreatic cancer, and pancreatic fistula (after pancreatic surgery). We evaluated patients characteristics, technical success, treatment success, and complications in these four groups. In particular, we compared the clinical features between the treatment success and failure to identify the factors that affect the treatment outcome in patients with WON managed by EUS-GTD alone. The long-term follow-up results of patients who underwent EUS-GTD were assessed and predictive factors for recurrence of PFC were identified.

MATERIALS AND METHODS

Patients

We retrospectively investigated 103 consecutive patients with PFC who underwent EUS-GTD between September 1999 and August 2015 at Chiba University Hospital (Chiba, Japan). Mean age of patients was 54.7 years, and a majority of patients were males. PFC caused by acute pancreatitis was classified according to the 2012 Atlanta classification and definition. We distinguished between WON and PPC by contrast-enhanced computed tomography (CT) 1-4 wk after the onset of pancreatitis and evaluated the existence and extent of pancreatic parenchymal necrosis in all patients with PFC. A chronic pseudocyst is defined as a well-demarcated fluid collection without solid debris occurring in the setting of known chronic pancreatitis and the absence of recent severe acute pancreatitis[14]. Indications for EUS-GTD were as follows: (1) infected cases (fever and leukocytosis) despite the administration of intravenous antibiotics; and (2) symptomatic cases, such as abdominal pain or obstruction of the gastric outlet, intestinal system, or biliary system. Informed procedural consents were obtained from all patients.

Procedures

Before EUS-GTD, a CT scan was obtained from all patients. The standard technique for EUS-GTD involved the following steps. A curved linear array EUS was used to visualize the extent of PFC and to determine the puncture site. Before puncturing, color Doppler was used to identify the regional vessels that needed to be avoided. The cavity was punctured with a 19-gage needle, and then PFC was performed for conducting blood biochemical tests and cultures. A guidewire was inserted through the puncture needle and coiled in PFC under fluoroscopic guidance. The punctured site was dilated by a dilator and a balloon dilator (sometimes an electric dilator was also used). Finally, a 7Fr double pigtail stent and a 7Fr nasocystic drainage catheter were emplaced. The nasocystic drainage catheter was removed after reduction of PFC, and the internal stent was removed within 6 mo after the treatment success.

We assessed the efficacy of EUS-GTD using CT. If the size of the cavity was not reduced after 1-2 wk, we performed additional procedures, such as the multiple transmural gateway technique, percutaneous drainage, or EN.

Outcomes

In this study, we defined technical success as achieving stent and/or nasocystic drainage catheter placement. We defined the treatment success as any reduction in the cavity size to less than 20 mm within 8 wk, as determined by a follow-up CT using EUS-GTD alone, without an additional treatment such as multiple transmural gateway technique, percutaneous drainage, or EN. The treatment success also included the improvement of symptoms. Recurrence was considered to have occurred if the size of the cavity increased to more than 20 mm, regardless of symptoms over 6 mo after EUS-GTD.

Statistical analysis

The factors associated with the clinical success and recurrence were determined using statistical comparisons. Continuous variables were presented as means (with standard deviations) and medians (with range) and compared using Mann-Whitney *U* test. Categorical variables were expressed as frequencies and proportions and compared using χ^2 tests with Yates' correction or Fisher's exact test. In patients with WON, multiple logistic regression analyses were performed to identify the predictor variables associated with the treatment success. The optimal cut-off value of the variables that differentiated between recurrence and no recurrence was determined by the receiver-operating characteristic analysis. In addition, the area under the curve was calculated. The statistical significance was determined as $P < 0.05$, and datasets were compiled using Microsoft Excel (Microsoft Corporation, Redmond, WA, United States). In addition, the IBM SPSS Statistics software version 20.0 (IBM Corporation, Chicago, IL, United States) was used to perform all the statistical analyses. The statistical methods of this study were reviewed by Kengo Nagashima, PhD from Department of Global Clinical Research, Graduate School of Medicine, Chiba University.

RESULTS

Patients

According to the 2012 Atlanta classification and definition for acute pancreatitis, each PFC was classified as WON (n = 40), PPC (n = 11), chronic pseudocyst (n = 33), and others (n = 19) for 103 patients who underwent EUS-GTD. More than 50% of PFCs were located mainly in the pancreatic body or tail. Mean

Table 1 Demographic and clinical characteristics of patients who underwent endoscopic ultrasound-guided transmural drainage *n* (%)

characteristics	Value
Age, yr	
mean (SD)	54.7 (15.5)
Range	15-89
Median	56
Gender	
Male	73 (70.9)
Female	30 (29.1)
Type of pancreatitis	
Acute pancreatitis	46(44.6)
Chronic pancreatitis	38 (36.9)
Other	19 (18.4)
Etiology of PFC	
Alcohol	51 (49.5)
Idiopathic	17 (16.5)
Gallstones	15 (14.5)
Trauma	6 (5.9)
Post-surgery (pancreatic fistula)	7 (6.8)
Post-ERCP	3 (2.9)
Pancreatic cancer	4 (3.9)
Category of PFC	
WON	40 (38.8)
Pancreatic pseudocyst	11 (10.7)
Chronic pseudocyst	33 (32.0)
Others (cancer/trauma/fistula)	19 (18.5)
Main location of cavity	
Head	19 (18.4)
Body or tail	84 (81.6)
Size of cavity, mm (long axis)	
mean (SD)	104.4 (49.0)
Range	30-246
Median	100

EUS-GTD: Endoscopic ultrasound-guided transmural drainage; PFC: Pancreatic fluid collection; SD: Standard deviation; ERCP: Endoscopic retrograde cholangio-pancreatogram; WON: Walled-off pancreatic necrosis.

cavity size was 104.9 mm (Table 1).

Technical success

Of 103 patients, 4 technically failed, resulting in the technical success rate of 96.1% (95%CI: 90.3%-98.9%). In the technically failed cases, each patient required one of the following additional treatments: surgery, transpapillary drainage, percutaneous drainage, extracorporeal shock wave lithotripsy (ESWL), and observation.

Treatment success

In the WON group (n = 40), a technical failure was reported in 1 patient and treatment success in 23 patients (57.5%; 95%CI: 40.9%-73.0%). The treatment success group comprised 2 patients who underwent percutaneous drainage for PFC distant from the main lesion treated by EUS-GTD. We could not achieve the treatment success by EUS-GTD alone in 16 patients, of whom 2 needed surgical treatment, 11 needed no surgical treatment, and 1 died. In the group treated without surgery, we performed EN in 5 patients, multiple transluminal gateway technique in 2 patients, percutaneous drainage in 5 patients, and continuing conservative treatment until reduction over 8 wk in 3 patients. After additional treatment, 2 patients died. All 3 patients who died after EUS-GTD or EN had a respiratory or renal failure.

In the PPC group (n = 11), there were no technical failures, and we achieved the treatment success in 10 patients (90.9%; 95%CI: 58.4%-99.8%). In the chronic pseudocyst group (n = 33), there were two technical failures, and of 31 patients, we achieved the treatment success in 30 patients (96.8%; 95%CI: 83.8%-99.9%). In the treatment success group of chronic pancreatitis, 3 patients underwent EUS-GTD for PFC distant from the main lesion treated by EUS-GTD. In the others group (n = 19), there was one technical failure, and we achieved the treatment success in 17 patients (94.4%; 95%CI: 73.2%-99.9%).

Complications

The procedural complications were encountered in 15 of 103 patients (14.6%), with cases of bleeding (n = 2), stent migration (n = 3), infection (n = 3), pneumoperitoneum (n = 2), localized peritonitis (n = 3), puncture into another organ (n = 1), and mediastinal emphysema (n = 1). All patients were managed conservatively without surgery.

Predictors of treatment success for WON

We compared the treatment success group with the treatment failure group. Patients with more than 50% pancreatic parenchymal necrosis (P = 0.004) and a PFC of more than 150 mm (P < 0.001) on CT were significantly associated with treatment failure based on the univariate analysis. However, PFC with infection was not significant (Table 2). Patients with more than 50% pancreatic parenchymal necrosis (OR = 17.0; 95%CI: 1.9-150.7; P = 0.011) and with a PFC of more than 150 mm (OR = 27.9; 95%CI: 3.4-227.7; P = 0.002) on CT were also significantly associated with treatment failure based on the multiple logistic regression analysis (Table 3).

The treatment success by EUS-GTD alone was not achieved in any patient with WON with more than 50% parenchymal necrosis and a with PFC of more than 150 mm diameter on CT but was achieved in 90% of patients with under 50% pancreatic parenchymal necrosis and with a PFC of less than 150 mm (Table 4). There were two cases of failure with fewer than 50% necrosis and with a PFC of less than 150 mm. In one case with a multilocular type of WON, the necrotic collection remained in the posterior pararenal extraperitoneal space, and we performed percutaneous drainage. In another case, extrapancreatic necrosis without pancreatic parenchymal necrosis extended widely, and EN was conducted.

Table 2 Comparison of patient demographics and clinical characteristics in patients underwent endoscopic ultrasound-guided transmural drainage of walled-off necrosis *n* (%)

		Treatment success		*P* value
		Yes (*n* = 23)	No (*n* = 16)	
Age, yr	Mean (SD)	57.8 (18.1)	55.8 (13.1)	
	Range	15-85	30-83	0.484
Gender	Male	16 (69.6)	14 (87.5)	0.359
Etiology of pancreatitis	Alcohol	8 (34.8)	6 (37.5)	0.862
Body mass index	Mean (SD)	23.3 (5.5)	24.2 (3.6)	0.203
ASA classification ⩾ 3	Yes	12 (52.2)	13 (81.2)	0.09
Pancreatic parenchymal necrosis ⩾ 50%	Yes	2 (8.7)	9 (56.3)	0.004
Duration from onset of pancreatitis to drainage, wk	Mean (SD)	11.1 (7.8)	9.4 (10.9)	
	Range	3.1-25.3	2.0-47.7	0.219
Size of cavity, mm (long axis)	Mean (SD)	109.9 (35.7)	156.9 (35.7)	
	Range	70-246	66-207	< 0.001
Size of cavity ⩾ 150 mm	Yes	2 (8.7)	11 (68.8)	< 0.001
PFC with infection	Yes	13 (56.5)	13 (81.3)	0.203
Follow-up durations, mo	Mean (SD)	26.9 (30.8)	28.1 (33.9)	0.808
	Range	0.7-133.5	1.3-128.3	

EUS-GTD: Endoscopic ultrasound-guided transmural drainage; WON: Walled-off pancreatic necrosis; PFC: Pancreatic fluid collection; SD: Standard deviation.

Table 3 Multiple logistic regression analysis examining factors associated with treatment success for walled-off necrosis

Multiple logistic regression	OR	95%CI	*P* value
Pancreatic parenchymal necrosis (< 50% *vs* ⩾ 50%)	17.0	1.9-150.7	0.011
Size of cavity (< 150 mm *vs* ⩾ 150 mm)	27.9	3.4-227.7	0.002

Table 4 Treatment success rate of walled-off necrosis according to two parameters; pancreatic parenchymal necrosis and size of cavity

	Size of cavity < 150 mm	Size of cavity ⩾ 150 mm
Pancreatic parenchymal necrosis < 50%	90.5% (19/21)	28.6% (2/7)
Pancreatic parenchymal necrosis ⩾ 50%	40.0% (2/5)	0% (0/6)

Recurrence at long-term follow-up

Overall, 75 patients were followed up for more than 6 mo, and the internal stent removal was confirmed. The median observation time was 38.8 mo. Of 75 patients, 10 patients suffered a recurrence, and the overall recurrence rate was 13.3%. The additional treatment for recurrence cases was additional EUS-GTD for 5 patients, transpapillary drainage for one patient, ESWL for one patient, surgery for 2 patients, and observation for one patient. In the recurrence group, the amylase level in the cavity was significantly higher than that in the no recurrence group (P = 0.01; Table 5) based on the univariate analysis. The amylase level at 63,100 in the cavity represented the most sensitive (83.3%) and specific (78.2%) point on the receiver-operating characteristic curve and corresponded to the largest area under the curve (0.820). No recurrence rate of the amylase level in the cavity ⩾ 63100 was significantly lower than that of the amylase level in the cavity < 63100 (P = 0.02; Figure 1).

DISCUSSION

In this study, we revealed the usefulness and feasibility of EUS-GTD for PFC; however, it was less effective in achieving the treatment success for WON as compared with other etiologies of PFC. The PFC size and the proportion of pancreatic parenchymal necrosis were related to the short-term outcomes of WON. In addition, mean amylase level in the cavity was associated with PFC recurrence, suggesting a prolonged stent placement for patients with predicted recurrence.

The treatment success rate of WON by EUS-GTD alone was 61.1%. Some retrospective studies have demonstrated a 45%-63% treatment success rate of EUS-GTD for WON[4,7]. A comparison of these results with our study is difficult because the definition of treatment success was different in each study, which in turn differed from our study, which was based on the 2012 Atlanta classification. A recent report, based on the 2012 Atlanta classification, revealed that the standard EUS-GTD using plastic stents or self-expandable metal stents resolved 70% of sterile WON and 40% of infected WON; however, the rest of WON required EN[15].

After treatment failure by EUS-GTD for WON, EN or other intervention, including surgical treatment, should be considered. A recent randomized controlled trial suggested that the IL-6 level following EN was significantly lower than that following surgical necrosectomy and that major complications or death occurred less frequently after EN compared to those after surgical necrosectomy[16]. Although EN should be

Table 5 Pancreatic fluid collection recurrence in patients followed-up over 6 mo and confirmed internal stent removal after successful endoscopic ultrasound-guided transmural drainage (*n* = 75) *n* (%)

		Recurrence		*P* value
		Yes (*n* = 10)	No (*n* = 65)	
Age, yr	Mean (SD)	54.4 (12.6)	55.1 (15.9)	
	Range	37-79	15-89	0.719
Gender	Male	9 (90)	45 (69.2)	0.266
Type of pancreatitis	Chronic	6 (60)	22 (33.8)	0.162
Main location of cavity	Head	1 (10)	16 (24.6)	0.677
External drainage only	Yes	2 (20)	9 (13.8)	0.634
Duration of internal stent, days (*n* = 64)	Mean (SD)	194.9 (106.1)	243.2 (217.3)	
	Range	86-399	21-1387	0.659
Spontaneous dislodgement of stent (*n* = 64)	Yes	4 (50)	17 (30.4)	0.421
Size of cavity, mm (long axis)	Mean (SD)	91.6 (38.0)	102.6 (49.3)	
	Range	44-167	30-230	0.612
Amylase in cavity, IU/L (*n* = 57)	Mean (SD)	96930 (55599)	44719 (53790)	
	Range	31380-188000	30-273700	0.011
	Median	83075	31200	
PFC with infection	Yes	2 (20)	28 (43.1)	0.298

PFC: Pancreatic fluid collection; SD: Standard deviation; EUS-GTD: Endoscopic ultrasound-guided transmural drainage.

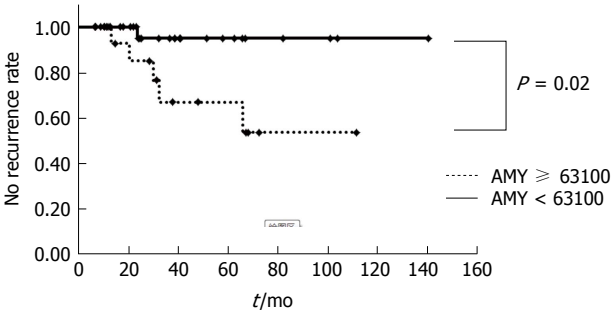


Figure 1 Kaplan-Meier curve comparing no recurrence rate of amylase in cavity ⩾ 63100 with that of amylase in cavity < 63100.

considered for WON that cannot be resolved by EUS-GTD, serious complications of EN have been reported. Recently, while three multiple-center trials revealed 75%-91% success rate of EN, the associated mortality and morbidity rated were 5.8%-11% and 26%-33%, respectively[17-19]. In our study, we performed EN on 4 patients, and one patient died due to multiple organ failure. Therefore, if possible, it is better to accomplish the resolution of WON by EUS-GTD alone.

Some factors associated with the failed resolution of WON by EN or standard drainage and EN have been reported, including body mass index > 32[18], American Society of Anesthesiologists physical status classification ⩾ 3[19], and multilocular morphology[15]. In this study, we elucidated that the treatment success rate of EUS-GTD alone for WON was significantly lower in patients with more than 50% pancreatic parenchymal necrosis or with a PFC of more than 150 mm on CT. All patients with WON with more than 50% parenchymal necrosis and with a PFC of more than 150 mm needed an additional treatment such as EN, whereas the treatment success was achieved in 90% patients with under 50% pancreatic parenchymal necrosis and within a PFC of 150 mm by EUS-GTD alone (Figure 2). If we can predict the treatment outcome by EUS-GTD with these parameters, we may be able to avoid unnecessary invasive therapy or make an earlier decision to perform additional treatment in a few days.

Instead of EUS-GTD, multiple transmural gateway technique for WON has been reported, and the treatment success rate of multiple transmural gateway technique was significantly better than that of EUS-GTD (94.4% *vs* 62.1%)[20,21]. More recently, novel, fully covered biflanged metal stents have been reported to be effective for the treatment of WON[9-12]. Regarding the use of these stents, high cost, stent migration, and other potential adverse events have been concerning[22]; therefore, optimal selection for using these stents is needed. According to our study, patients with PFC more than 50% pancreatic parenchymal necrosis or patients with more than 150 mm may be appropriate candidates for these treatments.

The proportion of pancreatic parenchymal necrosis could be associated with the amount of solid debris in the cavity of WON. Reportedly, the morphological findings of WON on EUS have therapeutic implications owing to the large size and more solid debris needing a more aggressive therapeutic method[23]. However, it is often challenging to estimate the necrotic component with the whole observation of PFC on EUS. In addition, even if rich debris seemed to exist in the cavity by EUS, some cases of WON could be resolved by EUS-GTD alone, as shown in Figure 2.

In this study, PPC was relatively rare (10.7%) based on the values in the 2012 Atlanta classification. Notably, 8 of 11 patients categorized as PPC were caused by acute pancreatitis occurring in the setting of known chronic pancreatitis and 3 were atypical etiology of pancreatitis (drug-induced in 2 patients and idiopathic in 1 patient). The treatment success rate of 90.9% for PPC in this study was higher than that of WON.

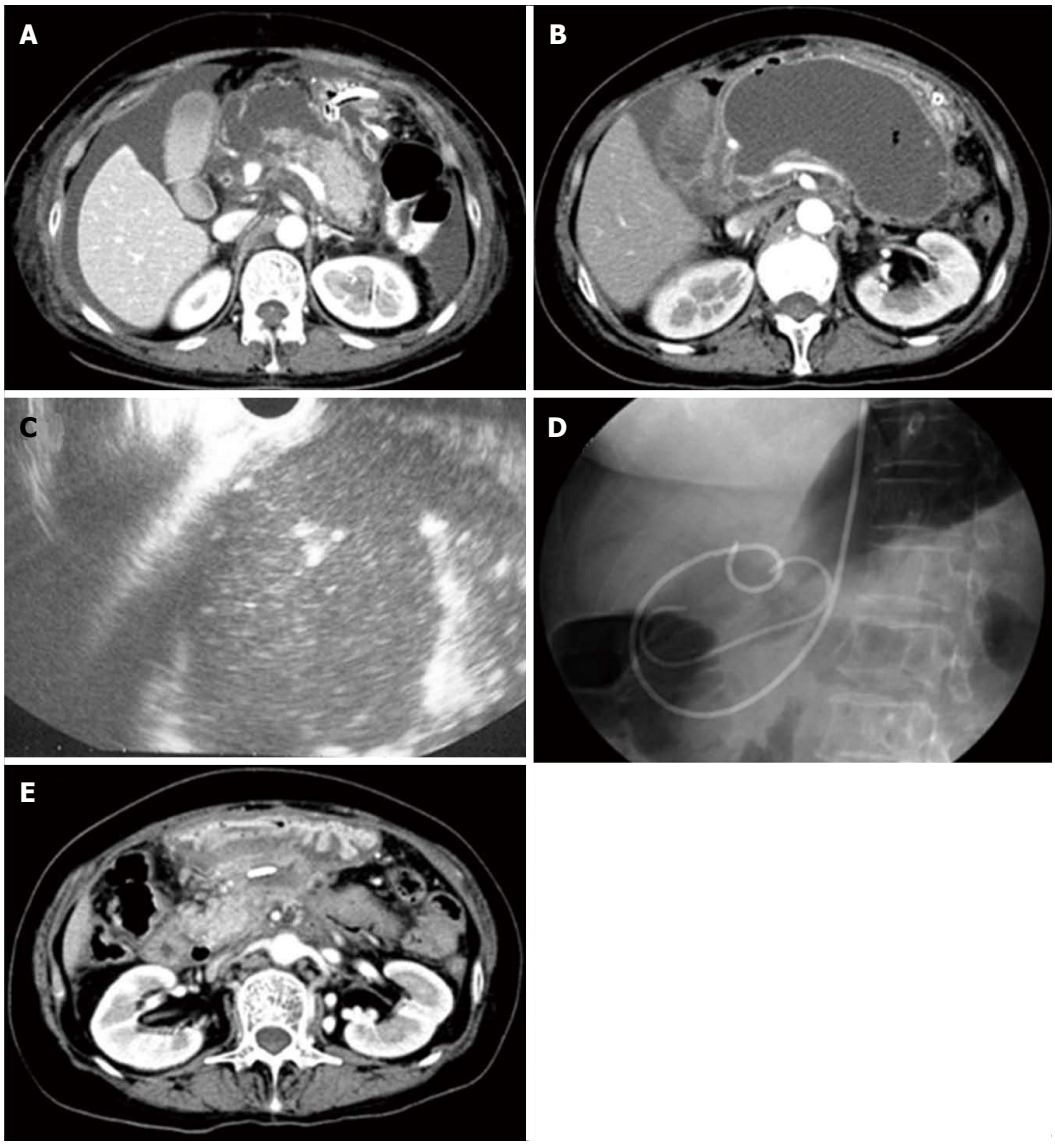


Figure 2 Representative case of walled-off necrosis resolved by endoscopic ultrasound-guided transmural drainage alone. A: Computed tomography of the abdomen (axial image) showing less than 50% pancreatic parenchymal necrosis; B: Pancreatic fluid collection was 135 mm in diameter; C: Rich debris seemed to exist in the cavity as shown by endoscopic ultrasound; D: Endoscopic ultrasound guided transmural drainage was performed and pancreatic fluid collection culture was positive; E: Cavity was reduced at 6 wk after intervention.

EUS-GTD for chronic pseudocysts was successful. In chronic pancreatitis, PFC often communicates with the main pancreatic duct and stricture of this duct exists. To manage chronic pseudocysts, a combination of EUS-GTD with transpapillary drainage or ESWL for pancreatic stones might be effective.

After successful initial treatment following EUS-GTD for PFC, recurrence of PFC in the long term was higher in patients with higher amylase level in the cavity, indicating the communication of PFC with the pancreatic duct such as chronic pseudocysts or DPDS. DPDS is characterized by the main pancreatic duct cut-off, with an inability to access the upstream pancreatic duct during an ERCP, and CT evidence of viable pancreatic tissue upstream (toward the spleen), in association with a persistent non-healing pancreatic fistula or PFC[24,25]. We routinely removed internal stents following the resolution of PFC almost 6 mo after EUS-GTD. Although a permanent stent placement significantly reduced PFC recurrence in comparison with scheduled stent removal, migrated stents caused bowel obstruction that required surgery[21]. In cases with higher amylase levels in the cavity, transpapillary treatment or prolonged stent placement for more than 6 mo should be considered. However, the timing of stent removal and permanent stent placement remains controversial, and further study is required.

There were some limitations in this study. First, this was a retrospective study conducted at a single tertiary center and the number of patients with WON was relatively less. Second, the study period was long (16 years) and a learning curve might have influenced the results, although the standard technique for EUS-GTD has not changed. Third, there may have

been a selection bias because we could not examine all patients with the amylase level in the cavity consecutively.

In conclusion, EUS-GTD is a successful and safe therapeutic technique in a majority of patients with PFC. The cavity size and proportion of pancreatic parenchymal necrosis are predictors for a successful treatment of WON. Higher amylase levels in the cavity might lead to PFC recurrence after stent removal. A prolonged stent placement should be considered in such cases.

ARTICLE HIGHLIGHTS

Research background

The 2012 Atlanta classification categorized PFC into four types. The revised classification suggests that many PFCs after acute pancreatitis that were treated as pancreatic pseudocyst (PPC) because of slight debris in the cavity should be addressed as walled-off necrosis (WON). Most patients with PPC achieved treatment success by EUS-GTD alone. Although endoscopic necrosectomy (EN) was performed in patients with no clinical improvement by EUS-GTD, serious complications of EN have been reported. However, the criteria for WON that should be treated by EN or other additional treatment remained unclear. Therefore, it is crucial to clarify the cases that can be resolved by EUS-GTD alone and those that should be treated with EN or other treatment in addition to EUS-GTD. Some PFCs develop the recurrent fluid collection in long term. Recommendations of permanent stent placement have been reported to reduce recurrence; however, stent migration or obstruction is concerned. A predictive factor of PFC recurrence should also be clarified.

Research motivation

The treatment success rate of WON by EUS-GTD alone was relatively lower than that of PPC. It is unclear that which patients with WON can be treated by EUS-GTD alone and which ones by EN or other additional treatment including metallic stents. Despite initial treatment success, some PFCs develop the recurrent fluid collection in the long term. It is also unclear which types of PFCs show recurrence.

Research objectives

We aimed to evaluate the short- and long-term results of EUS-GTD for PFC following a revision of the PFC framework by the 2012 Atlanta classification and identify the predictive factors of treatment outcome for WON managed by EUS-GTD alone and predictive factors for recurrence of PFC.

Research methods

The authors retrospectively investigated 103 consecutive patients with PFC who underwent EUS-GTD between September 1999 and August 2015 at Chiba University Hospital. The factors associated with clinical success and recurrence were determined using statistical comparisons. In patients with WON, multiple logistic regression analyses were performed to identify the predictor variables associated with the treatment success. In addition, PFC recurrence was examined in patients followed up over 6 mo and confirmed internal stent removal after successful EUS-GTD. The optimal cut-off value of the variables that differentiated between recurrence and no recurrence was determined by the receiver-operating characteristic analysis. In addition, area under the curve was calculated. The statistical significance was determined as $P < 0.05$.

Research results

The treatment success rate of WON, PPC, chronic pseudocyst, and others was 57.5%, 90.9%, 91.0%, and 89.5%, respectively. The treatment success rate of WON was significantly lower in patients with more than 50% pancreatic parenchymal necrosis (OR = 17.0: 95%CI: 1.9-150.7; $P = 0.011$) and in patients with more than 150 mm of PFC (OR = 27.9; 95%CI: 3.4-227.7; $P = 0.002$) on contrast-enhanced computed tomography using the multivariate logistic regression analysis. The recurrence of PFC in the long term was 13.3% (median observation time, 38.8 mo). Mean amylase level in the cavity was significantly higher in the recurrence group than in the no recurrence group ($P = 0.02$). In cases with higher amylase levels in the cavity, transpapillary treatment or prolonged stent placement for more than 6 mo should be considered. However, the timing of stent removal and permanent stent placement remains controversial, and further study is required.

Research conclusions

Reduction of WON by EUS-GTD alone was associated with the proportion of necrotic tissue and the extent of the cavity. Amylase level in the cavity may be a predictive factor for recurrence of PFC.

According to our study, additional treatments, such as EN, should be considered after EUS-GTD in patients with WON with more than 50% parenchymal necrosis and a PFC of more than 150 mm, whereas it may not be needed with under 50% pancreatic parenchymal necrosis and within a PFC of 150 mm.

After successful initial treatment following EUS-GTD for PFC, recurrence of PFC in the long term was higher in patients with higher amylase level in the cavity. This finding indicates the communication of PFC with the pancreatic duct such as chronic pseudocyst or disconnected pancreatic duct syndrome. Therefore, in cases with higher amylase levels in the cavity, transpapillary treatment or prolonged stent placement should be considered.

Research perspectives

In this study, we confirmed that the PFC size and the proportion of pancreatic parenchymal necrosis were related to the resolution of WON treated by EUS-GTD alone. If we can predict treatment outcome of WON by EUS-GTD alone, we might be able to avoid unnecessary invasive therapy or make an earlier decision to perform an additional treatment. Moreover, if we can predict recurrence of PFCs by the amylase level in the cavity, we might be able to reduce the recurrence of PFCs with prolonged stent placement or transpapillary treatment. Prospective studies with larger numbers of patients will be needed to confirm the reliability of these predictive factors.

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ORIGINAL ARTICLE

Retrospective Study

Laparoscopic finding of a hepatic subcapsular spider-like telangiectasis sign in biliary atresia

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Abstract

AIM

To assess the diagnostic value of a laparoscopic finding of a hepatic subcapsular spider-like telangiectasis (HSST) sign in biliary atresia.

METHODS

A retrospective study was conducted first and then a validation set was used to investigate the value of an HSST sign in predicting biliary atresia (BA). In the

retrospective study, laparoscopic images of the liver surface were reviewed in 126 patients with infantile cholestasis (72 BA patients and 54 non-BA cholestasis patients) and a control group of 38 patients with non-hepatic conditions. Analysis was first made by two observers separately and finally, a consensus conclusion was achieved. Then, the diagnostic value of the HSST sign was validated in an independent cohort including 45 BA and 45 non-BA patients.

RESULTS

In the retrospective investigation, an amplified HSST sign was found in all BA patients, while we were unable to detect the HSST sign in 98.1% of the 54 non-BA patients. There was no HSST sign in any of the control subjects. In the first review, the sensitivity and specificity from one reviewer were 100% and 98.1%, respectively, and the results from the other reviewer were both 100%. The consensus sensitivity and specificity were 100% and 98.1%, respectively. The HSST sign was defined as being composed of several enlarged tortuous spider-like vascular plexuses with two to eight branches distributed on all over the liver surface, which presented as either a concentrated type or a dispersed type. In the independent validation group, the sensitivity, specificity, positive predictive value and negative predictive value of the HSST sign were 100%, 97.8%, 97.8% and 100%, respectively.

CONCLUSION

The HSST sign is characteristic in BA, and laparoscopic exploration for the HSST sign is valuable in the diagnosis of BA.

Key words: Laparoscopic hepatic subcapsular spider-like telangiectasis sign; Infantile cholestasis; Biliary atresia; Infantile hepatitis; Laparoscopy; Diagnosis; Pediatric surgery

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Core tip: Color Doppler ultrasound finding of hepatic subcapsular flow has shown much potential for discriminating biliary atresia (BA). While in clinical practice, we have noticed a similar but more intuitive phenomenon - a laparoscopic finding of hepatic subcapsular spider-like telangiectasis (HSST) sign, which may be a specific marker for BA. Based on the current data, we found that the sensitivity and specificity of the HSST sign were each generally close to 100%.

Zhou Y, Jiang M, Tang ST, Yang L, Zhang X, Yang DH, Xiong M, Li S, Cao GQ, Wang Y. Laparoscopic finding of a hepatic subcapsular spider-like telangiectasis sign in biliary atresia. *World J Gastroenterol* 2017; 23(39): 7119-7128 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7119.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7119

INTRODUCTION

Biliary atresia (BA), as a common neonatal cholangiopathy that leads to cirrhosis[1], is the most common indication for liver transplantation in infants[2,3]. The incidence of BA ranges from 1 in 5000 (Asian countries) to 1 in 19000 (European countries) live births, and it presents with typical symptoms and signs such as persistent jaundice, acholic stools and hepatomegaly within the first months of life[2]. This disease is characterized by a progressive fibro-obliterative obstruction of extrahepatic bile ducts. A younger age (in the first 2 mo of life) at the time of Kasai portoenterostomy has been found to be life-saving in restoring bile flow and preventing the worsening of liver function[4,5]. Thus, early discrimination is vital for the prognosis. Nevertheless, it is still challenging to differentiate BA from other cholestatic diseases as there are no characteristic manifestations found in the early stage of BA.

Clinic features, such as prolonged jaundice, pale stools and high conjugated hyperbilirubinemia, are remarkable, but they are not specific[6]. The ultrasonographic findings of an abnormal gall bladder and triangular cord sign have been widely used in early discrimination of BA[6,7], but the accuracy of these measurements varies[8-15]. A recent study showed that the absence of a gallbladder and the triangular cord sign could both have the highest specificity, up to 99%, but a sensitivity of 28% and 80%, respectively[14]. Other examinations are also compelling, but the value is limited. A duodenal fluid test is difficult to perform. Definite biliary excretion shown by scintigraphy can exclude BA, however, the absence of excretion is not specific in BA because of it can be attributed to any form of severe cholestasis[2,16]. Due to the small diameter of and inadequate fluid in biliary ducts, magnetic resonance cholangiopancreatography (MRCP) plays a limited role in showing the biliary tree in normal infants younger than 3 mo[17]. Therefore, the high false positive rate in predicting BA is inevitable. Moreover, the pathological features of BA are bile ductular proliferation, canalicular and cellular bile stasis, bile plugs, and portal or perilobular fibrosis with preservation of the basic hepatic lobular architecture[5,18,19], which overlap with α-1-antitrypsin deficiency, and occasionally Alagille syndrome, cystic fibrosis, and total parenteral nutrition (TPN)-related cholestasis[5].

Cholangiography is the gold standard, and a definitive diagnosis of BA can be made when cholangiography fails to show the biliary tree[1]. Laparotomy cholangiography was previously used and was refused by some parents because of the abdominal trauma and incision complications, which finally led to the delay of diagnosis. Recently, with the application

of laparoscopy in pediatric surgery, laparoscopy-assisted cholangiography (LAC) has become possible. In our center, we performed laparoscopy-assisted cholangiography on infants who were highly suspected of having BA before the Kasai portoenterostomy was developed[20]. During the procedure, we found that a prominent pathological change on the liver surface - a hepatic subcapsular spider-like telangiectasis (HSST) sign - had a strong correlation with BA.

The current report includes a retrospective study and a prospective study, which aimed to investigate the significance of the laparoscopic finding of an HSST sign in differentiating BA from other causes of neonatal cholestasis.

The study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, and all parents of the patients signed the inform consent. Before the parents signed the consent form for surgery, they were informed that the procedure was minimally invasive and that the procedure would be carried out *via* a small incision at the umbilicus ring and one stab incision in the left abdominal wall. Parents were told that there was a chance of needing additional trocars or conversion to an open technique. Advantages and disadvantages were reviewed to ensure that the parents were fully educated about the procedure.

MATERIALS AND METHODS

Retrospective and clinical investigation

An HSST sign in laparoscopic images was evaluated in cholestasis cases and a control group.

Patients: Laparoscopic images of the liver surface were reviewed in 126 patients with infantile cholestasis who underwent laparoscopic cholecystocholangiography or laparoscopic exploration at our center between February 2011 and February 2015. Among the 126 patients, 72 (31 boys, 41 girls; mean age, 58 d ± 29 d) were diagnosed with BA on the basis of laparoscopic cholangiography or exploration (when cholangiography failed due to the presence of atrophic gallbladder), and the other 54 (24 boys, 30 girls; mean age, 59 d ± 21 d) were diagnosed with non-BA conditions, which included infantile hepatitis (n = 46), biliary hypoplasia (n = 5) and total parenteral nutrition (TPN) induced cholestasis (n = 3). Their final diagnoses were confirmed by LAC, pathological examination or clinical follow-up. Another 38 patients (19 boys, 19 girls; mean age, 63 d ± 22 d) without jaundice or liver disease who underwent laparoscopic surgery served as controls. Their clinical diagnoses were Hirschsprung's disease (n = 34) and intussusception (n = 4).

Laparoscopic findings: Laparoscopic images (5 mm or 10 mm optical laparoscopy, pediatric HOPKINS Ⅱ 26003BA, STORZ, Germany) of the liver surface were evaluated by two observers separately and blindly (Zhou Y and Jiang M) to screen for the presence or absence of the HSST sign. The HSST sign was preliminarily defined as enlarged tortuous spider-like vascular plexuses distributed on the liver surface.

Statistical analysis: The results are expressed as the mean ± SD or number (percentage). For quantitative data, statistical significance between groups was tested using a two-sample t test. For qualitative data (sex), significance between groups was tested by a χ^2 test. Sensitivity and specificity were calculated for the HSST sign with the method of Newcombe[21]. Individual readings were calculated first; consensus readings were calculated thereafter (by professor Tang ST). Meanwhile, we applied Cohen's κ coefficient to assess the agreement between the analyses conducted by the two observers. Logistic regression analysis was applied to determine whether the presence of the HSST sign was useful in discriminating BA. A P-value of < 0.05 was considered statistically significant. SPSS 13.0 software (SPSS, IL, United States) was used for data analyses.

Validation of the HSST sign

The diagnostic value of the HSST sign was validated in an independent cohort composed of 45 BA and 45 non-BA patients.

Patients: Between February 2015 and April 2017, we enrolled a consecutive series of patients who were highly suspected of having BA. The enrollment criteria in our series included the following: (1) icterus was gradually aggravated after the first 3-4 wk; (2) consistent depigmented stool; and (3) traditional diagnostic examinations (*i.e.*, physical examination, blood biochemical examination, ultrasonography, hepatobiliary scintigraphy, MRCP, *etc*.) failed to achieve a definitive diagnosis[20]. The children's general information, including gender, age and biochemical parameters [the level of total and direct serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gammaglutamyl transpeptidase (GGT)] were carefully documented.

Validation protocol: All enrolled patients underwent laparoscopic exploration for the HSST sign before LAC. The patient was considered to be BA when the HSST sign was detected. The final diagnosis was confirmed by LAC.

Pathologic evaluation: After surgery, patients underwent pathologic examination. The hepatic subcapsular spider-like vessels were confirmed by gross inspection, well preserved on the prepared tissue, and evaluated with a microscope.

Statistical analysis: The results are expressed as the mean ± SD or number (percentage). A two-sample *t* test was used to compare quantitative parameters between patients with BA and non-BA patients. A χ^2 test was used to compare the male-to-female ratio between patients with BA and non-BA patients. The cut-off values for optimal clinical performance (best sensitivity and best specificity simultaneously) of individual parameters were determined from the receiver-operating characteristic (ROC) curve. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the HSST sign using the method of Newcombe[21]. A *P*-value of < 0.05 was considered statistically significant. SPSS 13.0 software (SPSS, IL, United States) was used for data analysis.

RESULTS

Retrospective and clinical investigation

The two groups were age- and sex- matched (P > 0.05 for both). The mean levels of total bilirubin, direct bilirubin, AST and ALT in the BA group showed no significant difference compared to the non-BA group. The mean level of GGT was significantly higher in the BA group than in the non-BA group (P = 0.02). Total bilirubin, direct bilirubin, ALT, AST and GGT levels were normal in the controls (Table 1).

An amplified HSST sign was identified in all patients with BA (Figure 1A) and was not found in any of the control subjects by both observers (Figure 1 B). One observer (Jiang M) determined that there was no HSST sign in 53 (98%) non-BA patients (Figure 1C-E), except for one patient with cytomegalovirus (CMV) hepatitis (Figure 1F). The other observer (Zhou Y) had the opinion that there was no HSST sign in all patients with non-BA. After discussion, the discrepancy was resolved. The sensitivity and specificity of the HSST sign were 100% and 98.1%, respectively (Table 2).

The HSST sign was widely distributed on the surface of both left and right hepatic lobes, including on the visceral and diaphragmatic facies (Figure 2). Thus, we redefined the HSST sign as follows: it is composed of several enlarged tortuous spider-like vascular plexuses with four to eight branches distributed all over the liver surface, which presents as a dispersed type (radiating branches originate from more than one central point, but close to each other to form a spider-like sign) (Figure 3A) or a concentrated type (radiating branches originate from one central point) (Figure 3B).

Logistic regression analysis showed that the presence of the HSST sign was a significant predictor of BA (P < 0.01). In addition, the concordance between the two observers was also robust (κ = 0.94, 95%CI: 0.87–1.0).

Validation of the HSST sign

Characteristics of patients: The mean age of the BA group was 60 d ± 29 d. The mean age of the non-BA group was 61 d ± 23 d. In the non-BA group, the final diagnoses were infantile hepatitis (n = 43) and biliary hypoplasia (n = 2). As shown in Table 3, there was no significant difference between the BA and non-BA groups in age or male-to-female ratio (P > 0.05 for both). The mean levels of total bilirubin, direct bilirubin, AST and ALT in BA patients had no significant difference compared to those in the non-BA group. The mean level of GGT was significantly higher in the BA group than in the non-BA group (P < 0.001).

Diagnostic performance of the HSST sign: On high-definition laparoscopic images (5 mm or 10 mm optical laparoscopic, pediatric HOPKINS Ⅱ 26003BA, STORZ, Germany), the HSST sign in all BA patients was easily observed (Figure 4A). There were no HSST sign observed in non-BA patients, except for one patient (Figure 4B). The patient was considered to have BA due to the presence of the HSST sign, whose final diagnosis was biliary hypoplasia. The sensitivity, specificity, PPV and NPV of the HSST sign were 100%, 97.8%, 97.8% and 100%, respectively.

Pathologic evaluation: The hepatic subcapsular spider-like vessels were clearly identified in the stained tissue. The vessels in the BA group were revealed as dilated branches of hepatic arteries in the hepatic subcapsular area (Figure 4C) and the vessels in hypoplasia were revealed as hyperplastic capillaries in the hepatic subcapsular area (Figure 4D).

DISCUSSION

A large sample study focused on GGT showed that it was higher in the BA group than in other cholestatic disorders for all age groups[22], which was coincident with our results. In our validation set, we found that the area under the receiver operating characteristic curve was 0.745 and GGT at a cut-off value of > 404 U/L had a 64.4% sensitivity and 84.4% specificity in discriminating BA. A level of 300 U/L was also previously reported by Liu *et al*[16] with 85% accuracy. However high it may be, we cannot confirm BA directly by GGT alone.

In the entire study, there were 14 BA patients (6 in the retrospective study and 8 in the validation set) below the age of 40 d (with the minimum age of 20 d), in whom the HSST sign was observed but there was no liver cirrhosis at gross inspection (Figure 5A). The HSST sign was also found in 10 patients with BA (8 in the retrospective study and 4 in the validation set) who were older than 90 d (91-163 d) (Figure 5B). Based on the above data, the presence of the HSST

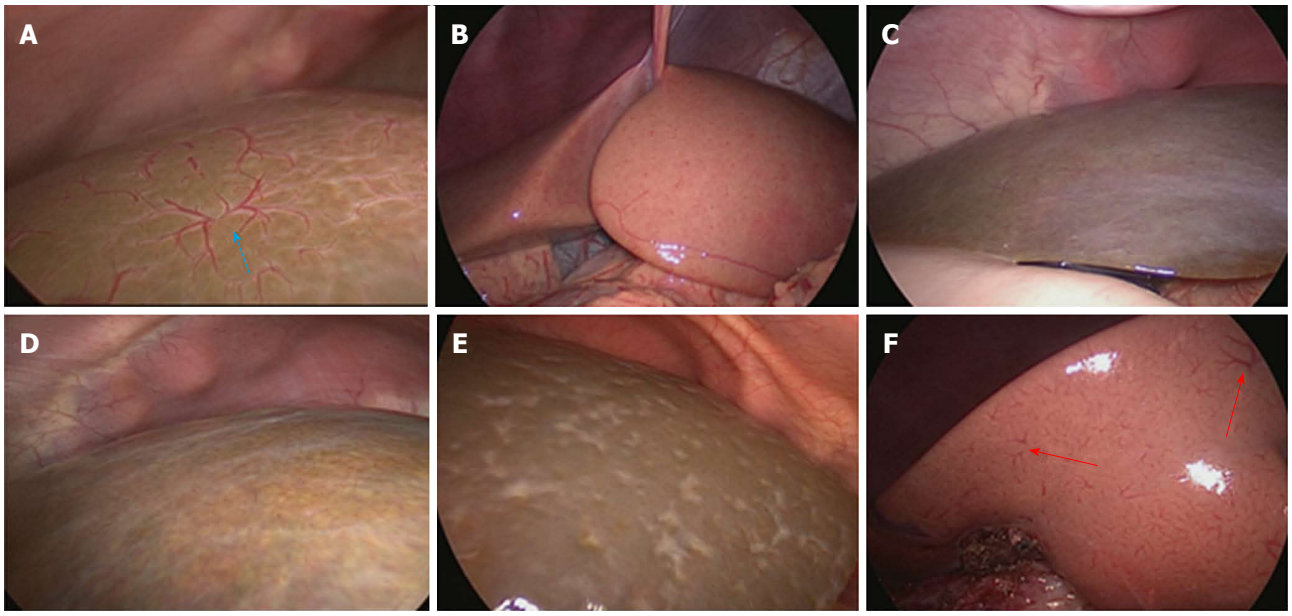


Figure 1 Laparoscopic images of the liver surface in the retrospective study. A: HSST sign in a 70-day-old boy with biliary atresia (blue arrow); B: Image of a 64-day-old boy with Hirschsprung's disease; C: Image of an 82-day-old boy with infantile hepatitis; D: Image of a 72-day-old girl with biliary hypoplasia; E: Image of a 70-day-old boy with total parenteral nutrition-induced cholestatic cirrhosis; F: Small vessel plexuses (red arrows) observed in a 55-day-old boy with cytomegalovirus hepatitis. The HSST sign does not exist in the images B-E. HSST: Hepatic subcapsular spider-like telangiectasis.

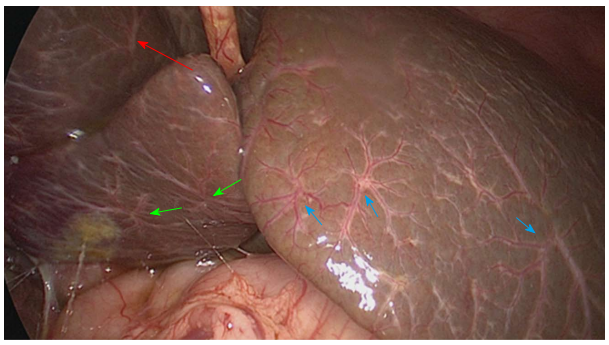


Figure 2 Hepatic subcapsular spider-like telangiectasis sign in a 68-day-old boy with biliary atresia. A hepatic subcapsular spider-like telangiectasis sign was widely distributed on the liver surface, including on the visceral facies of the right liver lobe (red arrow), on diaphragmatic facies of the left liver lobe (blue arrows) and on the surface of the caudate lobe (green arrows).

sign may not have a close relationship with hepatic fibrosis or cirrhosis. It is known that CMV infection was one causative factor of BA and may result in a delayed diagnosis and surgical treatment[23,24]. Mohanty *et al*[25] reported two cases of CMV infection, initially presenting with intrahepatic cholestasis, who subsequently developed BA. Whether the pathological change in the perivascular arterial tufts is the causative factor or results from the developmental of BA is still unknown and needs further investigation.

Ultrasonography (US) has been widely used in differentiating BA from other causes of jaundice. Hepatic subcapsular flow (HSF) in color Doppler US was documented as being useful to predict BA[8,15,18,26] and had the highest performance in discriminating BA from non-BA conditions among several parameters[18]. However, most of the neonates or infants were uncooperative during color Doppler US examination and other factors, such as the doctor's experience, machines, and patients' respiration intensities, also played an important role. Additionally, Takamizawa *et al*[9] debated that US alone could not rule out BA and laparotomy remained mandatory if BA was suspected. Therefore, we conducted this study and finally determined that the HSST sign was useful for predicting BA with a 100% sensitivity and 97.8% specificity. Here, we had a 53-day-old patient with a pre-operative icteric index of 149.9 but unobvious yellowing of the skin. As shown in the picture, the laparoscopic HSST sign (Figure 6A) and the result of LAC (Figure 6B) were easier to identify than distinguishing HSF in a color Doppler US image (Figure 6C).

A similar phenomenon was also observed in Lee's[8] and Ramesh's[27] studies. These authors reported that patients with BA who had hepatic subcapsular flow on color Doppler US images also had subcapsular telangiectatic vessels on the liver surface at the time of the Kasai procedure. In these reports, four patients with TPN-induced cholestasis and two with CMV hepatitis were considered as having HSF[8,15,18]. In our retrospective study, three patients with TPN-induced cholestasis cirrhosis were encountered, but no HSST sign was detected (Figure 2E). In our validation set, there were also two cases of infantile hepatitis who were considered as having HSF, while the HSST sign did not exist. The laparoscopic amplification effect on an intuitive view of the liver surface and ultrasonic

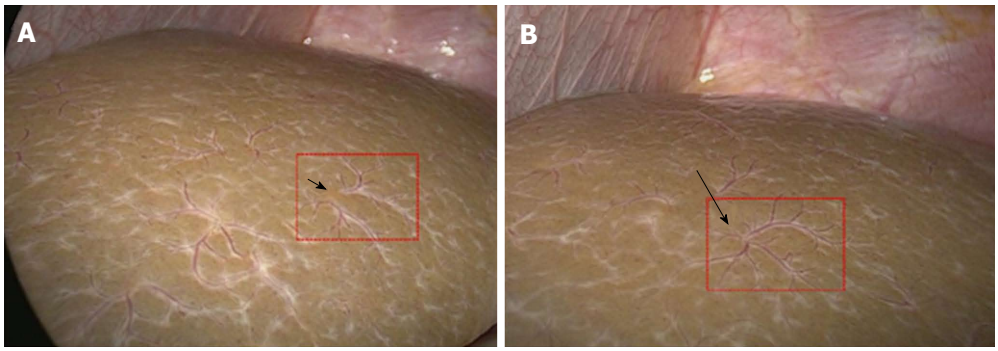


Figure 3 Different types of hepatic subcapsular spider-like telangiectasis signs. A: Dispersed type (short arrow) of the HSST sign in a 75-day-old boy with BA; B: Concentrated type (long arrow) of the HSST sign in a 77-day-old boy with BA. HSST: Hepatic subcapsular spider-like telangiectasis; BA: Biliary atresia.

Table 1 Clinical characteristics of patients with biliary atresia, non-biliary atresia patients and control subjects in the retrospective study

Characteristic	BA (n = 72)	Non-BA (n = 54)	P value[1]	Control (n = 38)	P value[2]	P value[3]
Age (d)	58 ± 29 (20-180)	59 ± 21 (35-125)	0.89	63 ± 22 (36-109)	0.35	0.34
Male-to-female ratio	31:41	24:30	0.88	19:19	0.49	0.60
Total bilirubin (μmol/L)	197.6 ± 72.8	163.6 ± 62.1	0.062	19.3 ± 18.3	<0.0001	<0.0001
Direct bilirubin (μmol/L)	132.8 ± 143.8	99.3 ± 50.8	0.29	5.7 ± 5.6	0.003	<0.0001
ALT (U/L)	162.9 ± 122.6	170.2 ± 362.6	0.91	21.3 ± 7.4	<0.0001	0.15
AST (U/L)	239.7 ± 156.7	183.5 ± 253.1	0.27	30.0 ± 11.1	<0.0001	0.037
GGT(U/L)	684.7 ± 450.6	454.2 ± 450.9	0.052	46.5 ± 22.4	<0.0001	0.003

[1]BA *vs* non-BA; [2]BA *vs* controls; [3]Non-BA *vs* controls. The results are expressed as the mean ± SD or number (percentage). HSST: Hepatic subcapsular spider-like telangiectasis; BA: Biliary atresia; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase.

Table 2 Diagnostic value of the hepatic subcapsular spider-like telangiectasis sign in first and consensus reviews by two reviewers in the retrospective study n (%)

	First review					
	Observer 1[1]		Observer 2[2]		Consistent result	
Imaging feature	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
HSST	72 (100)	54 (100)	72 (100)	53 (98.1)	72 (100)	53 (98.1)

[1]Observer 1 = Zhou Ying; [2]Observer 2 = Jiang Meng.

Table 3 Clinical characteristics of biliary atresia and non-biliary atresia patients in the validation group

Characteristic	BA (n = 45)	Non-BA (n = 45)	P value
Age (d)	60 ± 29	61 ± 23	0.90
Male-to-female ratio	21:24	25:20	0.40
Total bilirubin (μmol/L)	192.3 ± 63.5	173.6 ± 77.0	0.21
Direct bilirubin (μmol/L)	131.6 ± 140.8	104.5 ± 52.3	0.23
ALT (U/L)	172.8 ± 120.9	124.3 ± 264.1	0.27
AST (U/L)	255.2 ± 162.1	188.7 ± 193.9	0.08
GGT (U/L)	661.8 ± 466.3	320.7 ± 384.1	<0.001

The results are expressed as the mean ± SD. BA: Biliary atresia; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase.

artifacts may be the cause of this difference. Moreover, better concordance (κ = 0.937) was achieved through the amplified intuitive view.

Although the signal of hepatic subcapsular vessels was found with US, these vessels on laparoscopic images had their own characteristics: (1) intuitive and stable view of liver surface that was not influenced by interference (machines, doctors' experiences, thickness of the abdominal wall, *etc.*); (2) the HSST sign could be observed not only on the diaphragmatic surface but also on the visceral surface; and (3) images were magnified 4-8 times, which made the view of liver surface clear and vessels easily identified.

Microscopic examinations revealed dilated small arteries in the hepatic subcapsular area in patients with BA, which is consistent with Lee's[8] and El-Guindi's[15] studies. These vessels were branches of hepatic arteries, instead of capillaries in the hepatic capsule. Until now, this phenomenon was not mentioned for other liver diseases. The findings of arterial hyperplasia or hypertrophy in liver specimens and the enlargement of the hepatic artery on color Doppler US imaging in BA patients were also documented[15]. It has also been

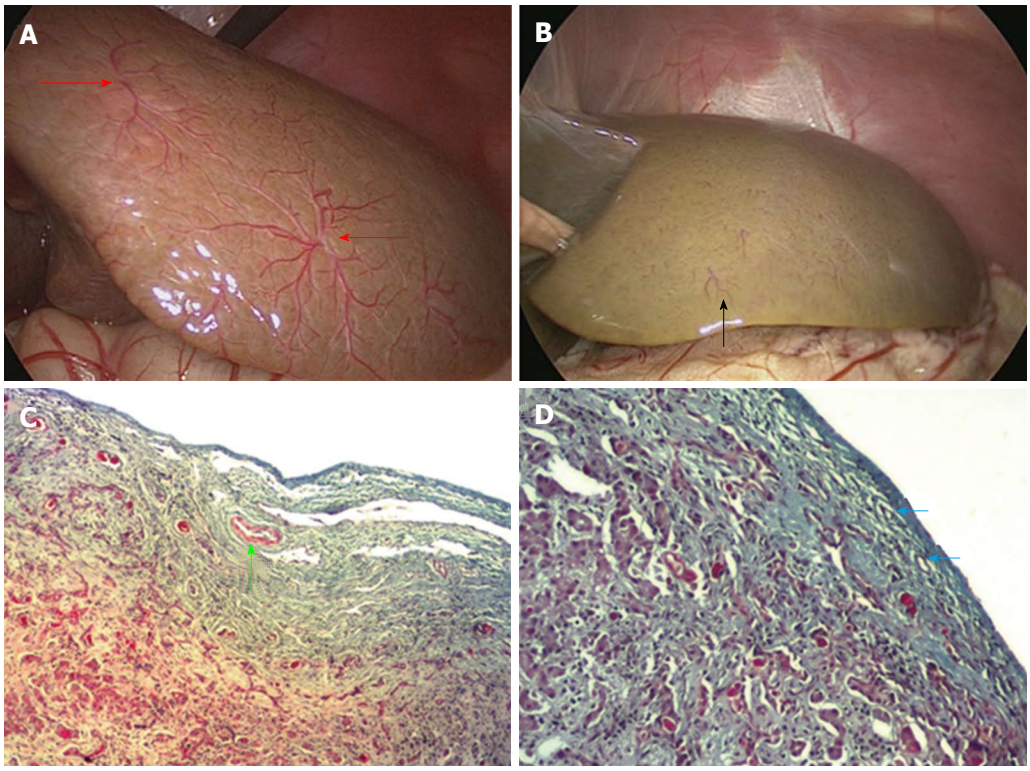


Figure 4 Hepatic subcapsular spider-like telangiectasis sign and pathologic evaluation in the validation set. A: Concentrated type of the HSST sign (red arrow) in a 68-day-old boy with BA; B: Vessels (black arrow) in biliary hypoplasia; C: Vessels in (A) was revealed as dilated small arteries (green arrow) in the hepatic subcapsular area; D: Vessels in biliary hypoplasia (B) was revealed as dilated capillaries (blue arrow) in the hepatic subcapsular area. (Trichrome staining; ×100). HSST: Hepatic subcapsular spider-like telangiectasis; BA: Biliary atresia.

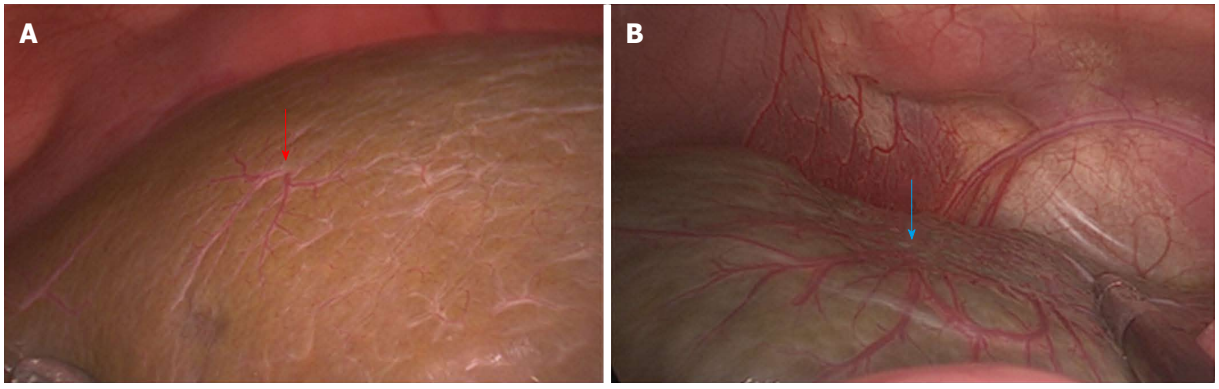


Figure 5 Laparoscopic images of hepatic subcapsular spider-like telangiectasis sign in biliary atresia patients at different ages. A: HSST sign (red arrow) in a 40-day-old boy with BA. There was no cirrhosis at gross inspection; B: HSST sign (blue arrow) in a 156-day-old boy with BA, who had hepatic cirrhosis with ascites and underwent liver transplantation. The HSST sign is obvious in both patients. HSST: Hepatic subcapsular spider-like telangiectasis; BA: Biliary atresia.

reported that the arterial changes might be driven by primary growth signals, or represent a response to a diseased micro-environment that might be rich in growth promoting signals that favor angiogenesis[28-32]. Similarly, the HSST sign is one of the arterial changes in an intuitive view. As with the HSST sign in images 1-5, they were not quite the same and this difference might help in predicting prognoses in the future. Whether it is a primary change of BA or secondary to the bile duct inflammation is unknown and will be our next focus of study.

At present, the procedure for addressing BA patients is a routine examination followed by laparotomy or laparoscopic cholangiography and a laparotomy Kasai operation. For the laparoscopic cholangiography, two or three 5-mm trocars and 30-45 min are needed with one or two instances of radiation exposure. However, with the application of the HSST sign, the

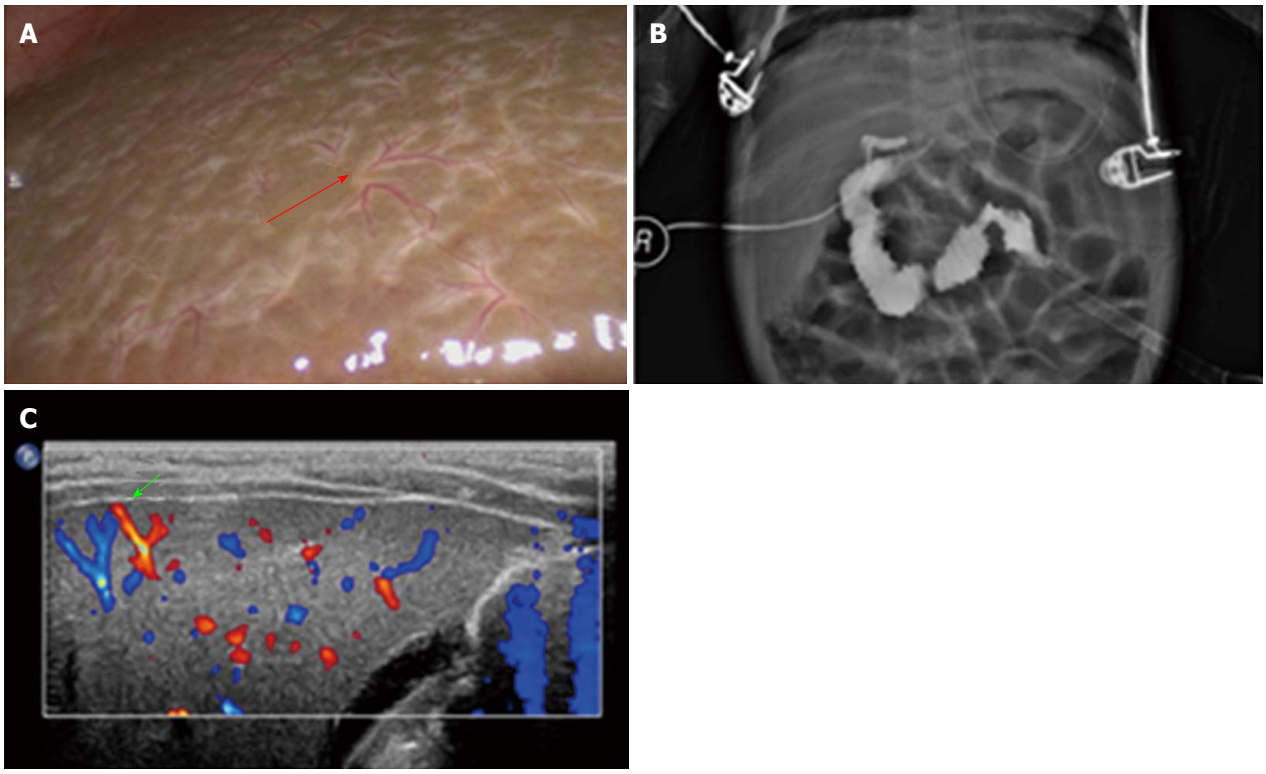


Figure 6 Images in a 53-day-old patient with biliary atresia. A: Dispersed type of the HSST sign (red arrow); B: Image of laparoscopic cholangiography indicates biliary atresia; C: Hepatic arterial flow extending to the hepatic surface on color Doppler US image (green arrow) indicates positive HSF. Laparoscopic HSST sign and the result of laparoscopic cholangiography were easier to identify than distinguishing HSF in a color Doppler US image. HSST: Hepatic subcapsular spider-like telangiectasis; US: Ultrasonography; HSF: Hepatic subcapsular flow.

procedure for addressing BA may become a routine examination followed by laparoscopic exploration for the HSST sign and a laparotomy Kasai operation in the future, which would shorten the operative time and radiation exposure and only needs one 5-mm trocar. The advantage is obvious especially for non-BA patients. In addition, it reflects the diagnostic function of laparoscopy in BA, similar to a laparoscopic diagnosis of hermaphroditism and inguinal hernias.

In conclusion, the HSST sign was preliminarily found to have a good performance in diagnosing or excluding BA with a 100% sensitivity and 97.8% specificity. According to an early laparoscopic exploration of the HSST sign, we can rapidly differentiate BA from non-BA cases and potentially further prevent the delay of a diagnosis of BA. However, given the limited sample size included in the analysis, the value of the HSST sign should be further confirmed in the future. Along with the further research, the HSST sign might be widely applied in clinical practice through a multicenter and large sample study in the future.

COMMENTS

Background

As a common neonatal cholangiopathy that leads to cirrhosis, biliary atresia (BA) is the most common indication for liver transplantation in infants. This disease is characterized by a progressive fibro-obliterative obstruction of extrahepatic bile ducts. A younger age (in the first 2 mo of life) at the time of Kasai portoenterostomy has been found to be life-saving in restoring bile flow and preventing the worsening of liver function. Nevertheless, it is still challenging to differentiate BA from other cholestatic diseases as there are no characteristic manifestations found in the early stage of BA. The demand for a specific and reliable test to diagnose BA has increased.

Research frontiers

Color Doppler ultrasound finding of hepatic subcapsular flow has shown much potential for discriminating BA. While in clinical practice, we have noticed a similar but more intuitive phenomenon - a laparoscopic finding of hepatic subcapsular spider-like telangiectasis (HSST) sign, which may be a specific marker for BA. This has not been published in previous literature.

Innovations and breakthroughs

According to these preliminary investigation, the HSST sign showed promising diagnostic performance for differentiating BA from any other cholestasis diseases, such as infantile cholestasis and cholestatic syndrome. The authors found that the sensitivity and specificity of the HSST sign were each generally close to 100%.

Applications

With the application of the HSST sign, the procedure for addressing BA may become a routine examination followed by laparoscopic exploration for the HSST sign and a laparotomy Kasai operation in the future, which would shorten the operative time and radiation exposure.

Terminology

The HSST sign is composed of several enlarged tortuous spider-like vascular

plexuses with four to eight branches distributed all over the liver surface, which presents as a dispersed type (radiating branches originate from more than one central point, but close to each other to form a spider-like sign) or a concentrated type (radiating branches originate from one central point).

Peer-review

Very interesting findings. It is worth to be published because of new findings of laparoscopic view for the surface of liver in biliary atresia

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ORIGINAL ARTICLE

Retrospective Study

Digestive tract reconstruction using isoperistaltic jejunum-later-cut overlap method after totally laparoscopic total gastrectomy for gastric cancer: Short-term outcomes and impact on quality of life

Ze-Ning Huang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jia-Bin Wang, Jian-Xian Lin, Jun Lu, Qi-Yue Chen, Long-Long Cao, Mi Lin, Ru-Hong Tu, Ju-Li Lin

Ze-Ning Huang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jia-Bin Wang, Jian-Xian Lin, Jun Lu, Qi-Yue Chen, Long-Long Cao, Mi Lin, Ru-Hong Tu, Ju-Li Lin, Department of Gastric Surgery, Fujian Medical University Union Hospital, Fuzhou 350001, Fujian Province, China

Author contributions: Huang ZN and Huang CM conceived the study, analyzed the data, drafted the manuscript, and made the video; Zheng CH, Li P, Xie JW and Wang JB helped revise the manuscript critically for important intellectual content; Lin JX, Lu J, Chen QY, Cao LL, Lin M, Tu RH and Lin JL helped collect the data and design the study; all authors read and approved the final manuscript.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Fujian Medical University Union Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM

To evaluate the short-term outcomes and quality of life (QoL) in gastric cancer patients undergoing digestive tract construction using the isoperistaltic jejunum-later-cut overlap method (IJOM) after totally laparoscopic total gastrectomy (TLTG).

METHODS

A total of 507 patients who underwent laparoscopic gastrectomy (D2) from January 2014 to March 2016 were originally included in the study. The patients were divided into two groups to undergo digestive tract construction using either IJOM after TLTG (group T, *n* = 51) or Roux-en-Y anastomosis after laparoscopic-

assisted total gastrectomy (LATG) (group A, n = 456). The short-term outcomes and QoL were compared between the two groups after 1:2 propensity-score matching (PSM). We used a questionnaire to assess QoL.

RESULTS

Before matching, age, sex, tumor size, tumor location, preoperative albumin and blood loss were significantly different between the two groups (P < 0.05). After PSM, the patients were well balanced in terms of their clinicopathological characteristics, although both blood loss and in-hospital postoperative days in group T were significantly lower than those in group A (P < 0.05). After matching, group T reported better QoL in the domains of pain and dysphagia. Among the items evaluating pain and dysphagia, group T tended to report better QoL ("Have you felt pain" and "Have you had difficulty eating solid food") (P < 0.05).

CONCLUSION

The IJOM for digestive tract reconstruction after TLTG is associated with reduced blood loss and less pain and dysphagia, thus improving QoL after laparoscopic gastrectomy.

Key words: Esophagojejunostomy; Overlap; Later-cut; Totally laparoscopic total gastrectomy; Quality of life

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Core tip: This paper used propensity score-matched analysis and questionnaire survey to evaluate the short-term outcomes and quality of life (QoL) in patients who underwent digestive tract reconstruction using the isoperistaltic jejunum-later-cut overlap method (IJOM) after totally laparoscopic total gastrectomy (TLTG) and in patients who underwent Roux-en-Y anastomosis after laparoscopic-assisted total gastrectomy. We found the IJOM for digestive reconstruction after TLTG is associated with reduced blood loss and less pain and dysphagia, thus improving the QoL after laparoscopic gastrectomy.

Huang ZN, Huang CM, Zheng CH, Li P, Xie JW, Wang JB, Lin JX, Lu J, Chen QY, Cao LL, Lin M, Tu RH, Lin JL. Digestive tract reconstruction using isoperistaltic jejunum-later-cut overlap method after totally laparoscopic total gastrectomy for gastric cancer: Short-term outcomes and impact on quality of life. *World J Gastroenterol* 2017; 23(39): 7129-7138 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7129.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7129

INTRODUCTION

Since Kitano *et al*[1] reported laparoscopic-assisted distal gastrectomy in 1991, laparoscopic techniques and instruments have improved substantially. Consequently, totally laparoscopic distal gastrectomy is increasingly employed and has a proven history of safety and feasibility[2-8]. However, although scholars have reported a variety of totally laparoscopic total gastrectomy (TLTG) methods[3,9-13], this technique has not been widely adopted because of the technological difficulty inherent in digestive reconstruction. Interest in improving the postoperative appearance and quality of life (QoL) in patients with gastric cancer has been increased. This goal, combined with the reduced trauma associated with TLTG, has heightened interest in developing ways to improve TLTG. Surgeons have primarily adopted the overlap or functional end-to-end method for digestive tract reconstruction after TLTG. However, these methods have drawbacks, such as jejunal freeness, which is seen particularly frequently with large anastomoses. Therefore, we devised the isoperistaltic jejunum-later-cut overlap method (IJOM), which involves esophagojejunostomy anastomosis after TLTG. Using this technique, we believe that the jejunum can be positioned with greater ease, thereby reducing the difficulties encountered with the anastomosis. However, little is known about the short-term outcomes and QoL of patients following the implementation of this IJOM for digestive reconstruction after TLTG. Thus, this study aimed to assess the short-term outcomes and QoL in gastric cancer patients undergoing digestive tract reconstruction with IJOM after TLTG and with Roux-en-Y anastomosis after LATG using propensity-score matching (PSM)[14,15] and a QoL assessment scale.

MATERIALS AND METHODS

Study population and inclusion/exclusion criteria

Between January 2014 to March 2016, data were collected from 703 patients who underwent laparoscopic gastrectomy at Fujian Medical University Union Hospital. The including criteria were: (1) pathologically proved gastric cancer by endoscopic biopsy specimen analysis; (2) the aforementioned examination indicated no evidence of distant metastasis; and (3) postoperative pathological diagnosis was curative R0. The exclusion criteria were: (1) intraoperatively proved distant metastasis; (2) T4b stage; (3) missing pathological data; (4) neoadjuvant therapy; and (5) comorbidities that could influence QoL (*e.g.*, previous or combined malignancies; cardiovascular disease; cerebrovascular disease; neurological conditions, such as dementia and seizure; and severe chronic obstructive pulmonary disease requiring persistent medical aid). A number of 507 patients were eligible. Group T consisted of 51 patients who underwent the IJOM after TLTG, and group A comprised 456 patients who received a Roux-en-Y anastomosis after LATG. The 1:2 PSM was performed. Ultimately, group T included 51 patients and group A included 102 patients (Figure 1).

Table 1 Characteristics, merit and demerit of different esophagojejunostomy anastomosis techniques

Anastomosis surgeon	Characteristics	Merit	Demerit
Uyama *et al*[12]	The anastomosis line is vertical with esophageal long axis Jejunum is located in the right side of the esophagus.	Anastomotic is large enough	The number of anastomosis linear staplers is much
Matsui *et al*[37]	Complete the anastomosis before severed esophagus Close the stoma and resect specimens at the same time Jejunum is located in the right side of the esophagus	The number of anastomosis linear staplers is reduced	Probably develop dysphagia 6 mo after operation
Lee *et al*[13]	Suture esophagus, jejunum and right angle of diaphragm after anastomosis Jejunum is located in the right side of the esophagus	Reduce the incidence of esophageal hiatal hernia and anastomotic fistula	Increase the operation time
Okabe *et al*[38]	Before anastomosis, the specimens was removed Jejunum is located in the left side of the esophagus	The size of anastomotic stoma is bigger	The technique is difficult
Inaba *et al*[29]	Overlap anastomosis Divide the jejunum before anastomosis	Isoperistaltic anastomosis meets the physiological needs	The jejunum is free and difficult for anastomosis
Matsui *et al*[39]	Overlap anastomosis Divide the esophagus after anastomosis	Isoperistaltic anastomosis meets the physiological needs	The jejunum is free and difficult for anastomosis

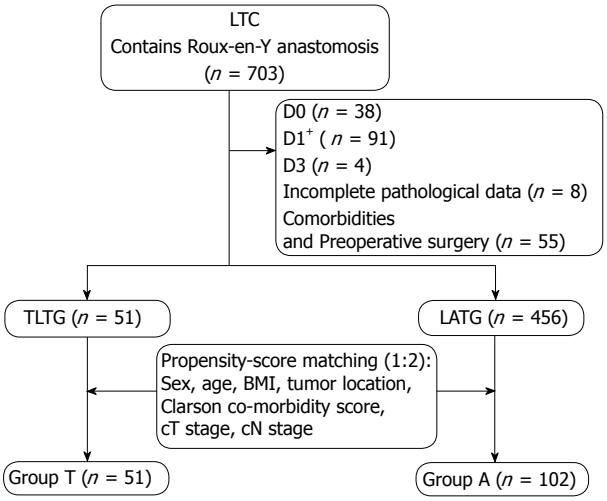


Figure 1 The flow chart of patient selection. LTG: Laparoscopic total gastrectomy; TLTG: Totally laparoscopic total gastrectomy; LATG: Laparoscopic assisted total gastrectomy.

Anastomosis step

In group T, the IJOM was performed after TLTG. After dissecting the lymph nodes laparoscopically and mobilizing the esophagus (Figure 2A) and the duodenum (Figure 2B), an endoscopic linear stapler was used to transect them sequentially in predetermined locations. Two small incisions were made on the left side of the resection margin of the esophagus (Figure 2C) and the antimesenteric border of the jejunum (Figures 2D) approximately 20 cm away from the ligament of Treitz, respectively.

Then, the two limbs of the stapler were inserted into each incision, respectively, and the forks of the stapler were closed and fired, achieving a side-to-side esophagojejunostomy (Figure 2E). After confirming that there was no bleeding *via* common stab incision (Figure 2F), the common stab was manually sutured (Figure 2G). Then, the jejunum was transected after baring the mesenteric border approximately 1 cm into the jejunum wall and approximately 3 cm away from the esophagojejunostomy (Figure 2H). After a small incision was made each on the antimesenteric border of the margin of the proximal jejunum and the distal jejunum roughly about 45 cm from esophagojejunostomy, the two limbs of the stapler were inserted into each incision, and the forks of the stapler were closed and fired to achieve a side-to-side jejunojejunostomy (Figure 2I). After confirming that there was no bleeding or damage to jejunal mucosa by common stab incision, the common stab incision was sutured laparoscopically (Figure 2J). Finally, we removed the specimen through a 3.5-cm incision on the lower abdomen. The differences between this method and other esophagojejunostomy anastomosis techniques are summarized in Table 1.

For patients in group T, the lymph nodes were dissected laparoscopically, and the esophagus and duodenum were mobilized. Then, the traditional open Roux-en-Y anastomosis was performed using a circular stapler[16].

Definition

All patients signed the informed consent form before operation. Preoperative computed tomography (CT) scanning, ultrasonography of the abdomen and endoscopic ultrasonography were routinely performed. When distant metastasis was suspected, positron emission tomography was performed. Preoperative morbidities were scored according to the Charlson score system[17]. Tumor staging was performed according to the 7th edition of the International Union against Cancer (UICC) classification[18]. Postoperative anastomosis-related complications were diagnosed by gastrografin esophagram or clinical manifestations and stratified using the Clavien-Dindo classification[19]. Perioperative death was defined as death that occurred during hospitalization. The Institutional Review Board

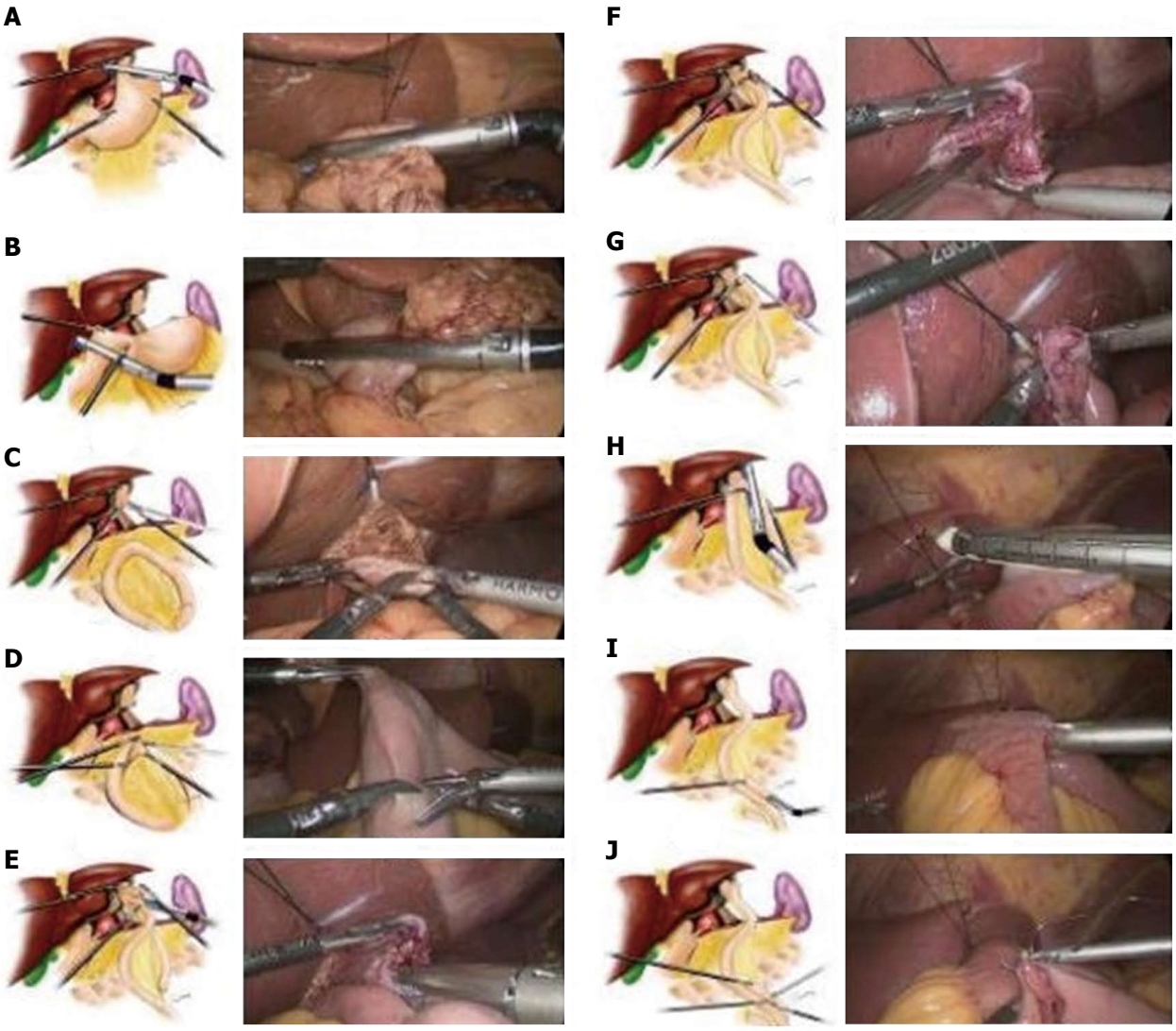


Figure 2 The schematic diagram of anastomosis.

of Fujian Medical University Union Hospital approved this study.

QoL questionnaire

We used the validated Chinese version of the European Organization for Research and Treatment of Cancer 30-item core QoL questionnaire (EORTC-QLQ-C30)[20] to assess QoL. The questionnaire includes a global health status, five functional items, three symptom dimensions, and six individual symptom items (Table 2). Furthermore, we used the validated Chinese version of the 22-item EORTC-QLQ gastric cancer module (EORTC-QLQ-STO22)[21] to assess tumor-specific QoL. This questionnaire includes a functional scale, five symptom dimensions, and three individual symptom scales. QoL is represented by a score ranging from 0 to 100 for every scale. Better QoL is indicated by the higher scores in the functional scales of the EORTC-QLQ-C30 and lower scores in the symptom scores of EORTC-QLQ-C30 and STO22 (Table 2).

Follow-up

The follow-ups were performed *via* mailing, telephone call, or outpatient service. We explained the content of each item of the questionnaire to the patients 6 mo postoperatively, and the patients chose their own responses. Most patients underwent physical examinations, laboratory tests, chest radiography, abdominal ultrasonography or CT, and annual endoscopic examinations.

Statistical analysis

The Statistical Package for Social Science version 18.0 (SPSS, Chicago, IL, United States) was used to perform statistical analyses. The *t* tests or paired *t* tests were performed to compare continuous variables. χ^2 tests were performed to compare categorical variables. $P < 0.05$ was considered statistically significant.

RESULTS

Demographics and clinical characteristics

Before matching, age, sex, tumor location, tumor size, and preoperative albumin level differed significantly between the two groups ($P < 0.05$). After 1:2

Table 2 Structure of EORTC QLC-C30 and EORTC QLQ-STO22

Scale	Number of constituting items
EORTC QLQ-C30	
Global health status/QoL scale[1]	2
Functional scales[1]	
Physical functioning	5
Role functioning	2
Emotional functioning	4
Cognitive functioning	2
Social functioning	2
Symptom scales[2]	
Fatigue	3
Nausea and vomiting	2
Pain	2
Dyspnoea	1
Insomnia	1
Appetite loss	1
Constipation	1
Diarrhoea	1
Financial difficulties	1
EORTC-QLQ-STO22[2]	
Dysphagia	3
Chest and abdominal pain	4
Reflux	3
Eating restrictions	4
Anxieties	3
Dry mouth	1
Taste problem	1
Body image	1
Hair loss	2

[1]Higher scores represent better QoL; [2]Higher scores represent worse QoL. EORTC QLQ indicates European Organization for Research and Treatment of Cancer Quality-of-life Questionnaire; QoL: Quality-of-life.

matching, 51 and 102 patients were included in groups T and A, respectively, and well balanced in their clinicopathological characteristics (Table 3).

Perioperative results

After matching, blood loss and postoperative days of hospital stay were significantly less in group T than in group A ($P < 0.05$). The number of harvested lymph nodes, operative time, time to first flatus, time to fluid diet, and hospitalization costs were similar in the two groups (Table 4).

Postoperative complications

Before matching, one patient had an anastomotic fistula in group T. In contrast, Group A included 22 patients with anastomotic fistula, one with anastomotic hemorrhage and four with anastomotic obstruction. The incidences of anastomosis-related complications were 2.0% and 5.9% in groups T and A, respectively; the two rates were similar. After matching, there was one patient with anastomotic fistula in group T, whereas there were four patients with anastomotic fistula, one with anastomotic hemorrhage, and two with an anastomotic obstruction in group A. The incidence of anastomosis-related complications had no statistical difference in the two groups, and no perioperative deaths occurred (Table 5).

Functional scales

After matching, six patients died and one was lost to follow-up 6 mo postoperatively in group T. Eventually, 44 patients joined the questionnaire survey. In group A, ten patients died, and three were lost to follow-up. A total of 89 patients from group A participated in the questionnaire survey. The functional scales of EORTC-QLQ-C30 were all similar in both groups (Figure 2).

Symptom scales

After matching, the symptom scales of EORTC-QLQ-C30 and STO22 were compared. Based on the pain scales of EORTC-QLQ-C30 and dysphagia scales of EORTC QLQ-STO22, group T reported better QoL (Figure 3). Subgroup analyses of two items in the pain scale ("Have you felt pain?" and "Has your life been affected by pain?"), group T tended to report better QoL in "Have you felt pain?"($P = 0.018$). In the dysphagia scale, subgroup analyses of three items ("Have you had difficulty eating solid food?", "Have you had difficulty swallowing liquid or eating soft food?", and "Have you had difficulty drinking water?") revealed that group T tended to report better QoL in response to the question "Have you had difficulty eating solid food?" than group A ($P = 0.039$) (Table 6).

DISCUSSION

Laparoscopy offers several advantages over traditional laparotomy, such as reduced trauma, faster recovery, fewer postoperative complications, and greater aesthetic appeal. These benefits are attributable to the minimal invasiveness of laparoscopy and the good clinical outcomes that have been reported[22-25]. Currently, the methods of digestive tract reconstruction employed after LG include LATG and TLTG. In LATG, the digestive tract reconstruction is performed *via* a small incision after lymphadenectomy, although this decreases the advantages of laparoscopic minimally invasive surgery. Since Uyama *et al*[12] reported totally laparoscopic digestive tract reconstruction, substantial research in Japan and Korea has revealed that TLTG has desirable short-term outcomes and is also safe and effective[11,26-28].

Currently, the digestive tract reconstruction methods used after TLTG include overlap[12] and functional end-to-end[29] techniques. However, jejunal freeness is one of several drawbacks associated with these methods and makes the anastomosis more difficult. Therefore, we devised the IJOM, which involves esophagojejunostomy anastomosis after TLTG. We believe that, using this technique, the jejunum can be more easily positioned, thereby reducing the difficulty in creating the anastomosis. Moreover, because the proximal jejunum is divided after the anastomosis, the length of the blind loops can be easily grasped. We found that blood loss was reduced in group T, and this may be attributed to the clearer,

Table 3 Demographic and clinical characteristics of patients in the two groups

	All patients			Propensity-matched patients		
	Group T (n = 51)	Group A (n = 456)	P value	Group T (n = 51)	Group A (n = 102)	P value
Age (mean ± SD, yr)	55.5 ± 12.1	61.6 ± 11.2	< 0.001	55.5 ± 12.1	55.9 ± 11.0	0.916
Gender			< 0.001			1.000
Male	34	345		34	68	
Female	17	111		17	34	
Charlson comorbidity index			0.281			0.608
0	48	418		48	92	
1-2	3	38		3	10	
BMI (mean ± SD, kg/m^2)	22.5 ± 13.1	22.3 ± 13.5	0.919	22.5 ± 13.1	22.6 ± 12.8	0.965
Tumor size (mean ± SD, cm)	4.5 ± 1.5	4.9 ± 1.3	0.041	4.5 ± 1.5	4.7 ± 1.7	0.142
Tumor location			< 0.001			0.177
Upper third	4	188		4	12	
Middle third	34	169		34	76	
Whole	13	99		13	14	
Histology type			0.453			0.482
Differentiation	47	416		47	97	
Undifferentiation	4	40		4	5	
Preoperative albumin (mean ± SD, g/L)	40.8 ± 4.3	39.1 ± 5.2	0.025	40.8 ± 4.3	40.6 ± 4.6	0.796
Depth of infiltration (T)			0.174			0.643
T1	15	82		15	23	
T2	8	83		8	18	
T3	10	135		10	16	
T4a	18	166		18	45	
Nodal status (N)			0.729			0.534
N0	21	190		21	34	
N1	11	77		11	18	
N2	5	66		5	10	
N3	14	123		14	40	
UICC stage			0.319			0.502
Ⅰ	13	78		13	18	
Ⅱ	17	159		17	40	
Ⅲ	21	219		21	44	

BMI: Body mass index; UICC stage: 7th edition of the International Union against Cancer.

Table 4 Operative variables of the patients

	All patients			Propensity-matched patients		
	Group T (n = 51)	Group A (n = 456)	P value	Group T (n = 51)	Group A (n = 102)	P value
Operative time, min (mean ± SD)	209.3 ± 41.0	203.6 ± 49.3	0.427	209.3 ± 41.0	200.5 ± 55.6	0.318
Blood loss, mL (mean ± SD)	48.3 ± 38.5	98.4 ± 149.1	0.017	48.3 ± 38.5	105.4 ± 147.9	0.008
Harvested LNs (mean ± SD)	44.5 ± 15.0	41.2 ± 14.2	0.237	44.5 ± 15.0	42.6 ± 15.2	0.465
Time to first flatus, days (mean ± SD)	3.8 ± 1.2	3.5 ± 1.7	0.220	3.8 ± 1.2	3.6 ± 1.2	0.332
Time to fluid diet, days (mean ± SD)	5.6 ± 1.4	5.6 ± 1.6	1	5.6 ± 1.4	5.5 ± 1.9	0.739
Postoperative days (mean ± SD)	12.6 ± 4.3	14.7 ± 8.9	0.097	12.6 ± 4.3	15.4 ± 8.9	0.035
hospitalization costs, yuan	75450 ± 20038	73308 ± 21932	0.505	75450 ± 20038	70407 ± 13254	0.065
Chemotherapy	33	310	0.635	33	78	0.123

LN: Lymph node.

amplified field of vision achieved during laparoscopic digestive tract reconstruction. As a result, blood vessels in the muscles and mesentery can be more readily identified and are less likely to be transected during the procedure. Consistent with our findings, previous studies have shown that reduced blood loss is associated with better postoperative recovery[30]. We found that the length of the postoperative stay in group T was significantly shorter than that in group A, confirming that less trauma and blood loss during TLTG can promote faster recovery.

Lee *et al*[31] showed that distal gastrectomy decreases the QoL because of problems with eating restrictions and body image. However, compared with open surgery, laparoscopic-assisted distal gastrectomy improves QoL, specifically by reducing the incidence of postoperative intestinal obstruction[32]. Fujii *et al*[33] suggested that this reduction in postoperative intestinal obstruction may be attributable to the less abdominal manipulation required for laparoscopic surgery, which may blunt the systemic cytokine and inflammatory responses[34,35]. Similarly, totally laparoscopic technology reduces the amount of intestinal manipulation required during digestive tract reconstruction and may also

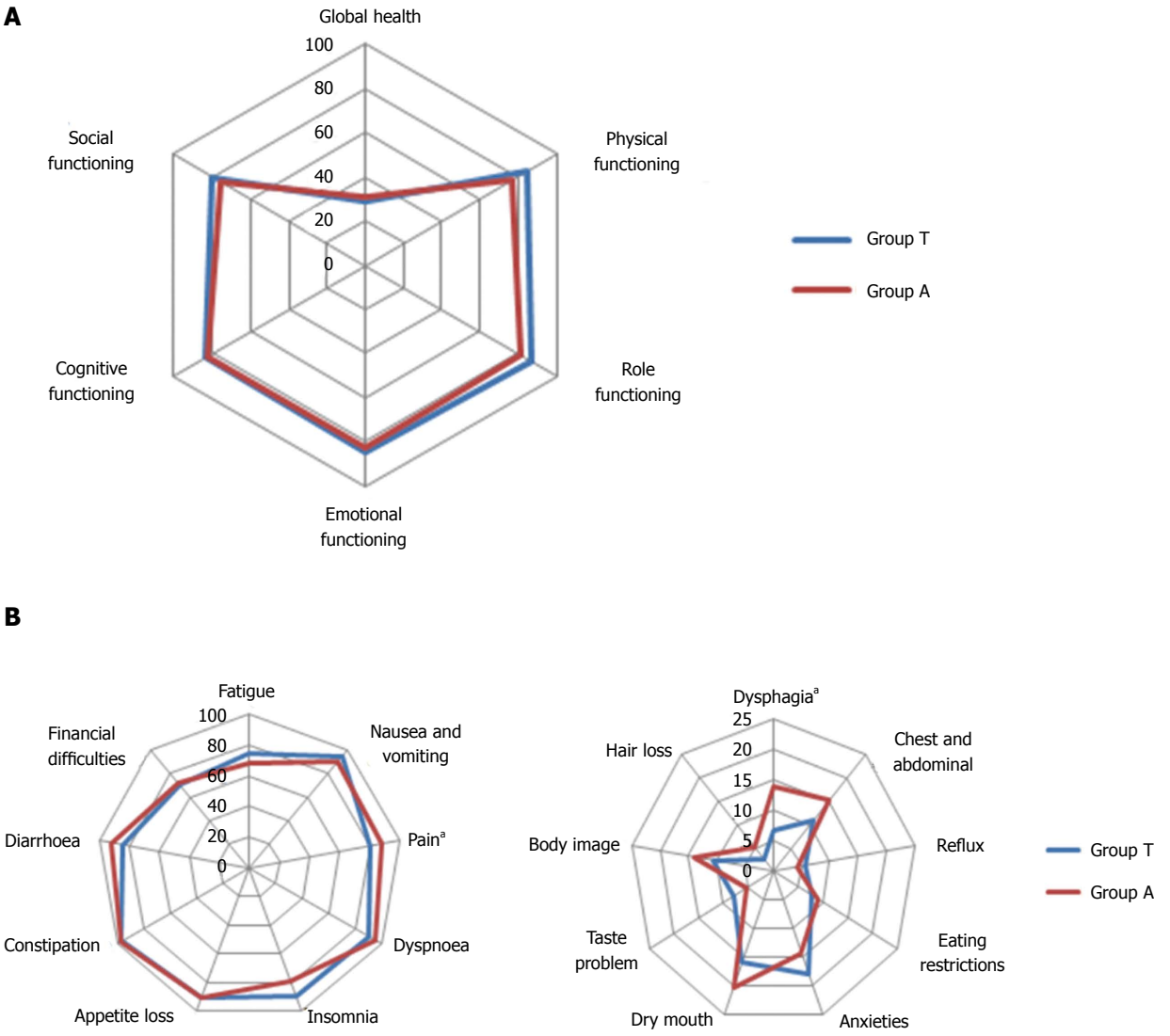


Figure 3 The functional scales. A: EORTC-QLQ-C30; B: EORTC-QLQ-C30 and EORTC-QLQ-STO22. [a]*P* < 0.05, EORTC-QLQ-C30 *vs* EORTC-QLQ-STO22.

Table 5 Morbidity and mortality associate with anastomosis

	All patients			Propensity-matched patients		
	Group T, % (*n* = 456)	Group A, % (*n* = 51)	*P* value	Group T, % (*n* = 102)	Group A, % (*n* = 51)	*P* value
Morbidity	1 (2.0)	27 (5.9)	0.893	1 (1.9)	6 (11.8)	0.552
Anastomotic fistula	1	22		1	4	
Anastomotic hemorrhage	0	1		0	0	
Anastomotic obstruction	0	4		0	2	
Mortality	0	0	/	0	0	/

reduce the incidence of postoperative intestinal obstruction. However, no studies have evaluated QoL following totally laparoscopic surgery. Schneider *et al*[36] reported that the diameter of the anastomotic stoma obtained using the linear stapler was significantly larger than that achieved with the circular stapler, which benefits the passage of food. At 6 mo post-surgery, we found that symptoms of dysphagia were better in group T, especially in terms of eating solid food. This finding indicates that using a linear stapler can expand the diameter of the anastomotic stoma and that the decreased intestinal manipulation involved in TLTG can reduce the incidence of intestinal obstruction.

Patients who underwent TLTG reported significantly less pain than those undergoing LATG. We believe that this is because the incision in the abdominal wall involved in TLTG is shorter, leading to less pain from inflammation and scar formation. In addition, less intra-abdominal manipulation likely contributes to decreasing the formation of intra-abdominal adhesions

Table 6 Constituent items of pain of EORTC-QLQ-C30 and dysphagia of EORTC-QLQ-STO22 as compared between group T and group A

	Propensity-matched patients		
	Group T (n = 44)	Group A (n = 89)	P value
Constituent items of pain of EORTC-QLQ-C30			
Have you felt pain?			0.018
Not at all	28	66	
A little	12	7	
Quite a lot	3	14	
Very much	1	2	
Have your life affected by pain?			0.271
Not at all	39	73	
A little	4	7	
Quite a lot	1	9	
Very much	0	0	
Constituent items of dysphagia of EORTC-QLQ-STO22			
Have you felt difficult to eat solid food?			0.039
Not at all	26	31	
A little	11	32	
Quite a lot	7	21	
Very much	0	5	
Have you felt difficult to eat liquid of soft food?			0.275
Not at all	38	67	
A little	5	15	
Quite a lot	1	7	
Very much	0	0	
Have you felt difficult to drink water?			0.194
Not at all	39	80	
A little	5	5	
Quite a lot	0	4	
Very much	0	0	

and, thus, the associated discomfort.

This is the first study investigating the differences in short-term outcomes and QoL between the IJOM after TLTG and Roux-en-Y anastomosis after LATG using PSM and a QoL assessment scale. The results show that utilizing the IJOM after TLTG can reduce intra-operative blood loss and relieve symptoms of pain and dysphagia. However, this study has several limitations. First, the follow-up period was short. Second, a retrospective, single-center design was used. Therefore, a prospective, multiple-center study with a longer follow-up period is needed.

COMMENTS

Background

Surgeons have primarily adopted several methods for digestive tract reconstruction after totally laparoscopic total gastrectomy (TLTG). However, these methods have drawbacks, such as jejunal freeness and difficult to perform. Therefore, we devised the isoperistaltic jejunum-later-cut overlap method (IJOM), but little is known about the short-term outcome and quality-of-life (QoL) in patients following the implementation of this digestive reconstruction.

Research frontiers

The QoL after distal gastrectomy was reported to be affected by eating restrictions and body image. For TLTG, scholars have reported a variety of digestive reconstruction methods which are safe and effective, but the QoL is uncertain.

Innovations and breakthroughs

The authors used propensity score-matched analysis and questionnaire survey to perform this research. The authors found that IJOM for digestive reconstruction can reduce blood loss compared with Roux-en-Y anastomosis and was associated with less pain and dysphagia, thus improving QoL after laparoscopic gastrectomy.

Applications

Through this study, we can focus the symptoms which probably happen after surgery and accordingly improve the QoL of patients. However, a prospective, multiple-center study with a longer follow-up period is needed.

Terminology

EORTC-QLQ-C30: Chinese version of the European Organization for Research and Treatment of Cancer 30-item core QoL questionnaire. EORTC-QLQ-STO22: the validated Chinese version of the 22-item EORTC-QLQ gastric cancer module. PSM: A statistical matching method which can reduce the bias of variables.

Peer-review

This study is a pioneer study about esophagojejunostomy anastomosis after TLTG.

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ORIGINAL ARTICLE

Observational Study

Adalimumab efficacy in enteropathic spondyloarthritis: A 12-mo observational multidisciplinary study

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Abstract

AIM

To report adalimumab (Ada) efficacy on articular-gastrointestinal disease and health-related quality of life (HRQoL) in patients with enteropathic spondyloarthritis (ES).

METHODS

A cohort of 52 patients with ES was evaluated in the departments of gastroenterology and internal medicine. At baseline, all patients underwent assessment by an integrated gastro-rheumatologic evaluation of articular and gastrointestinal activity, as well patient reported outcomes (PROs) of the HRQoL questionnaires. After this integrated evaluation and following a specific working flowchart, the Ada anti-tumor necrosis factor (TNF)-inhibitor was assigned to a cohort of 30 patients and its clinical efficacy was evaluated at baseline and

after 6-mo and 12-mo treatment by the following tests: (1) Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein (ASDAS-CRP); Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI) and Bath Ankylosing Spondylitis Metrology Index (BASMI) for articular activity; (2) Inflammatory Bowel Disease Questionnaire (IBDQ), Crohn's Disease Activity Index (CDAI) and partial Mayo (pMayo) score for gastrointestinal symptoms and activity; and (3) Health Assessment Questionnaire (HAQ), Patient Global Assessment (PGA) and Short Form-36 health survey (SF-36) questionnaires for PROs of the HRQoL.

RESULTS

Integrated evaluation and management of the patients affected by ES, carried out simultaneously by a gastroenterologist and a rheumatologist, allowed clinicians to choose the optimal therapeutic strategy. In a cohort of 30 ES patients affected by active articular and gastrointestinal disease, or axial active articular inflammation, Ada led to fast and sustained improvement of both articular and gastrointestinal disease activities. In fact, all the clinimetric evaluation tests exploring articular or gastrointestinal activity, as well as all the HRQoL scores, showed a significant improvement having been achieved at the earliest (6-mo) assessment. This important clinical improvement was maintained at the 12-mo follow-up. Importantly, global and gastrointestinal quality of life significantly correlated with articular disease activity, providing evidence to support that the integrated evaluation is the best option to manage patients with ES.

CONCLUSION

Ada treatment, upon multidisciplinary (gastro-rheumatologic) evaluation, significantly improves both articular and gastrointestinal inflammation, thereby improving the HRQoL in patients affected by ES.

Key words: Clinimetric assessment; Patient reported outcomes; Inflammatory bowel diseases; Enteropathic spondyloarthritis; Tumor necrosis factor-inhibitors; Multidisciplinary evaluation

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Core tip: Enteropathic spondyloarthritis (ES) is characterized by articular inflammation in patients with inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis. Correct management, especially covering both of the two clinical manifestations (gastro-rheumatologic), remains a challenge. In this study, we demonstrated that the integrated gastroenterological and rheumatologic evaluation of ES patients achieved the best therapeutic approach. In particular, we demonstrated that in a real-life cohort of ES patients, the tumor necrosis factor-inhibitor, adalimumab, led to fast and sustained improvement of articular and gastrointestinal inflammation, with a consequent improvement in the global and gastrointestinal quality of life.

Luchetti MM, Benfaremo D, Ciccia F, Bolognini L, Ciferri M, Farinelli A, Rossini M, Mosca P, Triolo G, Gabrielli A. Adalimumab efficacy in enteropathic spondyloarthritis: A 12-mo observational multidisciplinary study. *World J Gastroenterol* 2017; 23(39): 7139-7149 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7139.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7139

INTRODUCTION

Enteropathic spondyloarthritis (ES) is a seronegative spondyloarthropathy (SpA) characterized by the presence of articular inflammation in patients affected by inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) or ulcerative colitis (UC)[1,2]. Arthritis is the most frequent extra-intestinal manifestation in patients with IBDs[1] and it may primarily involve the axial joints, presenting with definite ankylosing spondylitis (AS) and/or isolated sacroiliitis, or peripheral joints and/or peri-articular structures, such as tendons and entheses[3]. The articular manifestations significantly affect health-related quality of life (HRQoL) of ES patients[4].

Although a link between inflammation of the joints and gut has been demonstrated[5,6], only half of ES patients are actually evaluated by a rheumatologist for proper diagnosis and, thus, for an integrated therapeutic approach through a coordinated action with the treating gastroenterologist[7]. Thus, an integrated clinical evaluation and therapeutic approach encompassing both the intestinal and articular features in ES patients, will likely be beneficial, particularly for the clinical outcomes. It has been demonstrated by recent studies that tumor necrosis factor (TNF)-alpha inhibitors could be effective therapeutic agents against ES[8], but few to date have reported on their real-life efficacy in this disease.

Herein, we have investigated the role of a gastro-rheumatologic multidisciplinary management and therapeutic approach in ES patients through evaluation of the efficacy of the TNF-alpha inhibitor adalimumab (Ada), assessing the efficacy on both gastrointestinal and rheumatologic disease activities and on the patient-reported HRQoL.

MATERIALS AND METHODS

Patients and study design

This study was carried out in a cohort of 52 patients with ES, including 31 affected by CD (59.6%) and 21 by UC (40.3%), collectively representing 23.6% of the 220 overall patient population with IBD in the

Table 1 Enteropathic spondyloarthritis patient features

	ES-AN, *n* = 52	ES-AN/Ada, *n* = 30
Crohn's disease:Ulcerative colitis	31 (60):21 (40)	19 (63):11 (37)
Males:Females	22 (42):30 (58)	17 (57):13 (43)
Age in years	47.2 ± 14.2	46.2 ± 14.4
Disease duration of IBD in years	11.3 ± 10.1	8.8 ± 7.9
Smokers:Ex-smokers	9 (17):20 (38)	
HLA-B27 positivity	5 (10)	4 (13)
Prior surgical intervention for IBD	13 (25)	5 (17)
Previous extra-intestinal disease	6 (11)	5 (17)
Eritema nodosum	2	1
Uveitis	3	3
Pioderma gangrenosum	1	1
Crohn's disease activity by CDAI		
Inactive	14 (45)	7 (37)
Moderate	10 (32)	8 (42)
Moderate-to-Severe	7 (23)	4 (21)
Ulcerative colitis activity by partial Mayo		
Mild	18 (86)	8 (73)
Moderate	3 (14)	3 (27)
Severe	0	0
Current medication at baseline		
Non-steroids anti-inflammatory drugs	3	0
Sulfasalazine	3	2
Mesalazine	25	12
Cyclosporine	1	1
Azathioprine	9	5
Oral steroids	12	7
Topical steroids	3	2
Metotrexate	2	1
Infliximab	6	4
Adalimumab	2	0
Spondyloarthritis features		
Ankylosing spondylitis according to Modified New York Criteria	16 (31)	10 (33)
Non-radiographic Axial-Spondyloarthritis by ASAS Criteria	13 (25)	10 (33)
Peripheral- Spondyloarthritis	23 (44)	10 (33)
Type of axial involvement	*n* = 29	*n* = 20
Syndesmophytosis	8 (28)	6 (30)
Bamboo spine	2 (7)	2 (10)
Sacroiliitis by MRI and/or X-ray	29 (100)	20 (100)
Type of articular involvement in Crohn's disease	*n* = 31	*n* = 19
Axial	16 (52)	11 (58)
Axial and peripheral	4	3
Peripheral only	15 (48)	8 (42)
Enthesitis	9 (29)	5 (26)
Type of articular involvement in ulcerative colitis	*n* = 21	*n* = 11
Axial	13 (62)	9 (82)
Axial and peripheral	9	5
Peripheral only	8 (38)	2 (18)
Enthesitis	4 (19)	2 (18)

Data are presented as *n*, *n* (%) or mean ± SD. CDAI: Crohn's Disease Activity Index; ES-AN: Patient cohort with enteropathic spondyloarthritis from the SPIB Program, Ancona, Italy; ES-AN/Ada: Patients of the ES-AN cohort treated with adalimumab; IBD: Inflammatory bowel disease; NSAID: Non-steroidal anti-inflammatory drug.

Spondyloarthritis in IBD Project (commonly referred to as SPIB), described elsewhere[9]. The patients' clinical and laboratory data are shown in Table 1, and the patient group is henceforth defined as the "ES-AN" cohort (Enteropathic spondyloarthritis from Ancona, Italy).

At each clinical observation, both the rheumatologist and the gastroenterologist collaborated in a shared session to develop the therapeutic strategy by applying a specifically designed algorithm (Figure 1) that was mainly based upon gastrointestinal-articular disease activity and the site of articular involvement at diagnosis (axial or peripheral arthritis). Briefly, the ES-AN patients were primarily separated into the following two groups for comparative analysis:

Group I (biological drugs-naïve group): This group encompassed three treatment subgroups. In the axial-ES-AN (Ax-ES-AN) subgroup, patients were administered Ada as first-line therapy, due to the absolute contraindication for a long-course treatment with nonsteroidal anti-inflammatory drugs (NSAIDs)

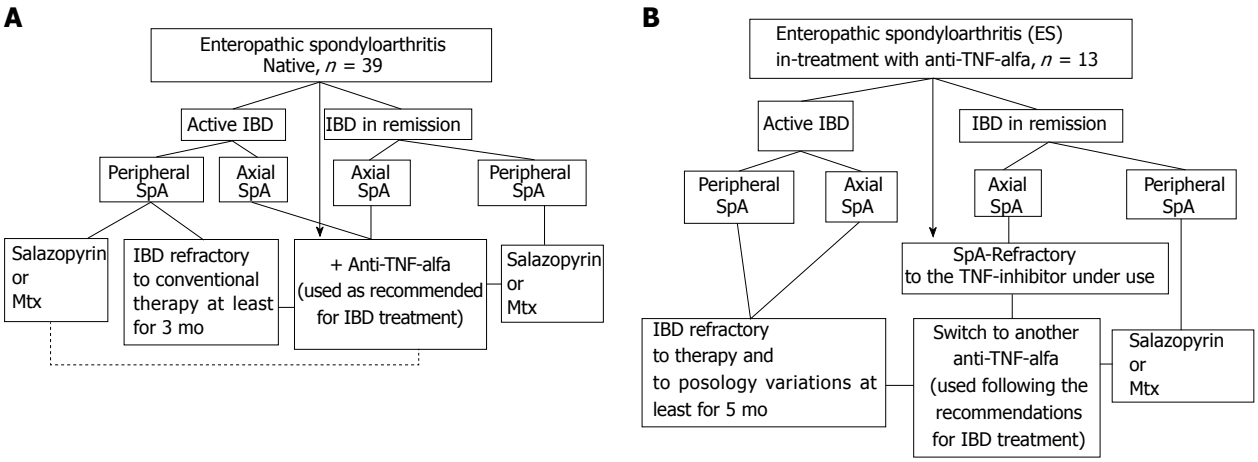


Figure 1 Therapeutic algorithm of the ES-AN patient cohort at the time of entry into the study. A: ES patients biological drugs-naïve were treated depending on IBD activity and site of the articular involvement, as follows: a) Ax-ES-AN with Ada as the TNF-inhibitor in first-line therapy, due to the absolute contraindication for a long-course treatment with NSAIDs in cases of IBDs; b) Per-ES-AN in cases of active IBD and in those patients who were non-responders to short-course corticosteroid treatment (not > 3 mo) or NSAIDs (not > 2 wk), with DMARD (methotrexate or sulfasalazine), or in cases of ESR > 30 mm/h and/or CRP > 0.5 mg/dL, and polyarticular inflammatory involvement with Ada; c) Per-ES-AN in inactive IBD, with steroids or DMARDs, depending on the number of inflamed joints and systemic inflammation (evaluated by ESR and/or CRP); B: ES patients already in treatment with infliximab for the IBD. In the ES-AN patients already treated with IFX: a) Per-ES-AN with active IBD were switched to Ada; b) Per-ES-AN with IBD in remission received a DMARD in addition to the IFX already in use; c) Ax-ES-AS were switched to Ada, regardless of IBD activity. Dashed line: Patients refractory to therapy. Therapeutic doses: DMARDs were prescribed at the standard dose regimens (salazopyrine 2 gr bid; methotrexate 10-20 mg once a week); Ada was used following the therapeutic dosage and indications for IBDs (160 mg at d 1 and 80 mg after 2 wk, followed by 40 mg every 2 wk). Ada: Adalimumab; AX-ES-AN: Patients with axial spondyloarthritis in the ES-AN cohort; CRP: C-reactive protein; ES: Enteropathic spondyloarthritis; ES-AN: Patients affected by ES in the Ancona's cohort; ESR: Erythrocyte sedimentation rate: DMARD: Disease modifying anti-rheumatic drug; IBD: Inflammatory bowel disease; NSAID: Non-steroidal anti-inflammatory drug; Per-ES-AN: Patients with peripheral spondyloarthritis in the ES-AN cohort.

in case of IBD. In the peripheral-ES-AN (Per-ES-AN) subgroup, patients with active IBD or who were non-responders to a short course of corticosteroids (not more than 3 mo) or NSAIDs (not more than 2 wk) were administered either a disease-modifying anti-rheumatic drug (DMARD), such as either methotrexate (MTX) or sulfasalazine (SSZ), or Ada if erythrocyte sedimentation rate (ESR) was > 30 mm/h and/or C-reactive protein (CRP) concentration was > 0.5 mg/dL and/or in the presence of polyarticular inflammatory involvement. In the Per-ES-AN in inactive IBDs subgroup, patients were administered steroids or DMARDs, depending on count number of inflamed joints and systemic inflammation (evaluated by ESR and/or CRP).

Group II (TNF inhibitor-treated group): This group also encompassed three treatment subgroups. For the first, the Per-ES-AN consisting of patients with still active IBD were switched to another TNF-inhibitor (Ada). For the second, the Per-ES-AN consisting of patients with IBD in remission were administered a DMARD in addition to the TNF-inhibitor already in use. For the third, the Ax-ES-AN patients were switched to Ada, regardless of IBD activity.

In all patients, the TNF-inhibitor Ada was used as recommended for IBD treatment: 160 mg in the first week, 80 mg for the next 2 wk, and thereafter 40 mg once every 2 wk.

Study measures and evaluation

All patients of the ES-AN cohort were assessed for clinical disease activity and HRQoL (Table 2). Briefly, articular (SpA) disease activity was assessed by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)[10] and the Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein (ASDAS-CRP) calculation[11]; gastrointestinal (IBD) disease activity was assessed with the Crohn's Disease Activity Index (CDAI) for CD[12] and the partial Mayo (pMAYO) score for UC[13].

Patient-reported outcomes (PROs) were assessed with tests specific for articular-related symptoms [*i.e.* the Bath Ankylosing Spondylitis Functional Index (BASFI)][14] and gastrointestinal-related symptoms [*i.e.* the Inflammatory Bowel Disease Questionnaire (IBDQ)][15]. Global wellness was assessed by use of the Health Assessment Questionnaire (HAQ), Patient Global Assessment (PtGA), and the Short Form-36 health survey (SF-36)[16].

Statistical analysis

Endpoints of this study were the disease activity indexes at baseline and at 6-mo and 12-mo follow-ups. Variables are presented as mean ± standard deviation. Comparisons between groups at baseline

Table 2 Clinimetric test for articular-gastrointestinal activity and patient reported outcomes of health-related quality of life

Test	Items and interpretation
Bath Ankylosing Spondylitis Disease Index[10]	6 items: (1) fatigue, (2) back pain, (3) peripheral pain/swelling, (4) discomfort at pressure, (5) morning discomfort, and (6) duration of morning stiffness; Range from 0 to 10, with lower number representing less severe disease activity; Score > 4 = active disease.
Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein[11]	5 items: (1) back pain, (2) morning stiffness, (3) patient global, (4) peripheral pain/swelling, and (5) C-reactive protein; Score < 1.3 = inactive disease; Score 1.3 to < 2.1 = moderate activity; Score 2.1 to ⩽ 3.5 = high activity; Score > 3.5 = very high activit; Change ⩾ 1.1 = clinically important improvement; Change ⩾ 2.0 = major improvement.
Crohn's Disease Activity Index[12]	8 items: (1) liquid stools, (2) abdominal pain, (3) general well-being, (4) extra-intestinal manifestations (including arthralgia), (5) use of anti-diarrheals, (6) abdominal masses, and (7) hematocrit, 8) weight; Final score is the sum of items, weighted by different factors; Score < 150 = non-active disease; Score > 150 = active disease; Score > 450 = extremely severe disease.
Partial MAYO score[13]	3 items: (1) stool frequency, (2) rectal bleeding, and (3) physician global assessment; Range from 0 to 9; Score < 2 = disease remission; Score 2-4 = mild disease activity; Score 5-7 = moderate disease activity; Score > 7 = severe disease activity.
Bath Ankylosing Spondylitis Functional Index[14]	10 questions designed to determine the degree of functional limitation; Final score ranges from 0 to 10, with lower score indicating less functional limitation.
Inflammatory Bowel Disease Questionnaire[15]	32 questions divided into 4 subscales: (1) bowel symptoms (10 questions); (2) systemic symptoms, including sleep disorders and fatigue (5 questions); (3) emotional function, such as depression, aggression and irritation (12 questions); and (4) social function, meaning the ability to participate in social activities and to work (5 questions); The patient is invited to choose from 1 to 7 for every question; Total score ranges from 32 to 224 points, with lower scores reflecting worse quality of life.
Patient Global Assessment (PtGA)	Collected on a numeric rating scale ranging from 0 to 10 for the question asking the patient: "Considering all the ways your disease affects you, how much do you think is active today?"
Short Form-36 health survey[16]	Generic health status instrument with 8 domains: (1) physical function, (2) body pain, (3) role limitations–physical, (4) general health, (5) vitality, (6) social function, (7) role limitations–emotional, and (8) mental health; Greater scores reflect better health status; Summarized in two summary scores defined as the (1) physical component score (Sf-36/PCS) and (2) mental component score (Sf-36/MCS).

and between baseline and subsequent assessments were performed, respectively, with unpaired and paired Student's *t*-tests. Correlations between variables were assessed using Pearson's correlation coefficient. Data were analyzed using the SPSS software (v22.0; IBM SPSS Statistics for Windows, Armonk, NY, United States).

RESULTS

Baseline assessment of the patients

In the ES-AN cohort, 29 (57.6%) patients were affected by predominant axial SpA (Ax-ES-AN) and 23 (42.3%) by peripheral SpA (Per-ES-AN) (Table 1). Only 5 patients showed positivity for human leukocyte antigen-B27 (9.6%), including 4 affected by Ax-ES-AN and 1 by Per-ES-AN. Sacroiliitis was found in all the Ax-ES-AN patients, but 17 (55%) patients fulfilled the Modified New York Criteria for AS, whereas 13 (45%) were affected by non-radiographic axial-SpA[2]. Syndesmophytosis was found in 8 (27.6%) of the Ax-ES-AN patients at different levels, but only 2 (6.9%) presented a bamboo spine radiologic feature at baseline. Concurrent peripheral arthritis and enthesitis were present in about half of the Ax-ES-AN patients (44.8%).

According to the established criteria, IBD was active in 63.2% of the CD patients and in 98% of the UC patients, although the degrees of activity varied. At baseline, no differences were observed between the axial and peripheral spondyloarthritis patients and between the CD and UC patients for the articular disease activity (BASDAI, ASDAS-CRP) and the PROs [IBDQ, BASFI, PtGA, HAQ, SF-36 Physical Component Score (PCS) and Mental Component Score (MCS)] (data not shown). Following findings from the integrated evaluation and according to the algorithm presented in Figure 1, the criteria employed in our study to guide the therapeutic choice were (1) the presence and/or absence of active IBD (evaluated by both the gastroenterologist and by the results of the gastrointestinal tests and PROs); and (2) the site of articular involvement (axial or peripheral joints). Thus, Ada was assigned to a cohort of 30 patients, henceforth defined as the ES-AN/Ada cohort. Among this cohort, 4 were switched from infliximab for inefficacy, and the total was comprised of 20 patients with Ax-ES-AN and 10 patients with Per-ES-AN (Table 1).

Evaluation of articular and gastrointestinal disease activity

In most of the ES-AN/Ada patients, articular disease activity, as assessed by ASDAS-CRP and BASDAI, was significantly improved at 6-mo compared to baseline

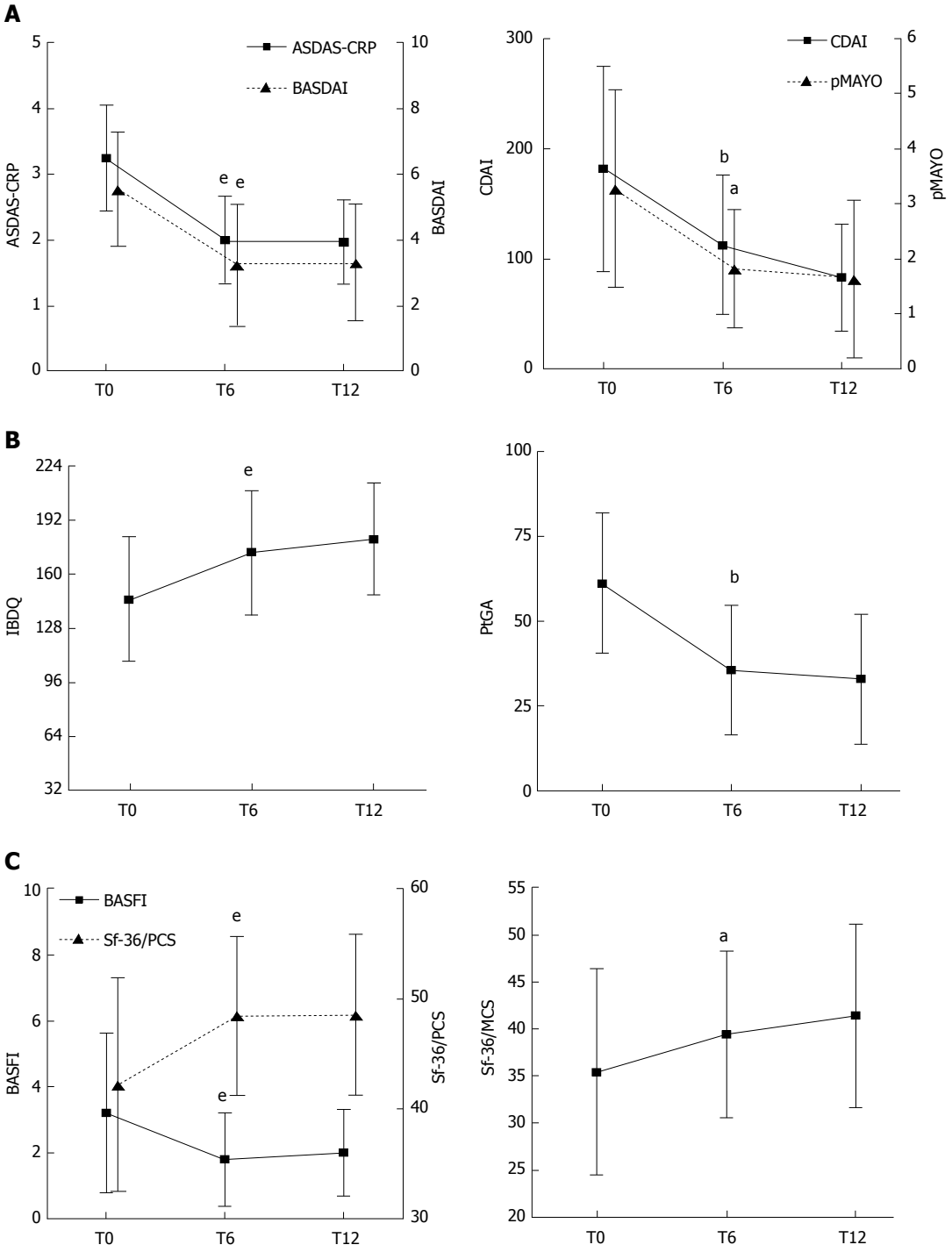


Figure 2 Evaluation of articular-gastrointestinal disease activity and HRQoL in the ES-AN patient cohort at baseline and after adalimumab therapy. All data of the ES-AN patients were collected at baseline (T0), and after 6 mo (T6) and 12 mo (T12) of therapy. A: Evaluation of the articular disease activity with BASDAI or ASDAS-CRP (Left), and of the gastrointestinal disease activity by CDAI for CD and pMAYO for UC (Right); B: Evaluation of PROs of gastrointestinal disease activity by IBDQ (Left), and of global wellness by the PtGA (Right). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$. ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score/C-Reactive Protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; CDAI: Crohn's Disease Activity Index; ES: Enteropathic spondyloarthritis; ES-AN: Patients affected by ES in the Ancona's cohort; HRQoL: Health-Related Quality of Life; IBDQ: Inflammatory Bowel Disease Questionnaire; pMAYO: Partial Mayo score; PROs: Patient reported outcomes.

(ASDAS-CRP: 3.2 ± 0.8 *vs* 1.9 ± 0.6, $P < 0.001$; BASDAI: 5.5 ± 1.7 *vs* 3.2 ± 1.8, $P < 0.001$). A major clinical improvement (ASDAS change of ⩾ 2) occurred in 21% and an important clinical improvement (ASDAS change of ⩾ 1.1) occurred in 52% (Figure 2A). The improvement of articular disease was significant in both the axial and the peripheral subgroups of patients ($P < 0.001$ for both comparisons). The clinical improvement at the articular level was maintained at the 12-mo examination (Figure 2A).

Regarding the gastrointestinal disease activity, in the CD patients the treatment led to a fast, consistent

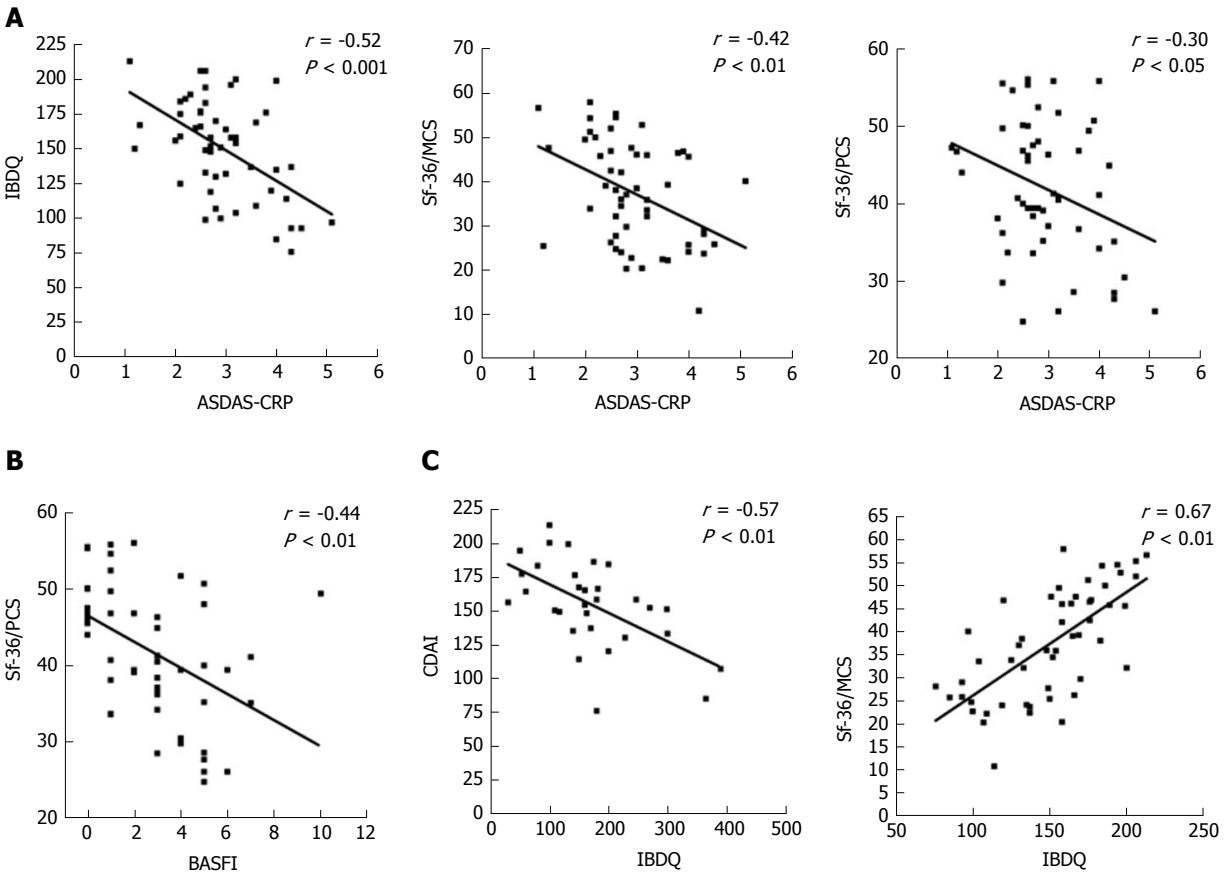


Figure 3 Correlations between articular and gastrointestinal disease activity and HRQoL scores in the ES-AN patient cohort at baseline. A: Correlation between the PROs of gastrointestinal disease activity (assessed by IBQD) scores and SF-36 summary scores (assessed by PCS and MCS) with articular activity scores (assessed by ASDAS-CRP); B: Correlation of the articular function (assessed by BASFI with SF-36/PCS); C: Correlation of the IBDQ with crohn's disease gastrointestinal disease activity (assessed by CDAI) and the SF-36/MCS. ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein; CDAI: Crohn's Disease Activity Index; ES: Enteropathic spondyloarthritis; ES-AN: Patients affected by ES in the ancona's cohort; HRQoL: Health-Related Quality of Life; IBDQ: Inflammatory Bowel Disease Questionnaire; PROs: Patient reported outcomes; SF-36/MCS: Summary of "Mental Component Score" of the Short Form-36 health survey; SF-36/PCS: Summary of "Physical Component Score" of the Short Form-36 health survey.

and significant improvement of the gastrointestinal symptoms at 6 mo, as assessed by the CDAI score (181.3 ± 93.2 *vs* 112.3 ± 63.0, $P < 0.01$; Figure 2A), and maintained at 12 mo, when the clinical remission was observed in almost all patients (Figure 2A). In parallel, a significant clinical improvement was observed also in the UC patients, as shown by the decrease of the pMAYO score at the 6-mo and 12-mo examinations (Figure 2A). All the values and comparisons are detailed in Table 3.

Evaluation of the HRQoL

ES-AN/Ada patients reported significant improvement, from baseline to 6 mo, in the IBDQ (145.3 ± 36.8 *vs* 172.8 ± 36.7, $P < 0.01$), PtGA (61.1 ± 20.6 *vs* 35.6 ± 19.1, $P < 0.01$; Figure 2B) and HAQ (4.8 ± 7.9 *vs* 2.0 ± 4.2, $P < 0.05$) scores, and this improvement was maintained at 12 mo. Moreover, regarding the PROs impacting articular function and global health wellness, significant improvements were observed from baseline to 6 mo in the BASFI (3.2 ± 2.4 *vs* 1.8 ± 1.4, $P < 0.01$), Sf-36/PCS (42.2 ± 9.7 *vs* 48.4 ± 7.3, $P < 0.01$) and Sf-36/MCS scores (35.4 ± 10.9 *vs* 39.4± 8.8, $P < 0.05$; Figure 2C); again, the improvements were maintained at 12 mo. The improvement of all the scores for PROs was similar in both the Ax-ES-AN/Ada and Per-ES-AN/Ada subgroups at each follow-up observation. All the values and comparisons are detailed in Table 3.

Correlations between variables

At baseline, it is noteworthy that a consistent significant correlation was observed between the IBDQ test (specific for the evaluation of the PROs related to the gastrointestinal disease activity) and the clinimetric scores of articular activity, as assessed by ASDAS-CRP (Figure 3A), BASDAI, and with most of the PROs of the HRQoL, as assessed by Sf-36/MCS and CDAI (Table 4). Moreover, articular disease activity at baseline was significantly correlated with worse results in the HRQoL tests, as shown by the Sf-36/PCS and Sf-36/MCS scores (Table 4). After 12 mo of Ada treatment, improvement of the articular activity (ASDAS-CRP) correlated significantly with decrease in the gastrointestinal disease activity scores, as assessed by CDAI in the CD patients (Figure 4A) and IBDQ in all of the patients, and with most of the PROs of the HRQoL, as

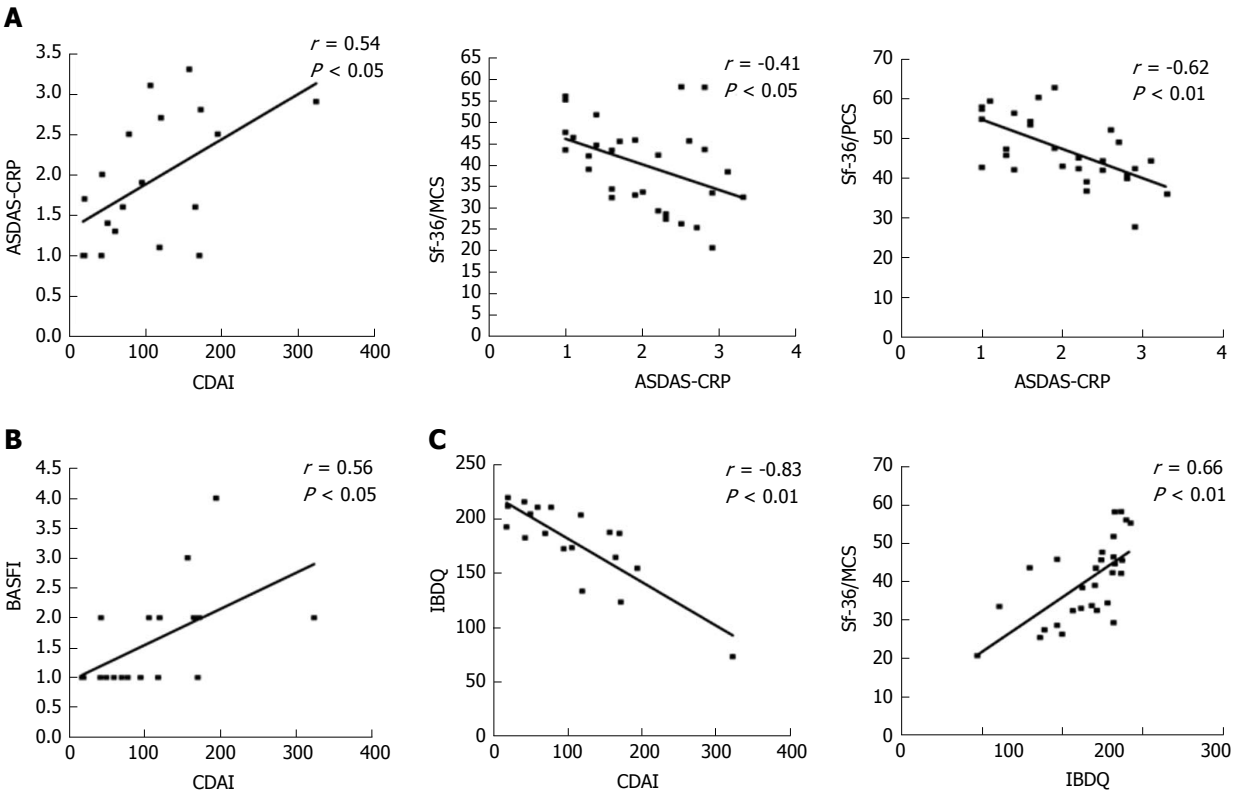


Figure 4 Correlations between articular and gastrointestinal disease activity and HRQoL scores in the ES-AN patient cohort after adalimumab therapy. A: Evaluation data collected for the 30 ES-AN/Ada patients after 12 mo of therapy, showing correlation between gastrointestinal and articular disease activity in Crohn's disease patients (assessed by CDAI) and ASDAS-CRP respectively, and between ASDAS-CRP and SF-36 summary scores (PCS and MCS); B: Correlation between CDAI and articular function (assessed by BASFI); C: Correlation between the gastrointestinal quality of life (assessed by IBDQ) and CDAI and SF-36/MCS. ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein; CDAI: Crohn's Disease Activity Index; ES-AN: Patients affected by ES in the Ancona's cohort; ES-AN/Ada: Patients of the ES-AN cohort treated with adalimumab; HRQoL: Health-Related Quality of Life; IBDQ: Inflammatory Bowel Disease Questionnaire; SF-36/MCS: Summary of "Mental Component Score" of the Short Form-36 health survey; SF-36/PCS: Summary of "Physical Component Score" of the Short Form-36 health survey; Sf-36/PCS: Summary of "Physical Component Score" of the short form-36 health survey; PROs: Patient reported outcomes.

Table 3 Scores of the clinimetric test for articular-gastrointestinal activity and patient reported outcomes of health-related quality of life

	Baseline	T6	T12
CDAI, *n* = 19	181.3 ± 93.2	112.3 ± 63.0[b]	82.7 ± 48.7[b]
pMAYO, *n* = 11	3.27 ± 1.79	1.81 ± 1.07[a]	1.63 ± 1.43[a]
IBDQ	145.3 ± 36.8	172.8 ± 36.7[e]	180.7 ± 33.0[e]
BASDAI	5.5 ± 1.7	3.2 ± 1.8[e]	3.3 ± 1.7[e]
BASFI	3.2 ± 2.4	1.8 ± 1.4[e]	2.0 ± 1.3[a]
ASDAS-CRP	3.2 ± 0.8	1.9 ± 0.6[e]	1.9 ± 0.6[e]
PtGA	61.1 ± 20.6	35.6 ± 19.1[b]	33.0 ± 19.1[b]
HAQ	4.7 ± 8.5	2.0 ± 4.2[a]	1.6 ± 3.5[a]
Sf-36/PCS	42.2 ± 9.7	48.4 ± 7.2[e]	48.5 ± 7.3[b]
Sf-36/MCS	35.4 ± 10.9	39.4 ± 8.8[a]	41.4 ± 9.7[a]
CRP in mg/dL	2.7 ± 3.7	0.8 ± 1.2[a]	0.5 ± 0.6[a]

[a]$P < 0.05$, [b]$P < 0.01$, [e]$P < 0.001$. ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; CDAI: Crohn's Disease Activity Index; CRP: C-Reactive Protein; pMAYO: partial Mayo score; HAQ: Health Assessment Questionnaire; IBDQ: Inflammatory Bowel Disease Questionnaire; PtGA: Patient Global Assessment; SF-36/MCS: Summary of "Mental Component Score" of the Short Form-36 health survey; SF-36/PCS: Summary of "Physical Component Score" of the Short Form-36 health survey; T6 and T12: Results of the evaluation after 6 mo and 12 mo of treatment with adalimumab respectively.

shown by the Sf-36/PCS (r = -0.30, $P < 0.05$) and Sf-36/MCS (r = -0.41, $P < 0.01$) scores (Figure 2C), as well as the BASFI (r = 0.57, $P < 0.001$), PtGA (r = 0.37, $P < 0.01$) and HAQ (r = 0.28, $P < 0.05$) scores. All the correlations for the values at 12-mo examination are detailed in Table 4.

Tolerability

Ada was well tolerated throughout the follow-up period. Side effects reported included recurrent upper respiratory tract infections (n = 2), bothersome hair loss (n = 1), and widespread itch (n = 2); but none necessitated suspension of the treatment. One patient suffered from a serious infection (axillary suppurative hidradenitis) that required prolonged antibiotic treatment. The other minor side effects reported were headache and fatigue.

DISCUSSION

The association between SpAs and IBDs has been known since the beginning of the last century[17]. However, gastroenterologists and rheumatologists continue to carry out their clinical evaluations and

Table 4 Correlations between the scores of clinimetric tests for articular-gastrointestinal activity and patient reported outcomes of health-related quality of life scores

	CDAI	pMAYO	IBDQ	BASDAI	BASFI	ASDAS-CRP	PtGA	HAQ	Sf-36/ PCS	Sf-36/ MCS	CRP
At baseline											
CDAI	1	1	-0.57[b]	0.19	0.29	0.21	0.14	0.12	-0.24	-0.33	0.35
pMAYO	1	1	-0.48[a]	0.34	0.38	0.37	0.15	0.18	-0.14	-0.11	0.28
IBDQ	-0.57[b]	-0.48[a]	1	-0.38[b]	-0.34[a]	-0.52[b]	-0.26	-0.19	0.27	0.67[b]	-0.26
BASDAI	0.19	0.34	-0.38[b]	1	0.64[b]	0.69[b]	0.24	0.26	-0.22	-0.31[a]	0.00
BASFI	0.29	0.38	-0.34[a]	0.64[b]	1	0.57[b]	0.37[b]	0.36[b]	-0.44[b]	-0.15	0.37[b]
ASDAS-CRP	0.21	0.37	-0.52[b]	0.69[b]	0.57[b]	1	0.37[b]	0.36[b]	-0.30[a]	-0.41[b]	0.32[a]
PtGA	0.14	0.15	-0.26	0.24	0.37[b]	0.37[b]	1	0.22	-0.14	-0.22	0.14
HAQ	0.12	0.18	-0.19	0.26	0.36[b]	0.36[b]	0.22	1	-0.46[b]	-0.03	0.07
Sf-36/PCS	-0.24	-0.14	0.27	-0.22	-0.44[e]	-0.30[a]	-0.14	-0.46[b]	1	0.05	-0.13
Sf-36/MCS	-0.33	-0.11	0.67[b]	-0.31[a]	-0.15	-0.41[b]	-0.22	-0.03	0.05	1	-0.08
After 12 mo of therapy with adalimumab (ES-AN/Ada cohort)											
CDAI	1	1	-0.83[b]	0.16	0.56[a]	0.54[a]	0.53[a]	0.53[a]	-0.71[b]	-0.67[b]	0.75[b]
pMAYO	1	1	-0.51	0.18	0.49	0.17	0.56	-0.29	-0.14	-0.25	0.6
IBDQ	-0.83[b]	-0.51	1	-0.23	-0.56[b]	-0.55[b]	-0.61[b]	-0.36[a]	0.53[b]	0.66[b]	-0.52[b]
BASDAI	0.16	0.18	-0.23	1	0.58[b]	0.68[b]	0.51[b]	0.39[a]	-0.52[b]	-0.11	-0.14
BASFI	0.56[a]	0.49	-0.56[b]	0.58[b]	1	0.56[b]	0.64[b]	0.54[b]	-0.52[b]	-0.45[a]	0.16
ASDAS-CRP	0.54[a]	0.17	-0.55[b]	0.68[b]	0.56[b]	1	0.67[b]	0.38[a]	-0.62[b]	-0.41[a]	0.31
PtGA	0.53[a]	0.56	-0.61[b]	0.51[b]	0.64[b]	0.67[b]	1	0.51[b]	-0.37[a]	-0.59[b]	0.26
HAQ	0.53[a]	-0.29	-0.36[a]	0.39[a]	0.54[b]	0.38[a]	0.51[b]	1	-0.47[b]	-0.40[a]	0.10
Sf-36/PCS	1	1	-0.83[b]	0.16	0.56[a]	0.54[a]	0.53[a]	0.53[a]	-0.71[b]	-0.67[b]	0.75[b]
Sf-36/MCS	1	1	-0.51	0.18	0.49	0.17	0.56	-0.29	-0.14	-0.25	0.60
CRP	-0.83[b]	-0.51	1	-0.23	-0.56[b]	-0.55[b]	-0.61[b]	-0.36[a]	0.53[b]	0.66[b]	-0.52[b]

Correlations between variables were assessed in the 30 patients with enteropathic spondyloarthritis from Ancona, Italy, at baseline and after 6 mo (data not shown) and 12 mo of therapy with adalimumab (ES-AN/Ada), using Pearson's correlation coefficient; [a]$P < 0.05$, [b]$P < 0.01$. ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score-C-Reactive Protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; CDAI: Crohn's Disease Activity Index; CRP: C-Reactive Protein; HAQ: Health Assessment Questionnaire; IBDQ: Inflammatory Bowel Disease Questionnaire; pMAYO: partial Mayo score; PtGA: Patient Global Assessment; SF-36/MCS: Summary of "Mental Component Score" of the Short Form-36 health survey; SF-36/PCS: Summary of "Physical Component Score" of the Short Form-36 health survey.

the therapeutic management of these diseases, now known collectively as ES, independently. In this regard, the different clinical guidelines employed by the two medical specialists may lead to different therapeutic decisions for the same clinical scenario, ultimately harboring potential for different clinical outcomes of the disease. A critical issue in the clinical management of ES is the correct therapeutic choice, and an important role has emerged recently for the TNF-alpha inhibitors in this regard. Infliximab was the first TNF-alpha inhibitor used in ES[18], but Ada has recently been applied to ES patients affected by CD and has achieved rates of clinical remission up to 50%[19].

To our knowledge, this is the first study demonstrating the efficacy of the integrated gastrorheumatologic approach for evaluation and therapeutic management of ES patients, with efficacy evidenced through assessments of disease activity and HRQoL over a 12-mo period. As dictated by our operative algorithm, both the gastroenterologist and the rheumatologist evaluated each of the patients in the study cohort, with both of the specialists collaborating to choose the optimal therapy. This integrated multidisciplinary approach, therefore, considered the gastrointestinal and articular disease activities simultaneously and, most importantly in the latter case, the site of articular inflammation (axial or peripheral). Thus, for example, in the case of axial spondyloarthritis, the therapy with anti-TNF-alpha inhibitors was mandatory, following the latest rheumatologic indications[20].

Since only few studies in the literature have so far evaluated the efficacy and safety of anti-TNF-alpha in ES, we employed the up and coming Ada treatment in our study. The results indicated that in ES patients, regardless of IBD type and/or site of articular involvement, Ada significantly improves the inflammation states of both the joints (assessed by ASDAS-CRP and BASDAI) and gut (assessed by CDAI or pMAYO score), as well as the HRQoL. Moreover, the benefits were already observable at 6 mo and the improvements were maintained at 12 mo. Ada also consistently improved not only the physical function in almost all ES patients (assessed by PtGA, BASFI, HAQ and Sf-36/PCS) but also their psychological function (assessed by Sf-36/MCS and IBDQ), from baseline to 6 mo.

It is noteworthy that in our study the strong linear relationship between articular-gastrointestinal disease activity and the PROs confirms the strong link between gut and joint inflammations. In fact, the IBDQ (specific for the gastrointestinal-related quality of life) strongly correlated with results of the clinimetric tests of articular activity (ASDAS-CRP, BASDAI and BASFI); additionally, the articular disease activity (assessed by ASDAS-CRP) strongly correlated with all of the PROs studied, even those specific for IBDs. To

the contrary, however, at baseline the gastrointestinal disease activity scores (except for those of the IBDQ) did not correlate well with the other variables, but at 12 mo of therapy the CDAI correlated with most of the articular and PROs of HRQoL. This latter finding may be consequent to the different degrees of disease activities reported by the patients at baseline, which, after the treatment, tend to be globally ameliorated both at the gastrointestinal and articular levels.

Thus, since only the ASDAS-CRP test proved to be the most reliable in all patients in our study, we think that it should be necessary to develop composite scores and HRQoL questionnaires specific and suitable for ES patients at diagnosis, similar to those developed for other multidisciplinary diseases, such as psoriatic arthritis[21]. In conclusion, in our work that was carried out in a real-life cohort of a significant number of ES patients, we demonstrated that Ada produced a fast and significant improvement of both the gastrointestinal and articular scores of disease activity and, moreover, of the HRQoL. This clinical result was achieved by employing an integrated outpatient clinic specific for ES patients, as recently endorsed, particularly with regard to early diagnosis[22,23]. The integrated approach provided the optimal management of both the multidisciplinary clinical evaluation and the therapy of these patients. Further studies are certainly warranted to assess the long-term outcomes, and tolerability, of Ada and other TNF-alpha inhibitors in patients affected by ES.

COMMENTS

Background

Enteropathic spondyloarthritis (ES) is characterized by articular inflammation in patients with inflammatory bowel diseases (IBDs), such as Crohn's disease or ulcerative colitis. Arthritis is the most frequent extra-intestinal manifestation found in IBD patients, yet only half of the ES patients are actually evaluated by a rheumatologist for a proper diagnosis and, thereafter, for receipt of an integrated therapeutic approach through a coordinated action between the two specialists.

Research frontiers

Considering the multidisciplinary intrinsic "face" of IBDs and the novel therapeutic opportunities that comprise the biological drugs, as in the case of anti-tumor necrosis factor (TNF)-alpha inhibitors, an integrated approach and evaluation of all ES patients should be routinely employed and strongly encouraged to obtain the best therapeutic efficacy on all the clinical manifestations of this disease.

Innovations and breakthroughs

In this work, the authors have demonstrated that the integrated (simultaneous) evaluation of patients affected by ES, through the coordinated efforts of a gastroenterologist and rheumatologist, led to the best therapeutic approach, thereby allowing the patients to achieve a consistent clinical remission of both the articular and gastrointestinal inflammations.

Applications

Through this study authors have been able to generate a simple working flowchart of the multidisciplinary clinical care process applied during the patients' integrated assessment. They suggest that in patients with ES and in consideration of the therapeutic choice, particular attention should be paid to the presence of active intestinal disease, presence of active articular (arthritis) and/or periarticular (enthesitis) disease and localization of the joints' inflammation (peripheral or axial, as in the case of sacroiliitis).

Terminology

ES belongs to the group of seronegative spondyloarthritis (SpA) and, as such, is characterized by the presence of arthritis in patients affected by IBDs; it is also known as SpA-IBD.

Peer-review

This is an interesting and well-conducted work.

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ORIGINAL ARTICLE

Observational Study

Presence of columnar-lined esophagus is negatively associated with the presence of esophageal varices in Japanese alcoholic men

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Abstract

AIM

To determine whether the presence of columnar-lined esophagus (CLE) is associated with the presence of

esophageal varices (EVs) in male Japanese alcoholics.

METHODS

The subjects were 1614 Japanese alcohol-dependent men (≥ 40 years of age) who had undergone upper gastrointestinal endoscopic screening. Digitalized records of high-quality endoscopic images that included the squamocolumnar junction and esophagogastric junction were retrospectively jointly reviewed by four expert endoscopists for the purpose of diagnosing CLE. The authors investigated whether and to what extent there were associations between the presence of CLE and the presence of EVs, especially in the group with liver cirrhosis (LC).

RESULTS

CLE ≥ 5 mm in length was found in 355 subjects (≥ 30 mm in 6 of them), LC without EVs in 152 subjects, LC with EVs in 174 subjects, and EVs without LC in 6 subjects. Advanced EVs, *i.e.*, nodular, large or coiled forms, red color sign, or post-treatment, were found in 88 subjects. The incidence of CLE ≥ 5 mm decreased in the following order ($P < 0.0001$): 23.3% in the group without EVs, 17.4% in the group with small and straight EVs, and 5.7% in the group with advanced EVs. The multivariate ORs (95%CI) for EVs and advanced EVs in the group with LC were lower when CLE ≥ 5mm was present [0.46 (0.23-0.93) and 0.24 (0.08-0.74), respectively, *vs* 0-4 mm CLE].

CONCLUSION

The presence of CLE in male Japanese alcoholics was negatively associated with the presence of EVs.

Key words: Alcohol; Columnar-lined esophagus; Hiatal hernia; Liver cirrhosis; Portal hypertension; Esophageal varices

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Core tip: A positive association between excessive drinking and the presence of short-segmental columnar-lined esophagus (CLE) has been reported in Asians. Endoscopic screening of 1614 Japanese alcohol-dependent men revealed the presence of CLE ≥ 5 mm in length in 355 subjects and esophageal varices (EVs) in 180 subjects. The presence of CLE was negatively associated with the presence of EVs, and even more negatively associated with the presence of advanced forms of EVs. Since the first resistance vessels to EVs are the mucosal palisade vessels and submucosal veins at the lower end of the esophagus, the development of CLE may impede the development of EVs.

Yokoyama A, Hirata K, Nakamura R, Omori T, Mizukami T, Aida J, Maruyama K, Yokoyama T. Presence of columnar-lined esophagus is negatively associated with the presence of esophageal varices in Japanese alcoholic men. *World J Gastroenterol* 2017; 23(39): 7150-7159 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7150.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7150

INTRODUCTION

Esophageal varices (EVs) develop as a result of portal hypertension, which is mainly due to liver cirrhosis (LC) in alcoholics, and excessive alcohol drinking increases the risk of variceal bleeding and mortality[1-3]. Excessive alcohol consumption has been reported to be associated with the presence of gastroesophageal reflux disease (GERD) and a short-segmental columnar-lined esophagus (CLE) in East-Asian studies[4-8]. Long-segmental CLE is rare in Asians[8], and a large pooled analysis of the cases in Western studies did not show a positive association between heavy drinking and GERD or long-segmental CLE[9]. The differences between the results in East Asia and the West may be attributable to the differences between East Asia and Western countries in abdominal obesity, incidences of *Helicobacter pylori* infection, and gene polymorphisms of alcohol-metabolizing enzymes.

Our empirical impression based on the results of endoscopic screening examinations of Japanese alcoholic men is that EVs are less common among men with short-segmental CLE. The development of CLE has been shown to be accompanied by several histological changes around the palisade vessels, including the development of a double muscularis mucosae[10,11]. Since dilatation of the palisade vessels at the lower end of the esophagus has been suspected of being one of the major initial events in the development of EVs secondary to portal hypertension[12,13], some of the histological changes accompanying the development of CLE may protect against the development of EVs.

It is widely accepted that genetic polymorphisms of alcohol dehydrogenase-1B (ADH1B, rs1229984) and aldehyde dehydrogenase-2 (ALDH2, rs671) affect the susceptibility of East-Asians to alcoholism[14-16], and the presence of fast-metabolizing ADH1B encoded by the *ADH1B*2* allele in Japanese alcoholics has been reported to be positively associated with the presence of advanced liver disease[17-19].

The aim of the present study was to determine whether and to what extent associations exist between the presence of EVs and the presence of CLE in Japanese alcoholic men based on the results of endoscopic screening examinations and their ADH1B/ALDH2 genotypes.

MATERIALS AND METHODS

Subjects

The reference population of this study consisted of 1902 Japanese alcoholic men 40 years of age and over who: (1) Came to the Kurihama Medical and Addiction Center for treatment of alcohol dependence for the first

time between May 2004 and December 2011; (2) Were evaluated for the presence of physical comorbidities; (3) Underwent routine upper gastrointestinal endoscopic screening; (4) And underwent ADH1B and ALDH2 genotyping[19]. After excluding the 194 subjects with a history of either gastrectomy or treatment for esophageal cancer and the 94 subjects without a digitalized record of high-quality endoscopic images that included the squamocolumnar junction and esophagogastric junction, 1614 patients were selected as subjects.

All of the alcoholics who participated in this study met the DSM-Ⅳ criteria for alcohol dependence[20]. Just before the endoscopic screening examination we asked each participant when he was in a sober state about his drinking and smoking habits. Usual alcohol consumption during the preceding year was expressed in grams of ethanol per day calculated by using standard conversion factors for alcoholic beverages. Beer and low-malt beer were assumed to be 5% ethanol (v/v); wine, 12%; sake, 16%; shochu, 25%; and whiskey, 40%.

The clinical diagnoses of comorbidities were made after alcohol detoxification. Patients received a routine examination that included a physical examination, blood tests, chest X-ray and abdominal X-ray, upper gastrointestinal endoscopy, abdominal ultrasound examination, and abdominal computed tomography. The clinical diagnosis of LC was made on the basis of the results of the physical examination, blood tests, and imaging studies or detection of esophagogastric varices during the endoscopic examination. The severity of LC was graded according to the Child-Pugh scoring system based on the findings at the first visit[21]. Hepatitis B surface (HBs) antigen and second generation anti-hepatitis C (HCV) antibody were measured with Abbott enzyme immunosorbent assays (Abbott Japan Inc., Tokyo).

Endoscopic procedure

Endoscopy was performed with an Olympus XQ230, Q240, Q240Z, or Q260 panendoscope (Olympus Optical Co. Ltd., Tokyo, Japan). Esophagogastric varices were diagnosed according to the grading system for esophagogastric varices adopted by the Japanese Society for Portal Hypertension[22]; *e.g.*, based on their form [small and straight (F1), nodular (F2), and large or coiled (F3)] and red color (RC) sign (Figure 1). The severity of portal hypertensive gastropathy (PHG) was evaluated according to Toyonaga's grading system, which has been adopted by the Japanese Society for Portal Hypertension (Figure 2)[23]: All PHG lesions exhibit a snake-skin (mosaic) pattern in their background mucosa; Grade 1, erythematous flecks or maculae; Grade 2, red spots or diffuse redness; and Grade 3, intramucosal or luminal hemorrhage. Three patients could not be evaluated for PHG because of food residue in the stomach.

Assessment for columnar-lined esophagus and hiatal hernia

The digitalized images that included the squamocolumnar junction and esophagogastric junction acquired before advancing the endoscope into the stomach were stored by the medical imaging communication system. The endoscopic digitalized images were retrospectively assessed during a joint review by four expert endoscopists (Yokoyama A, Hirata K, Nakamura R, Omori T) to diagnose CLE and hiatal hernia according to the classification system adopted by the Japan Esophageal Society[24]. The endoscopic esophagogastric junction was defined as the lower limit of the palisade longitudinal vessels (Figure 3)[10,11]. It was defined as the upper limit of the gastric mucosal folds when the lower limit of the palisade vessels was unclear. The greatest axial lengths of CLE were classified into four categories: 0-4 mm, 5-9 mm, 10-29 mm, and $\geqslant$ 30 mm. The axial length of a hiatus hernia was defined as the distance between the esophagogastric junction and the hiatus represented by the diaphragmatic pinch. The images were examined for the presence or absence of a hiatal hernia whose axial length was $\geqslant$ 10 mm.

Alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 genotyping

ADH1B and ALDH2 genotyping of every subject was performed by PCR-RFLP methods on a lymphocyte DNA sample[19].

Statistical analysis

The data have been summarized as means and standard errors or as percentage values. Student's *t*-test was used to compare normally distributed continuous variables between groups; the Mann-Whitney *U*-test was used to compare non-normally distributed continuous variables between groups; and Fisher's exact test or Cochran-Mantel-Haenszel test for trend was used to compare proportions between groups. Multiple logistic regression models were used to calculate adjusted odds ratios (ORs) and their 95% confidence intervals (CIs). A *P* value less than 0.05 was considered statistically significant. All analyses were performed by a biomedical statistician (Yokoyama T) using the SAS statistical package (version 9.4; SAS Institute, Cary, NC United States).

RESULTS

LC without EVs was diagnosed in 152 subjects, LC with EVs in 174 subjects, and EVs without LC in 6 subjects. The EVs were classified as F1 in 92 subjects, F2 or F3 in 41 subjects, RC-sign-positive in 21 subjects, and post-treatment in 26 subjects, 17 of whom were reported to have been treated for variceal rupture. All of the EVs in the subjects without LC were classified as F1. Table 1 shows the background factors of the 1614

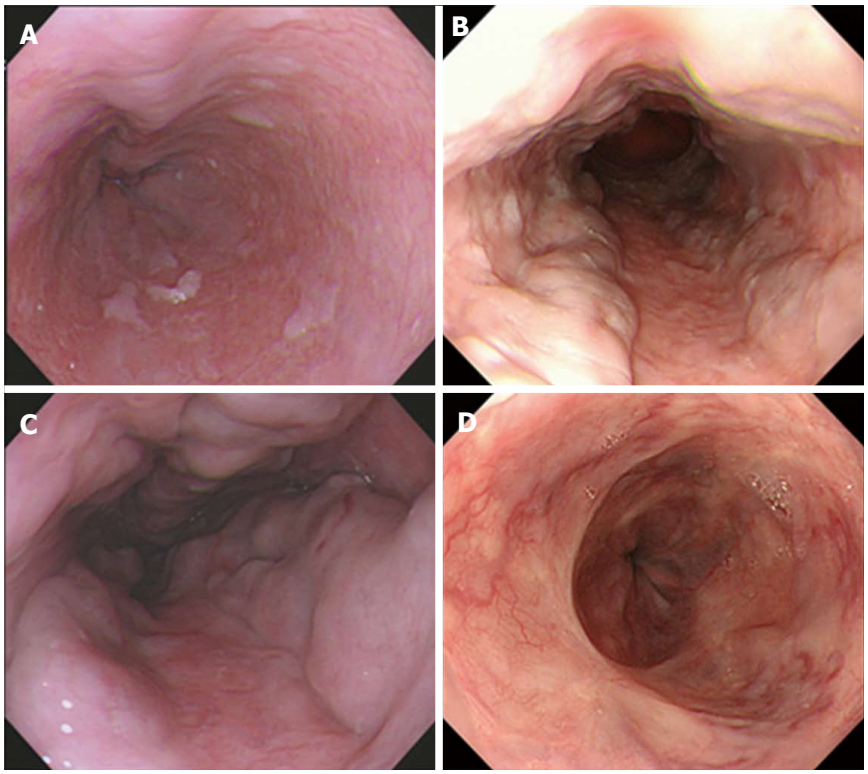


Figure 1 Classification of esophageal varices. A: F1 varices, small and straight; B: F2 varices, nodular; C: F3 varices, large or coiled; D: post-treatment varices.

Table 1 Background of the Japanese alcoholic men with and without esophageal varices *n* (%)

	Esophageal varices		
	Absent	Present	*P* value
n	1434	180	
Age (yr)			
mean ± SE	55.8 ± 0.3	54.4 ± 0.7	0.079
Alcohol consumption (g ethanol/d)			
mean ± SE	121 ± 2	133 ± 7	0.066
Ever smoker	1310 (91.4)	159 (88.3)	0.21
Liver cirrhosis			
Absent	1282 (89.4)	6 (3.3)	
Present	152 (10.6)	174 (96.7)	< 0.0001
Gastric Varices			
Absent	1418 (98.9)	147 (81.7)	
Cardia, cardia/fornix	2 (0.1)	23 (12.8)	
Fornix	14 (1.0)	10 (5.6)	< 0.0001
Portal hypertensive gastropathy	1432	179	
Absence	1172 (81.8)	59 (33.0)	
Grade 1	191 (13.3)	80 (44.7)	
Grade 2	53 (3.7)	25 (14.0)	
Grade 3	16 (1.1)	15 (8.4)	< 0.0001
Anti-HCV antibody positive	69 (4.8)	13 (7.2)	0.20
HBs antigen positive	17 (1.2)	4 (2.2)	0.28
ALDH2 genotype			
*1/*1	1216 (84.8)	161 (89.4)	
*1/*2	218 (15.2)	19 (10.6)	
*2/*2	0 (0.0)	0 (0.0)	0.12
ADH1B genotype			
*1/*1	406 (28.3)	41 (22.8)	
*1/*2	470 (32.8)	69 (38.3)	
*2/*2	558 (38.9)	70 (38.9)	0.20

ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; *P* values were calculated by Student's *t*-test for mean values, and Fisher's exact test for percent data.

subjects according to whether they had EVs. There were no significant differences between the group with EVs and the group without EVs in age, usual alcohol consumption, smoking, hepatitis C and B infection status, or ALDH2 and ADH1B genotypes. LC, gastric varices, and higher grade PHG were more common in the group with EVs than in the group without EVs ($P < 0.0001$). Most of the gastric varices in the group without EVs were found in the fornix alone, and gastric varices located in the cardia or cardia plus fornix predominanted in the group with EVs.

CLE $\geqslant$ 5 mm was found in 355 (22.0%) of the subjects (5-9 mm, 13.6%; 10-29 mm, 8.1%; and $\geqslant$ 30 mm, 0.4%; Table 2). When advanced forms of EVs were defined as F2 - F3 varices, RC-sign-positive varices, and post-treatment varices, the proportion of subjects with CLE $\geqslant$ 5 mm decreased in the following order ($P < 0.0001$): 23.3% in the group without EVs, 17.4% in the group with F1 varices, and 5.7% in the group with advanced varices, and the findings were similar in the subgroup of subjects with LC ($P = 0.0004$). The incidence of CLE $\geqslant$ 5 mm in the group of subjects with advanced EVs did not differ significantly according to whether they had or had not received endoscopic treatment (7.7% and 4.8%, respectively, $P = 0.63$). The proportions with a hiatal hernia $\geqslant$ 10 mm (24.4%, 20.7%, and 13.6%, respectively) decreased in the same order as the order in which the proportions of subjects with CLE $\geqslant$ 5 mm decreased ($P = 0.017$), but the trend was not significant in the subgroup of subjects with LC.

Table 2 Columnar-lined esophagus and hiatal hernia and the degree of esophageal varices *n* (%)

Esophageal varices	Absent	F1 varices	Advanced varices	*P* value
n	1434	92	88	
Columnar-lined esophagus				
0-4 mm	1100 (76.7)	76 (82.6)	83 (94.3)	
⩾ 5 mm	334 (23.3)	16 (17.4)	5 (5.7)	< 0.0001
5-9 mm	208 (14.5)	8 (8.7)	3 (3.4)	
10-29 mm	120 (8.4)	8 (8.7)	2 (2.3)	
⩾ 30 mm	6 (0.4)	0 (0.0)	0 (0.0)	
Hiatal hernia ⩾ 10 mm				
Absent	1084 (75.6)	73 (79.3)	76 (86.4)	
Present	350 (24.4)	19 (20.7)	12 (13.6)	0.017
Number of patients with liver cirrhosis	152	86	88	
Columnar-lined esophagus				
0-4 mm	117 (77.0)	73 (84.9)	83 (94.3)	
⩾ 5 mm	35 (23.0)	13 (15.1)	5 (5.7)	0.0004
5-9 mm	22 (14.5)	8 (9.3)	3 (3.4)	
10-29 mm	12 (7.9)	5 (5.8)	2 (2.3)	
⩾ 30 mm	1 (0.7)	0 (0.0)	0 (0.0)	
Hiatal hernia ⩾ 10 mm				
Absent	124 (81.6)	71 (82.6)	76 (86.4)	
Present	28 (18.4)	15 (17.4)	12 (13.6)	0.36

Advanced varices: F2-F3 varices, red-color-sign positive varices, or post-treatment varices. *P* values are for trend calculated by the Cochran-Mantel-Haenszel test.

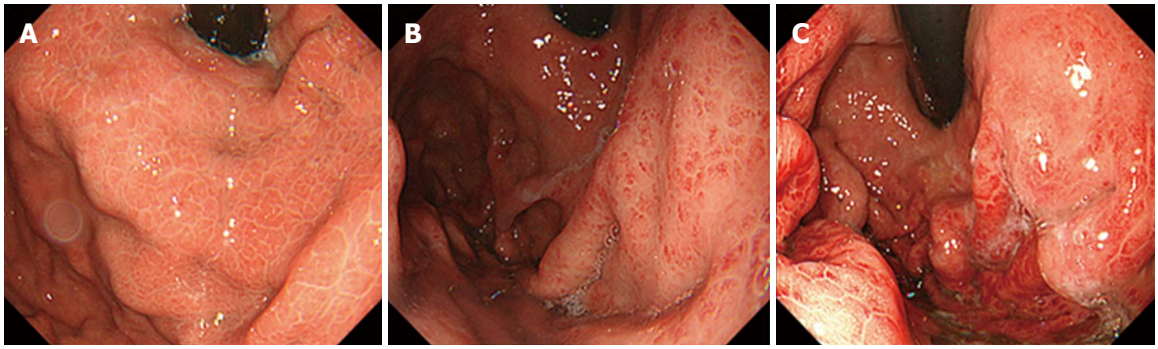


Figure 2 Portal hypertensive gastropathy lesions exhibiting a snake-skin (mosaic) pattern in their background mucosa. A: Grade 1, erythematous flecks or maculae; B: Grade 2, red spots or diffuse redness; C: Grade 3, intramucosal or luminal hemorrhage.

Table 3 shows the background factors of the 1614 subjects according to whether they had CLE ⩾ 5 mm, and there were no significant differences between the two groups in age, usual alcohol consumption, smoking, or ALDH2 and ADH1B genotypes. Hiatal hernia was more common in the group with CLE ⩾ 5 mm than in the other group (45.9% *vs* 17.3%, *P* < 0.0001). There was a significant difference between the group with CLE ⩾ 5 mm and the group without CLE ⩾ 5 mm in the proportions of 'no LC and no EVs' subjects, 'LC and no EVs' subjects, and the 'EVs' subjects they contained (*P* = 0.001). There were no significant difference between the group with CLE ⩾ 5 mm and group without CLE ⩾ 5 mm in the presence of gastric varices or the grade of PHG.

A multiple logistic regression analysis to predict the presence of CLE ⩾ 5 mm showed that the ORs (95%CI) for CLE ⩾ 5 mm increased with age per +10 years [1.15 (1.00-1.32)] and with the presence of hiatal hernia ⩾ 10 mm [4.33 (3.31-5.67)] and decreased with the presence of EVs [0.47 (0.26-0.87); Table 4]. The presence of LC, presence of gastric varices, and grade of PHG were not associated with the presence of CLE ⩾ 5 mm.

Table 5 shows the background factors of the 326 subjects with LC according to whether they had EVs, and there were no significant differences between the two groups in age, usual alcohol consumption, smoking, hepatitis C and B infection status, or ALDH2 and ADH1B genotypes. The absence of CLE ⩾ 5 mm (*P* = 0.003), presence of gastric varices (*P* < 0.0001), and higher grade PHG (*P* < 0.0001) were all more common in the LC group with EVs than in the LC group without EVs. Advanced Child-Pugh class (*P* = 0.011) was more common in the LC group with advanced EVs.

A multiple logistic regression analysis showed

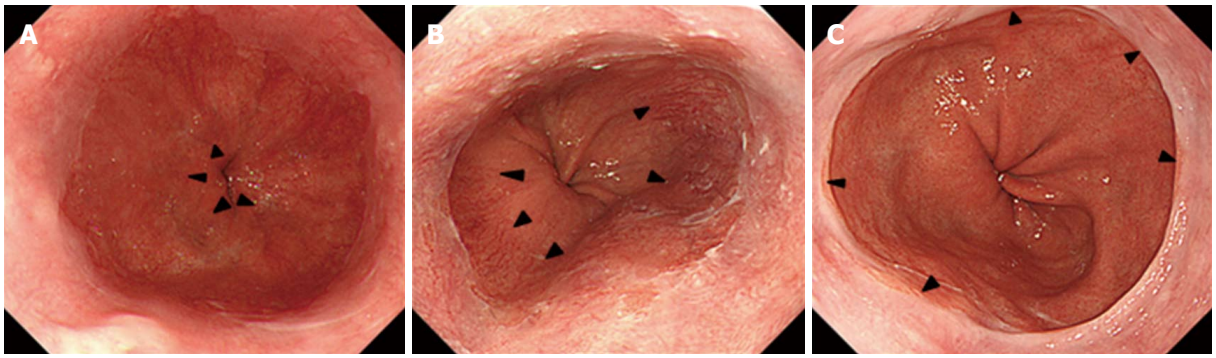


Figure 3 Columnar-lined esophagus and hiatal hernia. A: CLE 10-29 mm; B: CLE 5-9 mm; C: CLE 0-4 mm and hiatal hernia ⩾ 10 mm. The endoscopic esophagogastric junction was defined as the lower limit of the palisade longitudinal vessels (arrows). The axial length of a hiatal hernia was defined as the distance between the esophagogastric junction and the hiatus represented by the diaphragmatic pinch. CLE: Columnar-lined esophagus.

Table 3 Background of the Japanese alcoholic men with and without a columnar-lined esophagus *n* (%)

	Columnar-lined esophagus ⩾ 5 mm		
	Absent	Present	*P* value
n	1259	355	
Age (yr), mean ± SE	55.6 ± 0.3	55.8 ± 0.5	0.75
Usual alcohol consumption (g ethanol), mean ± SE	123 ± 2	121 ± 4	0.69
Ever smoker	1151 (91.4)	318 (89.6)	0.29
Hiatal hernia ⩾ 10 mm	218 (17.3)	163 (45.9)	< 0.0001
No liver cirrhosis and no EVs	983 (78.1)	299 (84.2)	
Liver cirrhosis and no EVs	117 (9.3)	35 (9.9)	
EVs	159 (12.6)	21 (5.9)	0.001
Gastric varices			
Absent	1215 (96.5)	350 (98.6)	
Cardia, cardia and fornix	23 (1.8)	2 (0.6)	
Fornix	21 (1.7)	3 (0.8)	0.13
Portal hypertensive gastropathy	1256	355	
Absent	947 (75.4)	284 (80.0)	
Grade 1	218 (17.4)	53 (14.9)	
Grade 2,3	91 (7.2)	18 (5.1)	0.17
ALDH2 genotype,			
*1/*1	1077 (85.5)	300 (84.5)	
*1/*2	182 (14.5)	55 (15.5)	
*2/*2	0 (0.0)	0 (0.0)	0.61
ADH1B genotype,			
*1/*1	348 (27.6)	99 (27.9)	
*1/*2	407 (32.3)	132 (37.2)	
*2/*2	504 (40.0)	124 (34.9)	0.15

ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; EVs: Esophageal varices; *P* values are according to the results of Student's *t*-test for age; the Mann-Whitney *U*-test for alcohol consumption; and Fisher's exact test for the other variables.

that the ORs (95%CI) for EVs and advanced EVs in the subgroup of subjects with LC decreased with the presence of CLE ⩾ 5 mm [0.46 (0.23-0.93) and 0.24 (0.08-0.74), respectively, *vs* CLE 0-4 mm; Table 6]. Child-Pugh class was not associated with EV status. The ORs for EVs and advanced EVs increased with progression of the grade of PHG, and the OR for advanced EVs increased with the presence of gastric varices.

DISCUSSION

The analysis of the results of endoscopic screening of 1614 Japanese alcoholic men in this study demonstrated that the presence of CLE was negatively associated with the presence of EVs, and even more negatively associated with the presence of advanced forms of EVs. The prevalence of CLE ⩾ 30 mm has been reported to be very low (0.0%-0.2%) in Asians[8,25-27]. The prevalence of CLE ⩾ 30 mm in the present study was 0.4%, and most of the CLE was short-segmental. The presence of a hiatal hernia was a strong determinant of the presence of CLE in this study, a finding that was consistent with the results of previous studies[5,7,8,26,28], suggesting that GERD in patients with a hiatal hernia contributes to the development of CLE. Asian studies have demonstrated positive associations between excessive alcohol consumption and both GERD and short-segmental CLE[4-8,26]. The prevalence of CLE ⩾ 10 mm has been reported to be 1.8% in unselected Taiwanese, 4.0% in symptomatic Koreans[27], and 5.3% in a group of Japanese who underwent an endoscopic examination in a university hospital[8]. Thus, the high prevalence (8.1%) of CLE ⩾ 10 mm in the population of alcoholics in our study was at least in part attributable to chronic heavy drinking.

As expected, the presence of EVs was positively associated with the presence of LC, gastric varices, and PHG. However, these factors were not associated with the presence of CLE, and adjustment for these factors revealed a negative association between the presence of CLE and the presence and severity of EVs. These findings taken together suggested a special relationship between CLE and EVs in the presence of LC and portal hypertension.

In the majority of LC patients the left gastric vein is the afferent vein to the EVs[29], and the first resistance vessels to the EVs are the mucosal palisade vessels and submucosal veins at the lower end of the

Table 4 Multivariate analyses to predict the presence of columnar-lined esophagus in Japanese alcoholic men

	CLE, ⩾ 5 mm *vs* 0-4 mm		
Independent variables	**OR**	**95%CI**	***P* value**
Age, per +10 yr	1.15	(1.00-1.32)	0.043
Usual alcohol consumption, per +22 g ethanol	0.99	(0.96-1.03)	0.75
Ever smoker *vs* never smoker	0.75	(0.49-1.14)	0.17
Hiatal hernia ⩾ 10 mm, presence *vs* absence	4.33	(3.31-5.67)	< 0.0001
Liver cirrhosis, presence *vs* absence	1.04	(0.68-1.60)	0.86
Esophageal varices, presence *vs* absence	0.47	(0.26-0.87)	0.015
Gastric varices, presence *vs* absence	0.59	(0.21-1.64)	0.31
Portal hypertensive gastropathy			
Absent	1.00	Referent	
Grade 1	1.23	(0.86-1.78)	0.26
Grade 2,3	0.91	(0.52-1.60)	0.74
Anti-HCV antibody, positive *vs*, negative	0.93	(0.53-1.66)	0.81
HBs antigen, positive *vs*, negative	0.56	(0.13-2.47)	0.44
ALDH2 genotype, *1/*1 *vs* *1/*2	0.94	(0.67-1.33)	0.73
ADH1B genotype, *2 carrier *vs* *1/*1	0.97	(0.73-1.28)	0.81

ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; OR: Multivariate odds ratio; CI: Confidence interval; CLE: Columnar-lined esophagus. ORs and CIs were calculated by using a multiple logistic regression model.

Table 5 Background of the Japanese alcoholic men with liver cirrhosis according to whether they had esophageal varices *n* (%)

	Esophageal varices				
	Absent	**All forms**	***P* value**	**Advanced forms**	***P*[1] value**
n	152	174		88	
Age (yr)					
mean ± SE	55.9 ± 0.7	54.5 ± 0.7	0.16	54.3 ± 1.0	0.21
Alcohol consumption (g ethanol/d)					
mean ± SE	124 ± 7	133 ± 7	0.37	142 ± 11	0.18
Ever smoker	132 (86.8)	155 (89.1)	0.61	83 (94.3)	0.081
Columnar-lined esophagus ⩾ 5 mm	35 (23.0)	18 (10.3)	0.003	5 (5.7)	0.0005
Hiatal hernia ⩾ 10 mm	28 (18.4)	27 (15.5)	0.55	12 (13.6)	0.37
Child-Pugh class					
A	93 (61.2)	88 (50.6)		41 (46.6)	
B	50 (32.9)	64 (36.8)		32 (36.4)	
C	9 (5.9)	22 (12.6)	0.053	15 (17.0)	0.011
Gastric varices					
Absent	137 (90.1)	141 (81.0)		65 (73.9)	
Cardia, cardia and fornix	2 (1.3)	23 (13.2)		17 (19.3)	
Fornix	13 (8.6)	10 (5.7)	< 0.0001	6 (6.8%)	< 0.0001
Portal hypertensive gastropathy	151	173			
Absent	88 (58.3)	55 (31.8)		27 (30.7)	
Grade 1	50 (33.1)	78 (45.1)		39 (44.3)	
Grade 2	10 (6.6)	25 (14.5)		12 (11.4)	
Grade 3	3 (2.0)	15 (8.7)	< 0.0001	15 (8.4)	< 0.0001
Anti-HCV antibody positive	8 (5.3)	13 (7.5)	0.50	8 (9.1)	0.29
HBs antigen positive	4 (2.6)	4 (2.3)	1.00	1 (1.1)	0.65
ALDH2 genotype					
*1/*1	134 (88.2)	156 (89.7)		77 (87.5)	
*1/*2	18 (11.8)	18 (10.3)		11 (12.5)	
*2/*2	0 (0.0)	0 (0.0)	0.72	0 (0.0)	1.00
ADH1B genotype					
*1/*1	32 (21.1)	36 (20.7)		15 (17.0)	
*1/*2	51 (33.6)	68 (39.1)		37 (42.0)	
*2/*2	69 (45.4)	70 (40.2)	0.55	36 (40.9)	0.41

[1]Advanced forms of EVs *vs* no EVs. ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; EVs: Esophageal varices; *P* values were calculated by Student's *t*-test for mean values, and Fisher's exact test for percent data.

esophagus, where there are many palisade vessels and few submucosal veins[12,13]. The palisade vessels penetrate the muscularis mucosae and connect to the submucosal veins 3-5 cm above the esophagogastric junction, the critical area for EV rupture[12,30,31]. Greatly enlarged palisade vessels disrupt the muscularis mucosae and form EVs across the proper mucosae and the submucosa[13], and they cause advanced EVs by

Table 6 Multivariate analyses to predict the presence of esophageal varices in Japanese alcoholic men with liver cirrhosis

	Cirrhosis with EVs *vs* cirrhosis without EVs			
	All forms of EVs		Advanced forms of EVs	
Independent variables	OR (95%CI)	*P* value	OR (95%CI)	*P*[1] value
Age, per +10 yr	0.88 (0.67-1.16)	0.35	0.90 (0.64-1.26)	0.55
Usual alcohol consumption, per +22 g ethanol	1.00 (0.94-1.06)	0.97	1.02 (0.95-1.10)	0.52
Ever smoker *vs* never smoker	1.04 (0.50-2.16)	0.93	2.13 (0.69-6.57)	0.19
Columnar-lined esophagus, ⩾ 5 mm *vs* 0-4 mm	0.46 (0.23-0.93)	0.030	0.24 (0.08-0.74)	0.013
Hiatal hernia ⩾ 10 mm, presence *vs* absence	1.10 (0.54-2.21)	0.80	0.97 (0.38-2.49)	0.95
Child-Pugh class				
A	1.00 Referent		1.00 Referent	
B	1.18 (0.70-1.98)	0.53	1.06 (0.55-2.05)	0.87
C	1.56 (0.64-3.82)	0.33	1.85 (0.67-5.16)	0.24
Gastric varices, presence *vs* absence	1.94 (0.96-3.91)	0.065	2.89 (1.29-6.47)	0.010
Portal hypertensive gastropathy				
Absent	1.00 Referent		1.00 Referent	
Grade 1	2.24 (1.32-3.80)	0.003	1.92 (0.97-3.77)	0.060
Grade 2,3	4.40 (2.11-9.15)	< 0.0001	5.29 (2.18-12.81)	0.0002
Anti-HCV antibody, positive *vs* negative	1.09 (0.41-2.93)	0.87	1.54 (0.49-4.88)	0.46
HBs antigen, positive *vs*, negative	1.16 (0.26-5.16)	0.85	0.61 (0.06-6.36)	0.68
ALDH2 genotype, *1/*1 *vs* *1/*2	1.02 (0.47-2.20)	0.96	1.00 (0.38-2.60)	1.00
ADH1B genotype, *2 carrier *vs* *1/*1	1.10 (0.61-1.99)	0.76	1.59 (0.72-3.51)	0.25

[1]Advanced forms of EVs *vs* no EVs, EVs: Esophageal varices; ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; OR: Multivariate odds ratio; ORs and CIs were calculated by using a multiple logistic regression model; Advanced forms of EVs were F2-F3 varices, red-color-sign positive varices, or post-treatment varices.

connecting with submucosal veins or sub- and intra-epithelial channels accompanied by 'varices on varices' and positive RC signs[12,13,32].

Although there is no difference between the maximal size of the palisade vessels in the CLE and normal lower esophagus[11], it is conceivable that the development of CLE may change the microenvironment of the interstitium around the palisade vessels, *e.g.*, increased interstitial fibrosis and the formation of the shallow muscularis mucosae of a double muscularis mucosae. A double muscularis mucosae was seen in 71% of the specimens obtained by endoscopic resection of a CLE[11]. The double muscularis mucosae in CLE divides the proper mucosae into two restricted compartments, and may increase the resistance to enlargement of palisade vessels and prevent the vessels from communicating with the submucosal vessels or sub- and intra-epithelial channels[13,32], thereby inhibiting the development of enlarged EVs and RC signs. Scars secondary to endoscopic ligation or sclerotherapy were present in some of the LC subjects with post-treatment varices and their presence may have influenced the development of CLE. However, the incidence of CLE in the LC group did not differ significantly according to whether there was a history of endoscopic treatment.

The presence of the *ADH1B*2* allele in Japanese alcoholics has been demonstrated to be positively associated with the presence of advanced liver disease and the progression of Child-Pugh class[17-19], but the results of the present study showed no significant associations between the genetic polymorphism and the presence of EVs. The effect of the *ADH1B*2* allele on EVs was probably eclipsed, because 46.6% of the LC subjects lacked EVs.

Our study had several potential limitations. The first potential limitation was that it was a cross-sectional study based on the results of the endoscopic screening, and the progression of CLE and EVs was not evaluated directly. Identification of a causal relationship between these endoscopic findings would require longitudinal follow-up examinations. The second potential limitation was that we evaluated the degree of CLE by retrospectively reviewing the endoscopic images, however, the review was performed jointly by four expert endoscopists. Pathological studies of autopsied cirrhotic subjects are warranted to clarify the pathological background underlying the negative association between the presence of CEL and the presence of EVs. We did not observe any effects of alcohol consumption during the preceding year on the presence of CLE or EVs, but that may have been because of the homogeneity of the study population in terms of their extremely high alcohol consumption. Generalization of the results obtained in our study based on investigations of alcoholic men treated in the Center requires confirmation in various drinking populations, including in a population with mild alcoholism.

Helicobacter pylori infection and chronic atrophic gastritis protect against the development of GERD and CLE[5,6,33]. If the observed associations between CLE and EVs and advanced EVs reflect causal relationships, the current trend toward lower *Helicobacter pylori* infection rates in Japan may result in lower EV rates and advanced EV rates in the future, and examination for CLE may benefit alcoholics with advanced liver disease by identifying their risk for the development or

progression of EVs.

In conclusion, this cross-sectional observational study revealed a negative association between the presence of CLE and the presence of EVs in Japanese alcoholic men. Further studies should be conducted prospectively in a longitudinal fashion to confirm this finding.

ARTICLE HIGHLIGHTS

Research background

Esophageal varices (EVs) develop as a result of portal hypertension, which is mainly due to liver cirrhosis (LC) in alcoholics. Excessive alcohol consumption has been reported to be associated with the presence of a short-segmental columnar-lined esophagus (CLE) in East-Asian studies. It has not been evaluated whether associations exist between the presence of EVs and the presence of CLE in alcoholics.

Research motivation

Our empirical impression based on the results of endoscopic screening examinations of Japanese alcoholic men is that EVs are less common among men with short-segmental CLE. The development of CLE is accompanied by several histological changes around the palisade vessels at the lower end of the esophagus. Some of the histological changes accompanying the development of CLE may protect against the development of EVs.

Research objectives

To determine whether and to what extent associations exist between the presence of EVs and the presence of CLE in Japanese alcoholic men based on the results of endoscopic screening examinations.

Research methods

The subjects were 1614 Japanese alcohol-dependent men (≥ 40 years of age) who had undergone upper gastrointestinal endoscopic screening. Digitalized records of high-quality endoscopic images that included the squamocolumnar junction and esophagogastric junction were retrospectively jointly reviewed by four expert endoscopists for the purpose of diagnosing CLE. The authors investigated whether and to what extent there were associations between the presence of CLE and the presence of EVs, especially in the group with LC.

Research results

CLE ≥ 5 mm in length was found in 355 subjects, LC without EVs in 152 subjects, LC with EVs in 174 subjects, and EVs without LC in 6 subjects. Advanced EVs, *i.e.*, nodular, large or coiled forms, red color sign, or post-treatment, were found in 88 subjects. The incidence of CLE ≥ 5 mm decreased in the following order ($P < 0.0001$): 23.3% in the group without EVs, 17.4% in the group with small and straight EVs, and 5.7% in the group with advanced EVs. The multivariate ORs (95%CI) for EVs and advanced EVs in the group with LC were lower when CLE ≥ 5 mm was present [0.46 (0.23-0.93) and 0.24 (0.08-0.74), respectively, *vs* 0-4 mm CLE]. To clarify the pathological backgrounds of the negative association between the presence of CEL and the presence of EVs, the further pathological studies of autopsied cirrhotic subjects may be warranted.

Research conclusions

The presence of CLE was negatively associated with the presence of EVs, and even more negatively associated with the presence of advanced forms of EVs. Since the first resistance vessels to EVs are the mucosal palisade vessels and submucosal veins at the lower end of the esophagus, the development of CLE may impede the development of EVs. *Helicobacter pylori* infection and chronic atrophic gastritis protect against the development of GERD and CLE. If the observed associations between CLE and EVs and advanced EVs reflected causal relationships, the current trend toward to lower *Helicobacter pylori* infection rates in Japan may influence EV rates and advanced EV rates in the future, and examination for CLE may benefit alcoholics with advanced liver disease by identifying their risk for the development or progression of EVs. The further studies should be conducted prospectively in the longitudinal fashion to test this finding.

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ORIGINAL ARTICLE

Observational Study

Characteristics and outcomes of cholangiocarcinoma by region in Thailand: A nationwide study

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Abstract

AIM

To identify the potential risk factors of cholangiocarcinoma, we determined the characteristics of cholangiocarcinoma patients among 5 different regions of Thailand.

METHODS

All patients diagnosed with cholangiocarcinoma between 2008 and 2013 were identified using the Nationwide Hospital Admission Data registry (n = 39421). Baseline characteristics, comorbidities and survival were abstracted.

RESULTS

The annual incidence during the study period was stable in all regions. Most patients lived in the Northeast (62.8%), followed by the North (16.9%), Central (12.3%), Bangkok (5.4%), and South (n = 2.6%) regions (P < 0.0001). Significantly more cholangiocarcinoma patients had diabetes, cirrhosis, and chronic viral hepatitis B/C infection than non-cholangiocarcinoma participants (diabetes: 11.42% *vs* 5.28%; cirrhosis: 4.81% *vs* 0.92%; hepatitis B: 0.74% *vs* 0.12%; and hepatitis C: 0.50% *vs* 0.10%, P < 0.0001 for all, respectively). The overall 1-year mortality rate was 81.7%, with a stable trend over time.

CONCLUSION

Diabetes and chronic liver diseases may be associated with cholangiocarcinoma in the Thai population.

Key words: Bile duct cancer; Population-based study; Epidemiology; Liver fluke infection

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Core tip: Cholangiocarcinoma is highly prevalent in Thailand, particularly in the Northeast region. The high cholangiocarcinoma incidence in this region is known to be associated with a high prevalence of liver fluke infection. Cirrhosis, diabetes, and chronic viral hepatitis B and C infections have been recently identified as risk factors for cholangiocarcinoma in Western countries. In this study, we found that diabetes and chronic liver diseases may be associated with cholangiocarcinoma in the Thai population. Further study to determine the magnitude of the impact of these factors on cholangiocarcinoma development in the Thai population is necessary.

Chaiteerakij R, Pan-ngum W, Poovorawan K, Soonthornworasiri N, Treeprasertsuk S, Phaosawasdi K. Characteristics and outcomes of cholangiocarcinoma by region in Thailand: A nationwide study. *World J Gastroenterol* 2017; 23(39): 7160-7167 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7160.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7160

INTRODUCTION

Cholangiocarcinoma (CCA), a malignancy of the biliary tract epithelium, is of increasing importance due to its continually increasing incidence worldwide[1-7]. The incidence of CCA varies substantially from region to region, with a high incidence in Asia [*e.g.*, 22.9, 7.5, and 5.6 cases per 100000 persons in Thailand[2], China[7] and South Korea[5], respectively] and a low incidence in the United States and Europe [*e.g.*, 3.4, 1.6, 1.3, and 1.2 cases per 100000 persons in Italy[3], the United States[6], France[8], and the United Kingdom[4], respectively]. Variation in the global incidence of CCA is due to variations in risk factors, particularly host and environmental risk factors. Within Thailand, the incidence of CCA also varies geographically[9]. The Northeast region of Thailand has the most CCA cases, with an incidence rate of 85 cases per 100000 persons per year, whereas the South region has the lowest number of cases at 5.7 cases per 100000 persons per year. The high incidence of CCA in Northeast Thailand is related to the high prevalence of liver fluke or *Opisthorchis viverrini* (OV) infection[10].

In addition to liver fluke infection, primary sclerosing cholangitis (PSC) is another major risk factor for CCA[11]. PSC is a major cause of CCA in Western populations and is associated with an 80- to 160-fold increase in CCA risk[12,13]. Although PSC markedly increases CCA risk, PSC is present in only 20% of all CCA patients[14]. Most CCA patients do not have identifiable risk factors.

Cirrhosis, chronic viral hepatitis B and C infection, diabetes, obesity, and smoking have recently been shown to be associated with increased CCA risk[12-14]. These factors were believed to contribute to the increasing incidence of CCA in the United States[15]. The question regarding whether or not these pre-existing conditions contribute to CCA development in the Thai population, particularly in people living in areas that are non-endemic for liver fluke infection, has not yet been studied. Accordingly, the objectives of this study were to compare the characteristics of CCA patients among the 5 different regions of Thailand. Secondarily, survival outcomes were determined. This population-based study was conducted using data from the National Health Security Office, Thailand.

MATERIALS AND METHODS

Ethical considerations

The protocol of this study was approved by the

Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 113/58).

Data source

The data were obtained from the Nationwide Hospital Admission Data (NHAD) registry. The data were already de-identified before the investigators accessed them. Briefly, the NHAD registry is a database that contains hospitalization data of Thai citizens who are covered by the national Medical Welfare Scheme (MWS). The MWS was established in Thailand in 2000 and is currently one of 3 major health insurance systems. During the study period, the MWS covered approximately 47 million of the 64 million total Thai people in all age groups across Thailand. Those who have health insurance coverage by the MWS can access health services in hospitals that are registered with the National Health Security Office. Patients have access to the hospitals that are assigned to each individual by the household registration system. At the time of data collection, there were 39421 hospitals in the registry, of which 14402 (36.5%) were primary or community hospitals that provide primary healthcare, 11277 (28.6%) were secondary hospitals that provide general healthcare, and 13742 (34.9%) were tertiary referral hospitals that provide specialized care for complex cases.

Study population

CCA cases: All patients diagnosed with CCA who were admitted to affiliated hospitals during the January 2008 to December 2013 study period were identified in the NHAD registry using the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) codes of C22.1 (intrahepatic cholangiocarcinoma), C24.0 (extrahepatic cholangiocarcinoma), C24.1 (cancer of the ampulla of Vater), C24.8 (cholangiocarcinoma whose subtype cannot be classified), and C24.9 (cholangiocarcinoma, unspecified subtype). Cancer of the ampulla of Vater was included in this study because it occurs within 2 cm of the distal end of the common bile duct. Demographic data, including age, gender, and residential area, were collected and recorded. Potential risk factors for CCA, including cirrhosis, diabetes, and chronic viral hepatitis B and C infection, were collected using the corresponding ICD-10 codes, as follows: HBV infection: B18.0 and B18.1; HCV infection: B18.2; cirrhosis: K74, K74.0, K74.1, K74.2, K74.6, K70.2, K70.3, and K70.9; and diabetes mellitus type 2: E11. Date of death was obtained from the death certificate.

Controls: There were 2 control cohorts in this study. First, to compare demographic differences between CCA patients and non-CCA individuals, all Thai citizens were used as controls (n = 64076033). Demographic data of the Thai citizens were obtained in the year 2011 (the midpoint of the study period) from the Department of Provincial Administration, Ministry of the Interior, Thailand (Table 1). Second, to explore the potential associations between cirrhosis, diabetes and chronic viral hepatitis B and C infections and CCA development in the Thai population, patients without CCA who were admitted to hospitals during the study period were used as controls (n = 18508448). The data of non-CCA controls were obtained from discharge summary notes in the same manner as CCA cases.

Statistical analysis

All data analyses were performed using PASW® Statistics version 18 (SPSS, Inc., Chicago, IL, United States) and STATA version 12.1 (StataCorp LP, College Station, TX, United States). The patients were divided into 1 of the following 5 geographic region groups: Northeast, North, Central, Bangkok, or South. Bangkok is the capital of Thailand and is located in the Central geographic region. However, we designated Bangkok as a separate region because it has several major university hospitals that may be able to provide more technologically advanced treatment options than most hospitals located in other parts of the country. The annual incidence rate of CCA was calculated by dividing the number of new patients who were admitted with a diagnosis of CCA annually by the general population in the same year. Underlying diseases and one-year mortality rates were compared among regions using Student's t-test for continuous variables and chi-square test for categorical variables.

Demographic data of CCA patients and all Thai citizens were compared using chi-square and t-tests. The proportions of individuals with cirrhosis, diabetes and chronic viral hepatitis B and C infections between CCA and non-CCA patients who were admitted to the hospital were compared during the study period using a χ^2 test. Data are presented as a number, number (%), or mean ± SD.

One-year mortality was calculated using the per-patient information in which the survival time was calculated from the subtraction between the date of death and the date of the first admission. One-year mortality was then defined as 1 if the survival time was less than a year and 0 otherwise. An analysis of the trend in CCA incidence and mortality was performed using "nptrend", the nonparametric test for trend across ordered groups developed by Cuzick (1985), which is an extension of the Wilcoxon rank-sum test. A P-value of < 0.05 indicated statistical significance.

RESULTS

Baseline characteristics

There were 39421 patients diagnosed with CCA who

Table 1 Baseline characteristics of cholangiocarcinoma patients diagnosed between 2009 and 2013 *n* (%)

Variable	CCA cases (*n* = 39421)	Thai population (*n* = 64076033)	*P* value
Gender			
Male	24120 (61.2)	31529148 (49.2)	< 0.001
Age (mean ± SD, yr)	64.1 ± 11.7	34.9[1]	
Geographic region			< 0.0001
Northeast	24239 (61.5)	21585883 (33.7)	< 0.001
North	6699 (17.0)	11783311 (18.4)	< 0.001
Central	5295 (13.4)	16060141 (25.1)	< 0.001
Bangkok	1114 (2.8)	5674843 (8.8)	< 0.001
South	1056 (2.7)	8971855 (14.0)	< 0.001

[1]Weighted average age calculated using the midpoint; Standard deviation was undefined; thus, a *t*-test was not performed. CCA: Cholangiocarcinoma.

Table 2 Incidence (per 100000 persons) of cholangiocarcinoma in Thailand during the 2009 to 2013 study period

Region	Year					P_{trend} value
	2009	2010	2011	2012	2013	
All	15.80	15.63	16.13	16.07	16.43	0.13
Northeast	28.71	28.33	28.96	29.30	28.83	0.25
North	19.59	18.80	19.46	18.80	20.84	0.70
Central	7.23	7.42	7.98	8.40	9.20	0.06
Bangkok	5.87	6.17	6.27	5.52	6.01	0.85
South	2.76	2.74	2.98	2.81	2.98	0.17

Table 3 Proportions of individuals with potential predisposing conditions to cholangiocarcinoma development *n* (%)

Conditions	CCA patients (*n* = 39421)	Non-CCA patients (*n* = 18508448)	*P* value
Cirrhosis	1896 (4.81)	170255 (0.92)	< 0.001
Chronic viral hepatitis B infection	291 (0.74)	21797 (0.12)	< 0.001
Chronic viral hepatitis C infection	196 (0.50)	18339 (0.10)	< 0.001
Diabetes	4502 (11.42)	977973 (5.28)[1]	< 0.001

[1]The number was estimated from the proportion of individuals with diabetes among 1900000 patients on whom data on diabetes were available, assuming the proportion of individuals with diabetes was similar between those with and without data on diabetes. CCA: Cholangiocarcinoma.

were admitted to the affiliated hospitals during the study period. The mean ± SD age was 64.0 ± 11.7 years, and most patients (61.2%) were male. When compared by geographic area, the number of CCA patients varied significantly. The Northeast region had the greatest number of CCA cases (24239; 61.5%), followed by the North (6699; 17.0%) and Central regions (5295; 13.4%) ($P < 0.0001$) (Figure 1). Bangkok had a relatively low number of CCA cases (1114; 2.8%), and the South region had the lowest number of CCA patients (1056; 2.7%) (Table 1). Differences in the demographic characteristics between CCA cases and the general population are shown in Table 1. During the study period, the annual incidence rate was stable over time, *i.e.*, 15.8, 15.6, 16.3, 16.1, and 16.4 cases per 100000 persons-year in the years 2009, 2010, 2011, 2012, and 2013, respectively, $P_{trend} = 0.13$. When classified by region, the annual incidence rate in each region remained stable over the course of the study period (Table 2).

Potential factors contributing to CCA in Thailand

Among the comorbid diseases potentially associated with CCA that were studied, diabetes was the most frequently detected, affecting 11.42% ($n = 4502$) of the entire CCA cohort. Cirrhosis and chronic viral hepatitis B and C infection were present in 1896 (4.81%), 291 (0.74%), and 196 (0.50%) patients, respectively (Table 3). CCA patients with cirrhosis ($n = 1896$) were significantly more likely to have hepatitis B infection, hepatitis C infection and alcoholic liver disease than CCA patients without cirrhosis ($n = 37525$), *i.e.*, 124 (6.5%) *vs* 132 (0.3%), 100 (5.3%) *vs* 84 (0.2%), and 197 (10.4%) *vs* 0 (0%) for hepatitis B infection, hepatitis C infection and alcoholic liver disease, respectively; $P < 0.0001$ for all.

In a comparison of the proportion of individuals with underlying diabetes and chronic liver diseases between CCA *vs* non-CCA patients, we found that a greater proportion of CCA patients had diabetes than non-CCA patients, *i.e.*, 4502/39421 (11.42%) *vs* 977973/18508448 (5.28%), $P < 0.001$, respectively. Similarly, a significantly greater proportion of CCA patients had viral hepatitis B and C infection and cirrhosis, *i.e.*, 291/39421 (0.74%) *vs* 21797/18508448 (0.12%), 196/39421 (0.50%) *vs* 18339/18508448 (0.10%), and 1896/39421 (4.81%) *vs* 170255/18508448 (0.92%) for viral hepatitis B, viral hepatitis C and cirrhosis, respectively; $P < 0.001$ for all comparisons (Table 3).

Compared to the Northeast region, all regions had a higher proportion of CCA cases with cirrhosis (5.39%, 6.84%, 7.46%, and 5.97% *vs* 3.97% for North, Central, Bangkok, and South *vs* Northeast, respectively; $P < 0.0001$ for all comparisons). Similarly, all regions except for Bangkok had a significantly greater proportion of CCA patients with chronic viral hepatitis B infection than the Northeast region (11.36%, 1.52%, and 1.11% in the North, South, and Central regions *vs* 0.44% in the Northeast, respectively; $P < 0.001$ for all comparisons) (Table 4). Bangkok had a borderline significantly greater proportion of individuals with chronic hepatitis B than the Northeast ($P = 0.05$) (Table 4). Taken together, these findings suggest that cirrhosis and chronic viral hepatitis B infection may be partly associated with the

Table 4 Proportion of cholangiocarcinoma patients who had underlying diabetes and chronic liver diseases *n* (%)

	Total (n = 39421)	Northeast (n = 24237)	North (n = 6696)	$P_{\text{North vs Northeast}}$	Central (n = 5294)	$P_{\text{central vs Northeast}}$	Bangkok (n = 1113)	$P_{\text{Bangkok vs Northeast}}$	South (n = 1055)	$P_{\text{South vs Northeast}}$	$P_{\text{for all regions}}$
Diabetes	4502 (11.42)	2803 (11.56)	572 (8.54)	< 0.0001	689 (13.01)	0.11	389 (18.36)	< 0.0001	133 (12.60)	0.25	< 0.0001
Cirrhosis	1896 (4.81)	963 (3.97)	440 (5.39)	< 0.0001	362 (6.84)	< 0.0001	83 (7.46)	< 0.0001	63 (5.97)	< 0.0001	< 0.0001
Chronic hepatitis B	291 (0.74)	106 (0.44)	91 (1.36)	< 0.0001	59 (1.11)	< 0.0001	18 (1.62)	0.05	12 (1.52)	0.002	< 0.0001
Chronic hepatitis C	196 (0.50)	92 (0.38)	57 (0.85)	< 0.0001	20 (0.38)	0.33	17 (1.53)	0.12	3 (0.28)	< 0.001	< 0.0001

P-value < 0.05 indicates statistical significance.

Table 5 One-year mortality rate in cholangiocarcinoma patients by region during the 2009 to 2013 study period *n* (%)

Year	Region					Total
	Northeast	North	Central	Bangkok	South	
2009	3825 (79.6)	1153 (85.5)	754 (80.6)	170 (78.7)	149 (74.5)	6051 (80.6)
2010	3928 (82.4)	1114 (86.1)	782 (81.0)	181 (80.4)	148 (74.0)	6153 (82.6)
2011	3988 (82.1)	1097 (83.0)	830 (80.0)	176 (78.2)	172 (78.5)	6263 (81.8)
2012	4087 (82.5)	1068 (82.3)	893 (79.7)	174 (81.3)	176 (83.4)	6398 (82.0)
2013	3973 (81.9)	1208 (84.2)	986 (79.9)	169 (72.5)	165 (73.3)	6501 (81.5)
P_{trend}	0.57	0.25	0.13	0.57	0.85	0.85

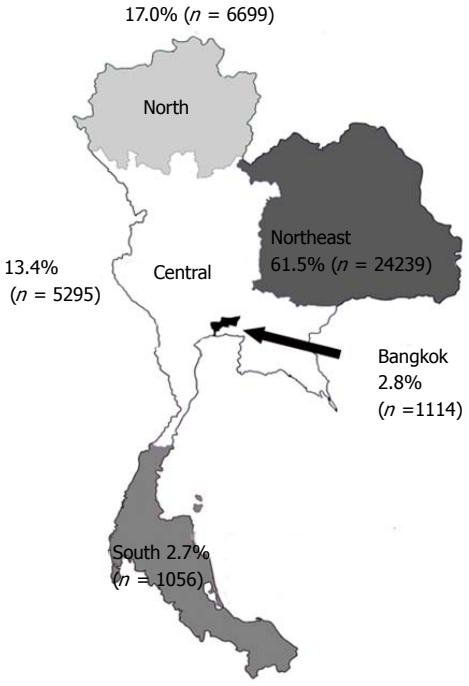


Figure 1 Northeast region had the greatest number of cholangiocarcinoma cases, followed by the North and Central regions.

development of CCA in our population.

Outcome of CCA patients

At the end of the study period, 35190 (89.27%) patients had died, of whom 31366 had died within 1 year after CCA diagnosis, corresponding to a 1-year mortality rate of 81.68%. The study period mean one-year mortality rates were stable (80.6%, 82.6%, 81.8%, 82.0%, and 81.5% for the years 2009 to 2013; P_{trend} = 0.85) (Table 5). A stable one-year mortality rate was also observed when evaluated by region (Table 5).

DISCUSSION

In this population-based study of 39421 Thai CCA patients, we observed substantial heterogeneity in the CCA incidence across various regions of Thailand. To a lesser degree, we also observed variation in mortality across the country. Our findings suggest that diabetes, liver cirrhosis, and chronic viral hepatitis B and C infection may contribute to CCA risk in the Thai population.

As expected, we found that approximately 62% of patients resided in Northeast Thailand, whereas only 3% of patients lived in the South region. The frequency of CCA cases in each region was correlated with the reported prevalence of OV infection in each area (as high as 67% in the Northeast and only 0.1% in the South)[16,17]. OV infection was shown to be associated with a 10-fold increased risk of CCA[10]. Accordingly, the eradication of OV infection could potentially reduce the incidence of CCA in Thailand. Indeed, a national program to control OV infection has been rigorously applied since 2000[17]. After the implementation of a comprehensive control program in a certain area in the Northeast in which the prevalence of OV infection was as high as 67%, the OV infection rate declined to 16%[17]. With the continuation of this control program, we may see a decline in CCA incidence in this particular study area over the next decade.

There is a body of evidence that suggests that chronic liver diseases and diabetes are potential risk factors for CCA[12-14,18,19]. Cirrhosis was shown to be associated with at least a 5-fold increase in CCA

risk[12-14,20]. Previous studies have shown that CCA patients had a higher proportion of individuals with cirrhosis than controls, with proportions ranging from 3% to 10% depending on the enrolled population, study design, and subtype of CCA studied[12-14,20]. In this study, a significantly greater proportion of CCA patients had cirrhosis than non-CCA patients. Additionally, the proportions of CCA cases with cirrhosis in non-endemic areas of OV infection (Central, Bangkok, and South regions) ranged from 5.6% to 7.5%, which were comparable to the rate of 7.8% reported in a Korean study[20]. Interestingly, the proportions of CCA patients with cirrhosis in the non-endemic areas of OV infection were significantly higher than the proportion in the Northeast region. Taken together, these findings suggest that cirrhosis may be a potential factor that contributes to CCA in Thai individuals, particularly in those who are not infected with OV.

Chronic viral hepatitis B and C infections have recently been recognized as potential risk factors for CCA[12-14,20-22]. In line with previous reports, we found that significantly more Thai patients with CCA had chronic viral hepatitis B and C infections than non-CCA patients. Two recent meta-analyses reported that hepatitis B and C infection confer risks of CCA with odds ratios of 2.0 and 5.4, respectively[21,22]. The magnitude of the effect of hepatitis B infection on CCA risk was more pronounced in Asian than in non-Asian populations. For example, the odds ratios for hepatitis B infection were 4.1 and 8.9 in Korean and Chinese cohorts[18,23], but only 2.8 in an American cohort[13]. Data related to the effect of hepatitis B and C infection on CCA risk in the Thai population are scarce[24]. The only report we were able to identify in the literature did not identify a significant association between hepatitis B/C infection and CCA; however, the study was conducted in an area endemic to OV infection, and the effect of hepatitis B and C on CCA risk may have been masked by the higher effect magnitude of OV infection[24].

We observed that an approximately 2-fold greater proportion of CCA patients had diabetes than non-CCA patients (11.4% *vs* 5.3%). A recent large case-control study of 2395 CCA cases and 4769 controls conducted in the United States reported that the proportion of subjects with diabetes among CCA cases and controls was 18.2% and 10.0%, respectively[13]. Accordingly, diabetes increased the risk of developing CCA by 2.7-fold, and this magnitude of risk was consistent with a reported odds ratio of 2.6 for developing CCA among Korean individuals with diabetes[13,18].

Taken together, our findings suggest that all of the studied underlying comorbid diseases potentially contribute to the risk of developing CCA in a Thai population. Future studies to confirm these findings and to determine the magnitude of the effect of these factors in a Thai population are warranted.

The major strength of this study is that our data were obtained from the Nationwide Hospital Admission Data (NHAD) registry, which is a database that includes the hospitalization data of 73% of all Thai citizens. As such, these findings most likely reflect an overall characterization of this cancer in Thailand. This study also has some mentionable limitations. First, the annual incidence estimated in this study might be underestimated due to the unique features of this database. The database solely includes information during hospitalization. Because data of patients seen at outpatient departments were not available, this could raise a concern that some patients, particularly those with advanced stage CCA who were only seen at outpatient departments and had never been admitted to a hospital, might be missed. We admit that this is possible; however, based on the current practice in our country, most CCA patients require at least one hospitalization, either for performing an investigation for a definite diagnosis or for receiving treatment by surgery, biliary drainage with palliative chemotherapy or even the best supportive care. Thus, we believe that the number of CCA patients who did not have any hospitalization was not large, and this number did not significantly affect the overall findings observed in this study. Second, the diagnosis of CCA was made by ICD codes that were obtained from hospital discharge summary notes and without histopathological confirmation. Consequently, the actual number of CCA patients during the study period could be higher than the number presented here. However, the proportions of CCA patients by region were consistent with the rates reported in a previous study[2]. As a result, it is likely that the different incidence rates among the 5 different regions are a valid finding. Another concern was that the proportion of CCA patients in each region shown in this study might not reflect the actual region of residence of patients because some local people might migrate to other regions; in particular, a portion of Northeastern people migrate to work in Bangkok. Thai people are covered by the national MWS only if they visit their primary hospital, which is designated by household registration. Patients who require hospitalization are usually referred back to their primary hospital. If necessary, the primary hospital will further refer patients to the nearby secondary or tertiary hospitals within the same region. Thus, the effect of migration across regions had a minimal impact on the proportion of patients in each region. Data on hepatitis B/C infection, diabetes, smoking, alcohol drinking, obesity, and metabolic syndrome were not available in the discharge summary notes of all participants, thus precluding us from estimating the effect of these conditions on CCA risk. Similarly, the prevalence of OV infection and the relationship between OV infection and CCA incidence cannot be

determined because the diagnosis of OV infection was not available in the medical records. The diagnosis of CCA was obtained from ICD codes, which group hilar and distal CCA together as extrahepatic CCA; thus, an analysis by CCA subtypes could not be performed. Choledocholiathiasis, hepatolithiasis, choledochal cysts, Caroli's syndrome and bile duct adenoma have been shown to be associated with CCA[1,14]. These conditions were not included in the analysis because this study aimed to explore the conditions that have recently been proposed to be related to CCA and may in part contribute to the increasing incidence of CCA worldwide.

In summary, CCA-related incidence and mortality among Thai patients vary by geographical region. Our findings yield indirect evidence that supports current epidemiological data that suggests that diabetes, cirrhosis, and chronic viral hepatitis B and C infection are associated with an increased risk of developing CCA. These conditions may be important factors contributing to CCA in Thai individuals.

COMMENTS

Background

The incidence of cholangiocarcinoma (CCA) has been increasing for a few decades for unclear reasons. Primary sclerosing cholangitis, liver fluke infection, and conditions with chronic biliary tract inflammation are established predisposing factors to CCA development. Accumulating evidence suggests that cirrhosis, chronic viral hepatitis B/C infection and diabetes are associated with CCA.

Research frontiers

The results of this study provide indirect evidence supporting the current epidemiological knowledge about risk factors of CCA.

Innovations and breakthroughs

This study used the Nationwide Hospital Admission Data (NHAD) registry to identify potential risk factors for CCA among 5 different regions of Thailand. The two key findings were that the incidence of CCA varied geographically in Thailand and that significantly more CCA patients had diabetes, cirrhosis, and chronic viral hepatitis B/C infection than non-CCA participants.

Applications

This study suggests that diabetes, cirrhosis, and chronic viral hepatitis B and C infection may contribute to CCA development in the Thai population. Further study to confirm these associations and determine the magnitude of the impact of these factors on cholangiocarcinoma development in the Thai population is warranted.

Terminology

Cholangiocarcinoma is a cancer of the bile duct epithelium. CCA can be categorized into 3 subtypes: intrahepatic, perihilar and distal cholangiocarcinoma. Although the three subtypes share some common features, each has its own characteristics, including genetic alterations, presentation, treatment and prognosis. Obtaining a diagnosis of cholangiocarcinoma using ICD codes precludes the analysis by subtypes because the ICD codes group perihilar and distal cholangiocarcinoma together as extrahepatic cholangiocarcinomas.

Peer-review

This study addresses an important healthcare problem in Thailand. This is an informative article about the risk factors of cholangiocarcinoma in Thailand using a nationwide database. This study aimed to identify potential risk factors of cholangiocarcinoma among 5 different regions of Thailand. The fact that even in Thailand, diabetes, viral hepatitis B/C infection and cirrhosis are risk factors of cholangiocarcinoma in a region where fluke infection is not endemic, is interesting.

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ORIGINAL ARTICLE

Observational Study

Expression of annexin A5 in serum and tumor tissue of patients with colon cancer and its clinical significance

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Abstract

AIM

To investigate the expression of annexin A5 in serum and tumor tissue of patients with colon cancer and to analyze its clinical significance.

METHODS

Ninety-three patients with colon cancer treated at our hospital between February 2013 and March 2016 were included in an observation group, and 40 healthy individuals were included in a control group. Enzyme-linked immunosorbent assay was performed to determine the serum level of annexin A5, while immunohistochemistry was performed to determine the expression of annexin A5 in cancer tissues.

RESULTS

The serum level of annexin A5 was 0.184 ± 0.043 ng/mL in the observation group, which was significantly higher than that in the control group ($P < 0.05$). Annexin A5 expression was detected in 79.31% of the patients with lymph node metastasis, which was significantly higher than that in patients without lymph node metastasis ($P < 0.05$). Moreover, annexin A5 expression was detected in 86.96% of the patients with stage Ⅲ to Ⅳ disease, which was significantly higher than that in patients with stage Ⅰ to Ⅱ disease ($P < 0.05$). The serum level of annexin A5 was 0.215 ± 0.044 ng/mL in patients whose tumors were positive for annexin A5 expression, which was significantly higher than that in patients whose tumors were negative for annexin A5 expression ($P < 0.05$). The serum level of annexin A5 was correlated with annexin A5 expression in colon cancer tissues (*r*

= 0.312, $P < 0.05$). When a cutoff value of > 0.148 ng/mL for serum level of annexin A5 was used in the diagnosis of colon cancer, the sensitivity was 83.90%, and the specificity was 57.50%.

CONCLUSION

For patients with colon cancer, annexin A5 expression in cancer tissues is related to lymph node metastasis and tumor grade. Serum level of annexin A5 is related to annexin A5 expression in cancer tissues and is of diagnostic relevance.

Key words: Immunohistochemistry; Annexin A5; Colon cancer; Serum

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Core tip: For patients with colon cancer, annexin A5 expression in cancer tissues is related to lymph node metastasis and tumor grade. Serum level of annexin A5 is related to annexin A5 expression in cancer tissues and is of diagnostic relevance.

Sun CB, Zhao AY, Ji S, Han XQ, Sun ZC, Wang MC, Zheng FC. Expression of annexin A5 in serum and tumor tissue of patients with colon cancer and its clinical significance. *World J Gastroenterol* 2017; 23(39): 7168-7173 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7168.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7168

INTRODUCTION

Colon cancer is a common malignancy of the digestive tract. Studies have shown that the incidence of colon cancer is ⩾ 0.005%[1] and that the incidence has continued to trend upwards in recent years because of risk factors such as diet and smoking[2,3] .

Basic cancer research has shown that changes in the levels of certain molecules affect tumor cell proliferation and differentiation, which in turn affect the development and progression of malignant tumors[4]. Annexin A5, first isolated from human placenta, was found to bind to phosphatidylserine in a calcium-dependent manner[5,6]. In the present study, 93 patients with colon cancer who were treated at our hospital between February 2013 and March 2016 were included to investigate the expression of annexin A5 in serum and in cancer tissues, with an aim to investigate its clinical significance.

MATERIALS AND METHODS

General information

Ninety-three patients with colon cancer (observation group) who were treated at our hospital between February 2013 and March 2016 were included in this study. The inclusion criteria were as follows: (1) pathologically confirmed colon cancer; (2) complete clinical and pathological data; and (3) being willing to provide informed consent. The exclusion criterion was incomplete clinical or pathological data. Forty healthy individuals who underwent a routine health checkup at our hospital were included as controls. No significant difference was observed with respect to age or gender between the two groups (Table 1).

Detection of serum level of annexin A5

A fasting venous blood sample was collected from each subject in the morning and centrifuged at 10000 *r*/min to separate the serum, which was then stored at -20 ℃ and tested within one week to determine the annexin A5 level. The Roche automated biochemical analyzer E170 module was used for testing, and the assay kit was purchased from Shanghai Taikang Biotechnology Co., Ltd. The assay was performed according to the instructions given in the package insert. Control serum or standard was included with the kit.

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and cut into 3 mm sections. The sections were incubated in 3% H_2O_2 at room temperature for 5 min, rinsed with deionized water (3 min × 3 times), blocked with 10% milk protein (1 g protein in 100 mL of purified water), and incubated at room temperature for 5 min. Next, the sections were incubated with a mouse anti-annexin A5 antibody (Nanjing Biyuntian Biotechnology Co., Ltd.) for 2 h at 37 ℃, followed by a PBS wash (5 min × 3 times). Then, the slides were incubated with a horseradish peroxidase-labeled rabbit secondary antibody (Roche) for 30 min at 37 ℃, followed by a PBS wash (5 min × 3 times). After that, the slides were incubated with NBT/BCIP reagent, which was used to develop the reaction, for 5 min. Finally, the sections were counterstained, dehydrated, cleared, mounted, and observed under an OLIPICS microscope (Shanghai Precision Instrument Co., Ltd). All the required reagents were purchased from Nanjing Taikang Biotechnology Co., Ltd.

Evaluation criteria for immunohistochemical staining

Immunohistochemical staining was considered positive if yellow granules were present in the cytoplasm of tumor cells or stromal cells. The staining intensity was graded as follows: 0, no staining; 1, light yellow; 2, yellow; and 3, brown. The percentage of positive cells was scored as follows: 0, < 5%; 1, 5% to 24%; 2, 25% to 50%; 3, 51% to 74%; and 4, and ⩾ 75%. The product of the staining intensity and the percentage of positive cells was either < 2 (negative) or ⩾ 2 (positive).

Table 1 General information (mean ± SD)

Group	*n*	M/F	Age (yr)
Observation group	93	54/39	53.29 ± 9.49
Control group	40	27/13	52.17 ± 8.14
t/χ^2		1.046	0.65
P value		> 0.05	> 0.05

Table 2 Relationship between Annexin A5 and clinicopathological features of patients with colon cancer *n* (%)

Clinicopathological feature	*n*	Positive	χ^2	*P* value
Gender				
M	54	31 (57.41)	0.023	> 0.05
F	39	23 (58.97)		
Age, yr				
≥ 55	47	29 (61.70)	0.516	> 0.05
< 50	46	25 (54.35)		
Lymph node metastasis				
Yes	29	23 (79.31)	7.812	< 0.05
No	64	31 (48.44)		
Tumor diameter (cm)				
≥ 5	51	29 (56.86)	0.067	> 0.05
< 5	42	25 (59.52)		
Tumor stage				
Ⅰ to Ⅱ	47	14 (29.79)	31.204	< 0.05
Ⅲ to Ⅳ	46	40 (86.96)		

Table 3 Serum levels of Annexin A5 in the two groups (mean ± SD, ng/mL)

Group	*n*	Annexin A5	*t*	*P* value
Observation group	93	0.184 ± 0.043	2.904	< 0.05
Control group	40	0.159 ± 0.051		

Statistical analysis

SPSS v19.0 was used for statistical analyses. Measurement data are expressed as mean ± SD and were analyzed by the *t*-test. Count data were analyzed by the χ^2 test. Spearman rank correlation analysis was performed to analyze potential correlations between variables. A receiver operating characteristic curve was used to analyze the diagnostic value of serum annexin A5 level. $P < 0.05$ was considered statistically significant.

RESULTS

Annexin A5 expression in cancer tissue

No significant difference was observed in the positive expression rates of annexin A5 among patients of different ages or genders, or those with different tumor diameters. Moreover, 79.31% of the patients with lymph node metastasis expressed annexin A5, which was significantly higher than the percentage of patients without lymph node metastasis ($P < 0.05$); 86.96% of the patients with stage Ⅲ to Ⅳ disease expressed annexin A5, which was significantly higher than the percentage of patients with stage Ⅰ to Ⅱ disease ($P < 0.05$) (Table 2).

Serum levels of annexin A5 in the two groups

The serum level of annexin A5 was significantly higher in the observation group than in the control group ($P < 0.05$) (Table 3).

Correlation between serum level of annexin A5 and expression of annexin A5 in tumor tissue

The serum level of annexin A5 was 0.215 ± 0.044 ng/mL in patients whose colon tumors were positive for annexin A5 expression, which was significantly higher than the corresponding value in patients whose colon tumors were negative for annexin A5 (0.180 ± 0.021 ng/mL) ($t = 4.599$, $P < 0.05$). A Spearman rank correlation analysis showed that the serum level of annexin A5 was related to the expression of annexin A5 in tumor tissues ($r = 0.312$, $P < 0.05$).

Diagnostic value of serum level of annexin A5

The ROC curve for the serum level of annexin A5 in the diagnosis of colon cancer showed an area under the curve of 0.732 ($P < 0.05$). At a cutoff value of 0.148 ng/mL, the sensitivity was 83.90%, and the specificity was 57.50% (Figure 1).

DISCUSSION

Changes in diet, excessive alcohol consumption, and genetic susceptibility factors promote the development and progression of colon cancer. In particular, among elderly male smokers aged 45 or older, the incidence of colon cancer is 0.005% or higher and has continued to trend upwards in recent years[7,8]. For colon cancer, the incidence of early metastasis is high, which results in poor patient outcomes: the five years survival rate is < 35%, and the median survival time is < 32 mo[9-11]. Studies on the genetic and biological mechanisms of the development and progression of colon cancer may provide new targets for immune therapy or strategies of comprehensive biological therapy for colon cancer[12,13].

Molecular changes play an important regulatory role in the development of malignant tumors. Cell surface Connexins or membrane proteins can induce the transcription initiation activity of downstream oncogenes, which promotes aberrant activation of the cell cycle in colonic epithelial cells and leads to excessive proliferation of cancer cells[14]. Accumulating experimental data indicate that phosphatidylserine exposition is associated with apoptosis and other cell death programs[15-17], which renders it an attractive target in imaging overall cell death. Annexin A5 is identified in blood vessel as a blood anticoagulation factor and it builds voltage-dependent calcium channel in phosphatidylserine bilayers[18,19]. Corsten *et al*[20] showed that through binding with strong affinity to phosphatidylserine, annexin A5 offers an interesting opportunity for visualization of aggregate

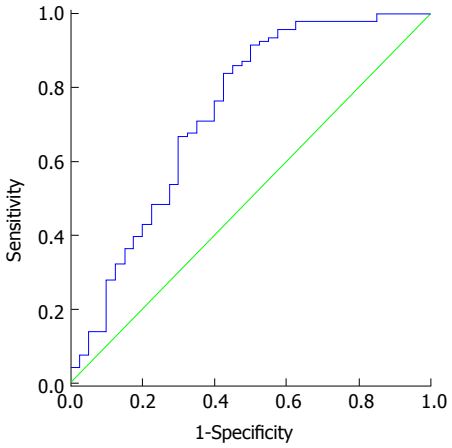


Figure 1 Receiver operating characteristic curve for the value of serum level of annexin A5 in the diagnosis of colon cancer.

cell death[21,22], thus providing a fit benchmark for *in vivo* monitoring of anticancer treatment[23-26]. Recently, annexin A5 has been reported as a new mediator of cisplatin-induced apoptosis by inducing voltage-dependent anion channel oligomerization in human kidney epithelial cells[27,28]. Annexin A5 forms N6-acetyllysine at specific positions of the amino-terminal region of the membrane protein, and as a result, it affects the formation of a transcriptional co-inhibitory complex and participates in transcriptional repression and silencing of tumor suppressor genes via H1 phosphorylation[29,30]. Previous studies have investigated the relationship between annexin A5 and liver cancer or esophageal cancer and showed that uH2B-related monotone generalization increased the risk of malignant digestive tumors and promoted clinical progression[31-34]. This study explored not only the expression of uH2B in colon cancer tissue but also the diagnostic value of its serum level in the diagnosis of colon cancer.

In this study, immunohistochemical staining showed significantly high expression of annexin A5 in colon cancer tissue and demonstrated that the positive expression rate of annexin A5 was significantly higher in patients with lymph node metastasis than in those without. This suggests that annexin A5 may play a role in the promotion of the invasion of lymph nodes by colon cancer cells. Furthermore, approximately 80% of the patients with late-stage (Ⅲ and Ⅳ) colon cancer expressed annexin A5, which was significantly higher than the percentage of patients with stage Ⅰ or Ⅱ disease, which suggests that annexin A5 significantly promotes the clinical progression and worsening of colon cancer. Annexin A5 induces the activation of second messengers in cancer cells, which promotes the production of cancer cell differentiation antigens, the proliferation and differentiation of colon cancer cells, and clinical progression.

In conclusion, annexin A5 is highly expressed in serum and tumor tissues of patients with colon cancer, and its expression is closely related to the clinical stage and presence of lymph node metastasis in patients with colon cancer. Nevertheless, this study has certain limitations. For instance, we did not investigate the relationship between the expression of annexin A5 and the long-term survival of patients with colon cancer.

COMMENTS

Background

Colon cancer is a common malignancy of the digestive tract. Studies have shown that the incidence of colon cancer is ⩾ 0.005% and that the incidence has continued to trend upwards in recent years because of risk factors such as diet and smoking.

Research frontiers

Basic cancer research has shown that changes in the levels of certain molecules affect tumor cell proliferation and differentiation, which in turn affect the development and progression of malignant tumors. Annexin A5 is a glycoprotein that contains a multiplex carboxyl terminus binding domain, which influences the differentiation of surface antigens on cancer cells and promotes tumor proliferation and invasion.

Innovations and breakthroughs

The objective was to investigate the clinical significance of annexin A5 expression in colon cancer.

Applications

For patients with colon cancer, annexin A5 expression in cancer tissues is related to lymph node metastasis and tumor grade. Serum level of annexin A5 is related to annexin A5 expression in cancer tissues and is of diagnostic relevance.

Peer-review

In this study, the authors investigated the expression of annexin A5 in serum and tumor tissues of patients with colon cancer and its clinical significance.

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CASE REPORT

Faecal microbiota transplantation in patients with *Clostridium difficile* and significant comorbidities as well as in patients with new indications: A case series

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Abstract

Fecal microbiota transplantation (FMT) is effective in recurrent *Clostridium difficile* infection (rCDI). Knowledge of the safety and efficacy of FMT treatment in immune deficient patients is scarce. FMT has been

suggested as a potential method for an increasing number of new indications besides rCDI. Among our FMT-treated rCDI patients, we reviewed those with major comorbidities: two human immunodeficiency virus patients, six haemodialysis patients, two kidney transplant patients, two liver transplant patients and a patient with chronic lymphatic leukaemia. We also reviewed those treated with FMT for indications other than rCDI: *Salmonella* carriage (two patients), trimethylaminuria (two patients), small intestinal bacterial overgrowth (SIBO; one patient), and lymphocytic colitis (one patient), as well as a common variable immunodeficiency patient with chronic norovirus infection and ESBL-producing *Escherichia coli* (*E. coli*) carriage. Of the thirteen rCDI patients treated with FMT, eleven cleared the CDI. The observed adverse events were not directly attributable to FMT. Concerning the special indications, both *Salmonellas* and ESBL-producing *E. coli* were eradicated. One trimethylaminuria patient and one SIBO-patient reported a reduction of symptoms. Three patients did not experience a benefit from FMT: chronic norovirus, lymphocytic colitis and the other fish malodour syndrome. There were no reported side effects in this group. FMT appeared to be safe and effective for immunocompromised patients with rCDI. FMT showed promise for the eradication of antibiotic-resistant bacteria, but further research is warranted.

Key words: Faecal microbiota transplantation; Antibiotic resistance; *Clostridium difficile* infection; Microbiota; Immunodeficiency; *Salmonella* infection

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Core tip: Knowledge of faecal microbiota transplantation (FMT) in immunocompromised patients and patients with conditions other than recurrent *Clostridium difficile* infection (rCDI) is scarce. We reviewed 13 FMT-treated patients with rCDI and major comorbidities as well as 8 patients with new indications. In our cohort, FMT appeared to be safe and effective for immunocompromised patients: dialysis patients, human immunodeficiency virus patients, solid organ transplant patients and a patient with chronic lymphatic leukaemia. Of the patients treated for indications other than rCDI, the most promising results were successful eradication of antibiotic-resistant bacteria. Eradication of chronic *Salmonella* carriage in two patients with FMT represents the first cases reported to date.

Lahtinen P, Mattila E, Anttila VJ, Tillonen J, Teittinen M, Nevalainen P, Salminen S, Satokari R, Arkkila P. Faecal microbiota transplantation in patients with *Clostridium difficile* and significant comorbidities as well as in patients with new indications: A case series. *World J Gastroenterol* 2017; 23(39): 7174-7184 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7174.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7174

INTRODUCTION

The intestinal microbiota is an area of active research. Knowledge of the human microbiota has been accumulating rapidly in recent years. The gut bacteria was previously regarded as passive or harmful waste, but the intestinal microbiota is currently respected as a well-orchestrated organism with an active role in the development of immunity and maintenance of health[1-4].

Microbial imbalance, dysbiosis, is suggested to play a role in many different diseases. The microbiota holds many expectations as a new treatment target. Faecal microbiota transplantation (FMT) is a straightforward way to change the microbial composition of the intestine[1-4]. FMT has been shown to induce profound and long-lasting changes in the microbiota, offering a means to modify the gut microbiota relatively permanently for the treatment of microbiota-associated diseases[5].

FMT has become a widely accepted treatment for recurrent *Clostridium difficile* infection (rCDI)[6-9]. We, among others, have shown that FMT through colonoscopy is an effective treatment for rCDI[5]. Experience in many centres has shown that FMT is also safe when performed to a high standard[10-13].

The risk of adverse events of FMT is low in well performed studies[6]. Rigorous screening of the donor is mandatory. Without proper screening, there is a possibility of transmitting infectious diseases and a possibility of transmitting conditions that FMT is suspected to ameliorate, such as obesity[14].

All FMT should be performed at a health care unit by professionals who are familiar with the procedure[15]. FMT is currently indicated only for rCDI[15]. If FMT is used for other indications, then patients should be carefully evaluated, and the results, whether positive or negative, should be reported. Our group has treated a few patients with conditions other than rCDI and outside of on-going study programmes. Such cases have been evaluated carefully on an individual basis by a gastroenterologist, an infectious disease specialist and a microbiologist.

Although FMT has been effective and generally safe in published studies, data concerning FMT in certain special groups are scarce[16]. More information is needed regarding the safety of FMT in immunosuppressed and other special groups such as patients undergoing haemodialysis[12]. In our FMT-treated patients, we gathered information on patients with significant comorbidities, such as immune-deficient patients, patients with an organ transplant and hae-

modialysis patients.

CASE REPORT

Study design

Of the patients treated with FMT in Helsinki University Hospital, we searched for those treated for rCDI and having major comorbidities and those treated for indications other than rCDI. We included dialysis patients and patients with known immune deficiencies in the rCDI group; human immunodeficiency virus (HIV), organ transplant or hematologic disease. We found 13 patients with rCDI and major comorbidities and 8 patients with conditions other than rCDI. We review the patient histories, the outcomes and adverse events of each patient.

Ethical aspects

Since FMT policy and legislation differ from country to country[17,18], we consulted the Finnish Medicines Agency (Fimea), which is the national competent authority for regulating pharmaceuticals and blood and tissue products. Fimea noted that stool does not fall under the category of pharmaceuticals and did not consider it relevant to the establishment of new regulations specifically for FMT. In Finland, FMT studies do not require approval of the drug authority Fimea.

In Finland, FMT may be used by doctors based on their own judgement. We recommend that clinicians using FMT follow international guidelines[15,19]. FMT is indicated for rCDI treatment; for other indications, it should be used only in a clinical trial setting.

FMT protocol

Some of the patients described in this article have been treated with stool from a donor familiar to them. Most of the described patients have been treated with stool from a universal donor - frozen and thawed stool from our local faecal bank. The faecal banking protocol is described in detail in our previous article[10]. Briefly, faecal material from a healthy donor is mixed with saline and glycerol and frozen at -80 ℃. In some cases, we used the faecal suspension stored at -20 ℃, which did not seem to affect the results or safety of FMT. The frozen suspension is thawed a few hours before FMT and collected into two 100-mL syringes. When necessary, the suspension is passed through a pre-sterilized, stainless steel tea strainer to remove larger particles.

Donor screening was performed as described in detail by Mattila *et al*[6] and Satokari *et al*[10]. Briefly, the donors were screened for hepatitis C and B, HIV, *Treponema pallidum* and common enteric pathogens. The donor was required to lack antibiotics for the previous six months and to present no gastrointestinal symptoms.

The preferred route of administration of FMT in our practice is colonoscopy. The suspension is administered into the cecum and ascending colon. Preceding the colonoscopy, the patients undergo bowel lavage by polyethylene glycol solution (between 3-5 L). The patients with rCDI underwent pre-treatment with vancomycin or metronidazole, and the treatment was stopped 36 h before the FMT. In some special cases, we administered FMT during gastroscopy, injecting the suspension into the duodenum as distally as possible.

Although FMT has become the routine treatment after two relapses for CDI in our hospital, the decision to assess FMT in immunosuppressed patients and in special indications is based on a very thorough consideration, especially of safety concerns, by gastroenterologist, specialist of infectious diseases and microbiologist with FMT experience.

Thirteen patients treated with FMT for rCDI and having significant comorbidities

In this article, we report the results of thirteen patients with major comorbidities who underwent rCDI and were treated with FMT (Table 1). Two of the patients had HIV, six were haemodialysis patients, two had a kidney transplant, two had a liver transplant and one had chronic lymphatic leukaemia (CLL).

Eleven of the thirteen rCDI patients (85%) treated with FMT successfully cleared the CDI. Six of the patients had major adverse events, of which two died at 2 and 5 mo post-FMT; however, these events were not directly attributable to FMT. A detailed description of each patient is published online as a supplement.

Eight patients treated with FMT and not having rCDI

Eight patients received FMT as an experimental form of treatment for various special indications. The patients and outcomes are compiled in Table 2 and described in detail below.

A carrier of salmonella #1

A 17-year-old male was found to be a carrier of *Salmonella* in a routine check-up on the 1st of September 2015. He had not had previous GI symptoms. According to Finnish health authority instructions, a *Salmonella*-positive person may not work in food processing. The patient was about to start his studies to become a cook and *Salmonella* was delaying his plans.

The *Salmonella* strain was resistant to doxycycline and ciprofloxacin. He had a mild knee symptom that was thought to be reactive arthritis. Ciprofloxacin was administered briefly and stopped as the sensitivity results of the *Salmonella* became apparent. He was receiving tetracycline for acne since the 18th of August, which was stopped on the 26th of October. In October, faecal salmonella was negative twice, but it was positive again on the 16th of November.

A 2-wk course of i.v. ceftriaxone was considered an

Table 1 The results of fecal microbiota transplantation treatments of thirteen patients with different comorbidities and *Clostridium difficile* infection

	Patient characteristics	Medical history	Post FMT situation	Adverse events in 1 mo
1	A patient with HIV, ulcerative colitis and rCDI	28-year-old male with HIV, antiviral medication and virus undetectable, previous suspicion of ulcerative colitis. Recurrent diarrhoea with *C. difficile* positivity, slow response to vancomycin.	No further relapses Two months after diarrhoea recurred at the same time with mild alcohol associated pancreatitis. In colonoscopy final diagnosis of ulcerative colitis was made. *C. difficile* remained negative.	No
2	A patient with HIV, alcoholism and rCDI	59-year-old female, depression, continuous heavy smoking and consumption of alcohol. HIV and antiviral therapy. rCDI after antibiotic treatment for respiratory infection.	No further relapses Diarrheal continued due to exocrine pancreatic insufficiency and excessive alcohol consumption 5 mo after FMT *C. difficile* reinfection treated with vancomycin and fidaxomicin	No
3	A Haemodialysis patient with rCDI #1	60-year-old female, rheumatoid arthritis and in haemodialysis due to amyloidosis. Chronic atrial fibrillation, polypectomies of rectum adenomas. Had *Enterococcus* sepsis 2012.	No further relapses. Half a year after FMT *Enterococcus faecalis* sepsis and an epidural abscess.	No
4	A Haemodialysis patient with rCDI #2	19-year-old female, haemodialysis due to Goodpasture syndrome complicated with pulmonary haemorrhage. Immunosuppressive therapy.	No further relapses.	No
5	A Haemodialysis patient with rCDI #3	77-year-old male, haemodialysis after renal carcinoma operation, diabetes Ⅱ and COPD. *Pseudomonas* septicaemia followed by rCDI.	No further relapses. One week after FMT hospitalized due to generalized enema and possible cystitis. Two months after FMT hospitalized due to gastroenteritis, faecal clostridium was negative.	One week after hospitalized due to enema and cystitis
6	A Haemodialysis patient with rCDI #4	80-year-old male. Haemodialysis because of chronic glomerulonephritis, type II diabetes, hypertension, epilepsy, AV-block and a pace maker. *Staphylococcus aureus* septicaemia followed by rCDI.	No further relapses. *Staphylococcus aureus* sepsis 5 mo after the FMT.	No
7	A Haemodialysis patient with rCDI #5	66-year-old male, haemodialysis due to microscopic polyangiitis. Chronic atrial fibrillation.	No further relapses	No
8	A Haemodialysis patient with rCDI #6	79-year-old female. Hypertension, dyslipidaemia, atrophic gastritis. TIA 2004 and 2005, a pace maker due to bifascicular block. Coronary disease. Haemodialysis due to an episode of rhabdomyolysis.	2 wk after FMT reinfection after an antibiotic treatment of cystitis. No further FMT's due to poor general condition. Patient died 2 mo after FMT to underlying diseases	2 wk after *C. difficile* reinfection
9	A Kidney transplant patient with rCDI #1	78-year-old female. Kidney transplant due to polycystic renal disease. Polycystic liver, type Ⅱ diabetes, hypertension and asthma. Operated for cholecystectomy and hysterectomy. *E. coli* sepsis and one month after another infectious episode treated with meropenem followed by severe rCDI.	No further relapses 3 d after FMT gastroenteritis, *Clostridium* was not tested. Restarted vancomycin for 2 d. 12 d after FMT the patient was hospitalized due to infection, CT scan did not reveal the aetiology.	Gastroenteritis 3 d after FMT Hospitalized 12 d after FMT
10	A Kidney transplant patient with rCDI #2	61-year-old female. A kidney transplant due to polycystic renal disease. rCDI after clindamycin for dental infection.	No further relapses	No
11	A Liver transplant patient with rCDI	56-year-old female. Liver transplant due to mushroom intoxication, a moderate renal failure.	No further relapses	No
12	A Patient with a liver transplant, renal insufficiency, haemodialysis and rCDI	69-year-old male. Liver transplantation due to alcohol cirrhosis, followed by renal insufficiency and haemodialysis.	No further relapses	No
13	A Patient with chronic lymphatic leukaemia, chronic norovirus infection and rCDI	65-year-old female. Chronic lymphatic leukaemia since 1996. Autologous stem cell transplantation in 2003. Cytostatic interventions from 2009-2011, after which she had prolonged pancytopenia, infections and hypogammaglobinaemia. In summer 2011, she had chronic norovirus infection and recurrent CDI, several vancomycin courses and gammaglobulin infusions. March 2012 FMT	No primary complications Hospitalized 2 wk after FMT due to diarrhoea. Both norovirus and *Clostridium difficile* stayed positive in stool samples. Patient died in August 2012, 5 mo after FMT for complications of her haematological disease.	CDI and norovirus related diarrhoea continued.

FMT: Fecal microbiota transplantation; HIV: Human immunodeficiency virus; rCDI: Recurrent *Clostridium difficile* infection; *C. difficile: Clostridium difficile*; *E. coli: Escherichia coli*.

Table 2 The results of fecal microbiota transplantation treatments of eight patients with different new indications

	Patient and diagnosis	Age at 1st FMT and gender	Route of administration	Outcome	FMT related complications
1	A carrier of *Salmonella* #1	17-year-old male	Colonoscopy	Successful eradication of Salmonella	No
2	A carrier of *Salmonella* #2	52-year-old female	Colonoscopy	Successful eradication of Salmonella	No
3	A patient with TMAU #1	24-year-old male	Gastroscopy	Moderate self-reported benefit up to 6 mo, at 12 mo symptoms had recurred to former severity	No
4	A patient with TMAU #2	49-year-old female	Gastroscopy	No change in self-reported symptom severity	No
5	A patient with SIBO	66-year-old male	Gastroscopy (treated 3 times using 2 donors)	Self-reported decrease in symptom severity	No
6	A patient with lymphocytic colitis	21-year-old female	Colonoscopy	Two week decrease in self-reported symptoms, then recurrence of symptoms to former severity	No
7	A carrier of norovirus	32-year-old female	Colonoscopy	No change in self-reported symptom severity, no success in virus eradication	No
8	A carrier of ESBL-producing	31-year-old female	Colonoscopy	Successful eradication of ESBL-producing *E. coli*	No

FMT: Fecal microbiota transplantation; TMAU: Trimethylaminuria; SIBO: Small intestinal bacterial overgrowth; *E. coli: Escherichia coli*.

option, but the patient did not accept this treatment due to his needle phobia. A two-week course of trimethoprim -sulfadiazine 160 mg/500 mg 1 × 2 per orally was started on the 26th of November since the *Salmonella* strain was found to be sensitive. Unfortunately, the faecal *Salmonella* test was still positive after this treatment.

On the 29th of January 2016, the patient was given FMT as described in detail in the FMT protocol section. Preceding the FMT, he received a 5-d course of ceftriaxone 2 g × 1 i.m. The colonoscopy findings were normal, as well as the histology of routine biopsies. There were no complications during the procedure, but the patient fainted soon afterward, which might have been caused in part by the pain and anxiety-relieving medications used during the colonoscopy. He recovered rapidly.

An upper abdomen ultrasound was performed and did not reveal any gallstones. Gallstones are a known risk factor for resistant *Salmonella*.

On the 9th of February - less than two weeks after FMT - the stool test was salmonella-positive and the treatment was considered a failure at first. However, the subsequent three tests (the 2nd, the 8th and the 11th of May) were all negative. According to the instructions of Finnish health authorities, the patient is considered free from *Salmonella* after having three negative samples in a row, and our patient could continue his studies.

There were no reported side effects of the FMT treatment. We consider it likely that the transplanted new gut microbiota played a role in the eradication of *Salmonella*, although we cannot exclude the possibility that the *Salmonella* would have been eradicated spontaneously.

A carrier of salmonella #2

A 52-year-old woman had *Salmonella* enteritis in March 2016. Her symptoms ceased, but she remained a chronic carrier. She had been treated with courses of trimethoprim-sulphadiatzine and amoxicillin. She had also undergone a two-week course of intravenous ceftriaxone, but the *Salmonella* culture remained positive. The bacterial strain was resistant to ciprofloxacin. She was on sick leave during this time because of her work in food production. FMT treatment was administered through colonoscopy on the 17th of November 2016. Prior to FMT, a course of ceftriaxone 2 g 1 × 1 i.v. was administered for six days. The three subsequent faecal tests after FMT (the 2nd, 12th and 19th of December 2016) for *Salmonella* were all negative, and she could return to work. No side effects were observed.

The rationale for treating salmonella carriage with FMT

The prevalence of chronic *Salmonella* carriage is estimated to be 2%-5% in endemic areas. Symptomless carriage of *Salmonella,* especially of individuals working in food production, is considered to be the main route of distribution of the disease among people. Furthermore, persistent carriage of *Salmonella* is associated with gallstones. Fluoroquinolones are the drug of choice for the treatment of chronic carriage of *Salmonella*[20], but strains that are resistant to ciprofloxacin pose a special challenge.

In animal models, *Salmonella* carriage is associated with changes in the intestinal microbiota[21]. It is not known whether people with *Salmonella* carriage possess alterations of the gut microbiota. To the best of our knowledge, there are no reported cases of eradication of *Salmonella* with FMT. FMT has been shown to reduce antibiotic resistance genes in the gut microbiota[22,23]. FMT has shown potential in eradicating faecal carriage of different multidrug-resistant bacteria in case reports[24]. Therefore, changing or diversifying the intestinal microbiota through FMT is a promising

new option to eradicate chronic *Salmonella* in cases where antibiotics have failed.

A patient with trimethylaminuria (fish malodour syndrome, TMAU) #1

A 24-year-old male had been diagnosed with fish malodour syndrome (trimethylaminuria, TMAU) two years earlier, but the symptoms had started at the age of 16 years. Choline loading resulted in a TMA/TMA-n-oxide-ratio of 0.43 mg/mmol creatinine (reference range 0.05-0.21). He had a severe odour problem, especially when sweating. He had been treated with riboflavin and activated charcoal without effect. A choline restricted diet and occasional two-week courses of metronidazole followed by lactobacilli treatment had a slight positive effect. Copper chlorophyllin was prescribed, but he did not initiate the treatment. After metronidazole pre-treatment, he was given experimental FMT through gastroscopy on the first of December 2015. Six weeks after FMT, he reported a slight reduction of the odour. Six months after the treatment, he reported fewer odour problems, but after one year, the malodour had returned to its former severity. He did not report any side effects.

A patient with TMAU #2

A 49-year-old female with TMAU. Odour problems started at the age of 12 when menstruation began. The odour problem was at its worst 7-10 d post-ovulation. The diagnosis was confirmed based on the urine TMA-oxide and TMA ratio. TMA-oxide was 59.1 mg/mmol creatinine (reference 17-147), and TMA was 16.5 mg/mmol creatinine (reference 2.5-10.8) ratio 0.28 (reference 0.05-0.21).

She was in the perimenopausal phase with hot flashes and excess paroxysmal sweating, causing the odour problem to worsen, but it was partly in control with hormonal treatment. Two-week metronidazole courses only helped temporarily. She had used a strict choline-restricted diet, vitamin B2 and high doses of lactobacilli. Copper chlorophyllin and activated charcoal had been ineffective. She had previously subjectively felt less of an odour problem for a few weeks after bowel cleansing for colonoscopy. FMT was given as an experimental therapy though colonoscopy. As a pre-treatment, the patient was prescribed metronidazole 400 mg three times per day for 7 d to facilitate engraftment of the donor's microbiota. Metronidazole was stopped 36 h prior to FMT. No relief of the malodour was achieved after FMT.

The rationale for treating fish malodour syndrome with FMT

Trimethylaminuria (TMAU) is a condition in which body odour resembles that of a dead fish. In TMAU, trimethylamine (TMA) accumulates in the body. Primary trimethylaminuria is genetic and caused by an inability to convert the fish smelling TMA into non-odorous trimethylamine-N-oxide (TMAO) in the liver due to a deficiency of the hepatic microsomal flavin-containing monooxygenase (FMO3). Secondary TMAU is defined as an accumulation of TMA without inherited FMO3 deficiency. The aetiology of secondary TMAU is not fully known. One causal factor may be the gut microbiota, which can produce TMA through the metabolism of certain food compounds such as TMAO and choline[25]. Thus, altering TMA metabolism may be possible through manipulating the intestinal microbiota. To our knowledge, there are no previous reported cases of TMAU treated with FMT.

A choline-restricted diet and copper chlorophyllin are the recommended treatments for TMAU[26]. Some patients experience partial relief for symptoms by using antibiotics followed by high doses of lactobacilli, riboflavin or charcoal tablets. Our hypothesis is that TMAU can be ameliorated by manipulating the gut microbiota through FMT. Some short-term positive effects were achieved in one patient, but the bacterial spectrum of the present single FMT did not seem to be effective for the treatment of TMAU. More data concerning the effect of FMT on TMAU are needed.

A patient with small intestinal bacterial overgrowth

The patient was 66 years old in January 2015 when he received his first FMT. He had colectomy and ileal pouch-anal anastomosis (IPAA) surgery in 2008 for ulcerative colitis and adenocarcinoma of the caecum. He had experienced bloating and flatulence during his adult life, but it had become worse since IPAA. He had bowel movements on average 6 times per day.

Endoscopic examination of the pouch showed no inflammation. On the small bowel passage X-ray, there was a small bowel dilatation of 10 cm on the left side of the abdomen. Small bowel MRI did not show an indication for surgery.

Before FMT, the patient underwent several treatments with inadequate results. He was treated with dietary changes and dimethicone to decrease bloating and flatulence. A probiotic - *Escherichia coli* (*E. coli*) *Nissle* up to 2 × 2 capsules (2.5×10^9-25×10^9 CFU/capsule) was administered to remediate dysbiosis. Antibiotics were given to decrease small intestinal bacterial overgrowth (SIBO). He received a course of metronidazole and two courses of rifaximin 200 mg 1 × 4, with one course lasting two weeks and the other four weeks with a tapering dosage. He reported a slight benefit from all of these treatments, but continued to suffer from flatulence and bloating.

On the 20th of January 2015, the patient received FMT *via* gastroscopy as an experimental treatment. For this treatment, 200 mL of the frozen and thawed faecal material was infused through a gastroscope deep into the descending duodenum. Biopsies were obtained *via* gastroscopy and revealed *Helicobacter*-negative atrophic gastritis.

Six weeks after FMT, the patient reported that his

symptoms and bowel movements decreased 50% and that the scent of his flatus was milder. The patient was considered to have reached a partial response and was scheduled for a new FMT.

The second FMT *via* gastroscopy was performed on the 2nd of October using the stool of the same donor as in the first FMT. This time, macrogol bowel preparation was used. The patient reported some benefit from the second treatment, but since disturbing flatulence continued, a third FMT was scheduled with a one-week course of per oral penicillin pre-treatment because the patient had previously experienced relief of his symptoms when using penicillin for a dental infection.

The third FMT *via* gastroscopy was performed on the 29th of January 2016. The transplant was from another donor who was an unfamiliar, tested and generally healthy person, whose stool was frozen and thawed on the day of the transplantation. The last contact with the patient was on the 21th of June 2016, *i.e.*, five months after the third FMT. He reported having fewer symptoms, but some flatulence persisted, though with a milder scent than previously.

The patient was considered to have gained a partial response to his SIBO symptoms from these three FMT treatments.

The rationale for treating SIBO with FMT

A SIBO is defined as an increase in the number or alterations of the type of bacteria in the small bowel. It may be associated with several features, such as alterations of the small bowel anatomy, motility, and immunity, among others. Alterations of the gut microbiota are associated with SIBO by definition. SIBO causes bloating and diarrhoea. Malabsorption, malnutrition and weight loss may also be present. SIBO can most accurately be diagnosed with jejunal aspirate, but this is not widely used due to the invasiveness of the procedure[27]. Hydrogen or methane breath tests are used more widely, but in many centres, including our own, these tests are not in clinical use. We diagnose SIBO clinically based on symptoms and signs. Our SIBO patient had an altered GI anatomy due to a J-pouch and altered immunity due to ulcerative colitis. He received three FMTs through gastroscopy and reported reduced symptoms. For a more objective evaluation of the FMT effect on SIBO, hydrogen breath tests before and after the treatments would have been valuable. More research on the effect of FMT on SIBO is warranted.

A patient with lymphocytic colitis

A female patient who was diagnosed with microscopic colitis in 2013 at the age of 18 had diarrhoea up to 20 times per day. Faecal calprotectin was constantly negative. She had an inadequate response to medications - mesalamine 2.4 mg/d, budesonide 9 mg/d for two months, loperamide or fibres. She had tried various diets to relieve the symptoms. The patient wished to be treated with FMT. For her case, there were no on-going scientific study protocols to follow.

After repeated requests from the patient and with no other rational treatment options available, FMT through colonoscopy was administered as an experimental treatment on the 21st of June 2016.

In the follow-up telephone conversation on the 7th of July, the patient reported to have gained a benefit from the procedure for two weeks, after which the diarrhoea recurred as before. The outcome was considered negative and no further transplants were given.

The rationale for treating lymphocytic colitis with FMT

Lymphocytic colitis is a subtype of microscopic colitis. It is a cause of diarrhoea that is more common in elderly people, but it may even affect children. Microscopic colitis may be associated with an altered gut microbiota. In a small study, patients with microscopic colitis had a decrease in *Akkermansia* species compared with the healthy controls. *Akkermansia* is considered to have a protective effect on the intestinal epithelium[28,29]. Our patient with lymphocytic colitis was treated once with FMT through colonoscopy. She experienced short-term (two weeks) relief of her symptoms, after which the symptoms recurred. The outcome was considered negative. In possible future studies, it might be worth considering a recurrent treatment-protocol with FMT, which has shown some promising results in IBD patients[30,31] and in a single case of collagenous colitis[32], as well as pre-treatment with antibiotics prior to FMT, which may facilitate engraftment of the donor´s microbiota[5].

A carrier of norovirus

A 32-year-old woman was treated with FMT for being a chronic carrier of norovirus. As a long-term diagnosis, she had common variable immunodeficiency (CVI), coeliac disease and osteoporosis. She had chronic diarrhoea since 2009, malabsorption since 2012 and partial parenteral nutrition since March 2015. Her norovirus infection was diagnosed in September 2013. Previously, she did not have acute gastroenteritis. Several medications for her norovirus infection had been attempted without success: interferon alfa, interferon with ribavirin and nitazoxsanide.

FMT was administered in March 2016 through gastroscopy. She was susceptible to bacterial infections due to CVI and bronchiectasis. She had received trimethoprim -sulfamethoxazole as a long-term prophylactic treatment, which was ceased 36 h before FMT. Bowel lavage was not administered prior to FMT. Routine biopsies of the gastroscopy revealed partial villus atrophy.

After FMT, the symptoms of the patient remained unchanged, with four to six bowel movements per day.

Faecal norovirus remained positive. Thus, the patient did not benefit from the experimental FMT treatment. No side effects related to the FMT were observed.

Rationale for treating chronic norovirus infection with FMT

Approximately 5% of CVI patients have enteropathy. In some case reports, chronic norovirus infection has been the cause of CVI-associated enteropathy, and eradication of the virus has cured the symptoms. It has even been hypothesized that chronic norovirus could be a key player in most of these cases[33].

It is suspected that the gut microbiota plays a role in regulating norovirus infection and its pathogenesis[34], and the relationship between the gut bacteria and norovirus infection is undergoing active analysis using murine models. We did not find any published cases of chronic norovirus or CVI enteropathy treated with FMT, and to our knowledge, the patient presented herein is the first reported case.

A carrier of ESBL-producing E. coli

A 31-year-old female patient with asthma was a carrier of the multidrug-resistant *E. coli*-extended spectrum beta-lactamase producing strain (ESBL). She had received pyelophritis caused by ESBL-producing *E. coli* two times, in October 2015 and June 2016. Both episodes of pyelonephritis had been treated with intravenous ertapenem. The duration of the second ertapenem treatment was ten days.

She had studied scientific literature concerning ESBL and *E. coli* virulence factors. After her second pyelonephritis, a consultant infectious disease specialist recommended ESBL eradication with FMT. Prior to FMT, faecal cultures for ESBL-producing *E. coli* were collected five times between August 2016 and February 2017. All the cultures were positive with ESBL-producing *E. coli*.

The *E. coli* strain was resistant to amoxicillin-clavulanic acid, ampicillin, cephalexin, ceftriaxone, cefuroxime, levofloxacin and trimethoprim-sulfamethoxazole, susceptible to ertapenem, meropenem, tobramycin, fosfomycin and nitrofurantoin and showed intermediate susceptibility to ceftazidime and piperacillin-tazobactam.

The patient was hospitalized for meningitis in November 2016. She was treated with ceftriaxone and acyclovir. The meningitis was shown to be caused by an enterovirus. She recovered fully, but the episode delayed her FMT treatment.

FMT was performed *via* colonoscopy on the 31st of January 2017. The endoscopic finding was normal, as were the routine biopsies. Six weeks later, on the 20th of March, the faecal culture of ESBL-producing *E. coli* was negative. The patient had symptoms of cystitis, and the urine test showed elevated leukocytes and *E. coli*, but this time they were susceptible to all the tested antibiotics. She was treated with a two and a half day course of nitrofurantoin 75 mg twice a day.

The rationale for treating ESBL-producing E. coli carriage with FMT

Antibiotic resistance is an emerging global health problem. One of the most common and clinically relevant types of antibiotic-resistant bacteria are the ESBL-producing enterobacteria, especially *E. coli*, which occurs worldwide[35]. Antibiotic resistance is largely caused by excessive use of wide spectrum antibiotics. We, among other authors, have reported the reduction of antibiotic resistance genes in the intestinal microbiome of patients with rCDI after FMT[22,23].

FMT has been successfully used for the eradication of ESBL-producing *E. coli* and other multidrug-resistant bacteria in a small number of published case reports[36-38]. Clinical use of FMT for eradicating resistant bacteria requires further study in larger groups of patients.

DISCUSSION

We report the results obtained for 21 FMT-treated patients; thirteen of the patients had rCDI with a significant underlying comorbidity, and eight of the patients had a condition other than rCDI. The 21 reviewed patients consisted of a heterogeneous group with many comorbidities. This establishes a limitation to our study; definitive conclusions cannot be drawn for the patients as a group. The strength of our study is that we review real life patients who are often excluded from studies due to their comorbidities.

There remains a paucity of data about FMT treatments of patients with different comorbidities. In particular, immunocompromised patients have been excluded from many studies due to the suspected risk of infectious complications. Published data from case series to date suggest that FMT is acceptably safe and effective, even for immunocompromised patients[11,12,39].

Of our thirteen patients with rCDI, *Clostridium difficile* (*C. difficile*) was successfully eradicated from eleven patients. Of those eleven, a patient with HIV and alcoholism experienced reinfection four months after FMT. One patient had gastroenteritis symptoms three days after the FMT and took vancomycin for two days without consulting a doctor. Faecal *C. difficile* was not tested. Her CDI relapses before FMT had been severe. There were no relapses of CDI documented over 8 mo of follow-up, and thus the outcome was considered positive. Two patients experienced a relapse, of which one had received antibiotics less than a week after FMT.

At one month of follow-up after FMT, two of the thirteen rCDI patients had relapsed. Two of the patients were hospitalized due to infections that were

not related to FMT. Two dialysis patients had sepsis in the months following FMT. One dialysis patient died two months after FMT. A patient with CLL and chronic norovirus did not clear the CDI or norovirus; she died due to complications of CLL five months after FMT. The HIV patient resolved the *C. difficile* infection through FMT but experienced an activation of underlying ulcerative colitis two months after FMT. The patient group had many comorbidities, and all the adverse events were considered likely to be unrelated to the FMT.

We also report eight cases of patients treated with FMT for a reason other than rCDI. These patients had prolonged *Salmonella* infection (two cases), ESBL-producing *E. coli* carriage, fish malodour syndrome (two cases), chronic norovirus infection, small bowel bacterial overgrowth and lymphocytic colitis. The acknowledged indication for FMT is recurrent *C. difficile* infection. When performed outside of this indication, FMT should preferably be conducted in a clinical trial setting[19]. However, experimental treatment in carefully considered cases is justified when other treatment options are limited. Such cases also provide preliminary results regarding the use of a specific treatment for new indications.

In the past few years, an increasing number of diseases have been shown to be associated with alterations of the gut microbiota, yet the causality is in most cases undefined. FMT has been suggested to be investigated in many of these diseases[1-4]. Although promising data about FMT in new indications such as autism[40], constipation[41] and epilepsy[42] have been reported, careful consideration of the associated risks is necessary.

The eight patients treated for causes other than rCDI all had a condition in which disruption of the gut microbiota was a possible etiological factor. All eight patients expressed a strong wish to try FMT for their condition, for which other treatments had previously failed. The patients were informed of the experimental nature of the procedure. The justification of each treatment was considered by at least three specialists, including the performing gastroenterologist, the referring physician and the head of the Gastroenterology Department.

The carriers of *Salmonella* and ESBL-producing *E. coli*, and the SIBO patient seemed to have benefitted from FMT. One of the fish malodour syndrome patients received only short-term relief for malodour, and the other did not gain any benefit. The patient suffering from lymphocytic colitis and the CVI patient with chronic norovirus infection did not gain a benefit from FMT. The positive outcome of the carriers of *Salmonella* and ESBL-producing *E. coli* was objectively defined with a laboratory test, as was the negative outcome for the norovirus patient. The outcomes of the other three patients was based on self-reported symptoms and thus were less objective. None of these patients reported any side effects.

The eradication of antibiotic resistant bacteria with FMT has been studied by many research groups, and the results to date are promising. Successful eradications have been described with several multidrug-resistant bacteria, such as ESBL-producing and carbapenemase-producing *Enterobacteriaceae*, vancomycin-resistant *Enterococci*, or methicillin-resistant *Staphylococcus aureus*[36-38]. To the best of our knowledge, the two eradications of *Salmonella* carriage are the first reported cases. The efficacy of FMT for chronic norovirus infection and fish malodour syndrome has also not been reported previously. The treatment options for multidrug-resistant organisms are scarce - eradication and increasing colonization resistance by FMT may offer a new means to counter the problem.

We think it necessary to further study the effect of FMT in conditions where other treatment options are limited. Placebo-controlled trials should be preferred due to the high risk of a placebo effect in conditions in which the diagnosis relies mostly on symptoms, although randomized controlled trials may not be an option for infrequent conditions due to the small number of patients. Thus, case series provide valuable guidance for clinical practice and future clinical trials.

In conclusion, in our cohort, FMT appeared to be a safe and effective treatment for rCDI for patients with significant comorbidities, although further conclusions cannot be drawn due to the small sample size. FMT also shows promise for the eradication of antibiotic-resistant bacteria, for which further research is warranted. FMT is only indicated for rCDI; for other indications, FMT should still be performed only in a clinical trial setting.

COMMENTS

Case characteristics

The authors reviewed 21 fecal microbiota transplantation (FMT)-treated patients, of which 13 had recurrent *Clostridium difficile* infection (rCDI) and major comorbidities: two human immunodeficiency virus patients, six haemodialysis patients, two kidney transplant patients, two liver transplant patients and a patient with chronic lymphatic leukaemia. In addition, the authors reviewed 8 patients treated with FMT for new indications: *Salmonella* carriage (two patients), trimethylaminuria (two patients), small intestinal bacterial overgrowth, lymphocytic colitis, ESBL-producing *Escherichia coli* carriage and a common variable immunodeficiency-patient with chronic norovirus infection.

Treatment

The patients were treated with FMT. Most of the patients received FMT *via* colonoscopy, and stool from a universal donor was mainly used. In a minority of cases, FMT was administered through gastroscopy.

Related reports

Immunocompromised patients have been excluded from the majority of FMT studies, but case reports and series have started to emerge. The number of case reports of patients treated with FMT for indications other than *Clostridium*

difficile is growing. To our knowledge, eradication of *Salmonella* carriage with FMT has not been reported previously.

Experiences and lessons

FMT is acceptably safe for the treatment of rCDI in immunocompromised patients. FMT is promising as a treatment for the eradication of antibiotic-resistant bacteria. There is a great demand for further research on FMT for many new indications.

Peer-review

This article gives a clear description of 21 cases and reasonable discussion, and it can provide a good reference in the daily performance of FMT. The case description in the article is very detailed, the analysis is also very thorough. The article has a good clinical significance.

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CASE REPORT

Oval mucosal opening bloc biopsy after incision and widening by ring thread traction for submucosal tumor

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Author contributions: Mori H was responsible for devising the research and writing the manuscript; Kobara H, Guan Y, Goda Y, Kobayashi N and Nishiyama N participated equally in the work; Masaki T provided a critical revision of the manuscript for intellectual content and was responsible for final approval of the manuscript.

Institutional review board statement: This study was approved by the ethics committees of Kagawa University Hospital (approval No. 51), and it is in accordance with the Declaration of Helsinki.

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Abstract

Gastric submucosal tumors (SMTs) less than 2 cm are generally considered benign neoplasms, and endoscopic observation is recommended, but SMTs over 2 cm, 40% of which are gastrointestinal stromal tumors (GISTs), have malignant potential. Although the Japanese Guidelines for GIST recommend partial surgical resection for GIST over 2 cm with malignant potential as well as en bloc large tissue sample to obtain appropriate and large specimens of SMTs, several reports have been published on tissue sampling of SMTs, such as with endoscopic ultrasound sound fine needle aspiration, submucosal tunneling bloc biopsy, and the combination of bite biopsy and endoscopic mucosal resection. Because a simpler, more accurate method is needed for appropriate treatment, we developed oval mucosal opening bloc biopsy after incision and widening by ring thread traction for submucosal tumor (OMOB) approach. OMOB was

simple and enabled us to obtain large samples under direct procedure view as well as allowed us to restore to original mucosa.

Key words: Gastric submucosal tumors; Gastrointestinal stromal tumor; Reversible opening biopsy; Endoscopic ultrasonography; Large sample

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Core tip: Gastric submucosal tumors (SMTs) less than 2 cm are generally considered benign neoplasms, and endoscopic observation is recommended, but SMTs over 2 cm, 40% of which are gastrointestinal stromal tumors (GISTs), have malignant potential. Although partial surgical resection for GIST over 2 cm with malignant potential as well as en bloc large tissue sample to obtain appropriate and large specimen of SMTs is recommended, several reports have been published on tissue sampling of SMTs. Because a simpler, more accurate method is needed for appropriate treatment, we developed oval mucosal opening bloc biopsy after incision and widening by ring thread traction approach.

Mori H, Kobara H, Guan Y, Goda Y, Kobayashi N, Nishiyama N, Masaki T. Oval mucosal opening bloc biopsy after incision and widening by ring thread traction for submucosal tumor. *World J Gastroenterol* 2017; 23(39): 7185-7190 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7185.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7185

INTRODUCTION

Gastric submucosal tumors (SMTs) less than 2 cm are generally considered benign neoplasms, and endoscopic observation is recommended[1]; however, SMTs over 2 cm, 40% of which are gastrointestinal stromal tumors (GISTs), have malignant potential[2]. The Japanese Guidelines for GIST over 2 cm with malignant potential recommend removal by partial surgical resection as well as en bloc large tissue sample collection to obtain an accurate diagnosis before surgery[3]. To obtain appropriate and large specimens of SMTs and diagnose them accurately, there have been several reports related to tissue sampling of SMTs, such as endoscopic ultrasound sound fine needle aspiration (EUS-FNA)[4,5], submucosal tunneling bloc biopsy (STB)[6], and the combination of bite biopsy and endoscopic mucosal resection (CB-EMR) by which the crown of SMTs was partially resected by EMR[7]. Because a simpler, more accurate method is needed for appropriate treatment, we developed oval mucosal opening bloc biopsy after incision and widening by ring thread traction for submucosal tumor (OMOB) approach.

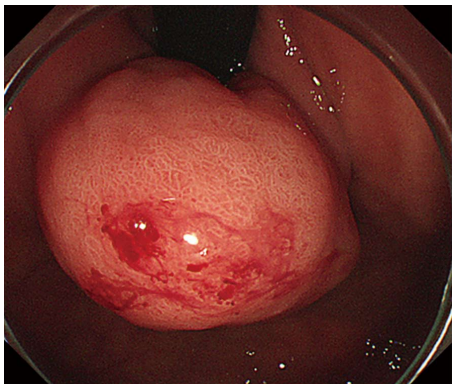


Figure 1 Endoscopic findings of gastric submucosal tumor. A gastric submucosal tumor (30 mm in diameter) is shown in the fornix of the stomach.

CASE REPORT

A forty-seven-year-old woman was diagnosed with a gastric SMT that was 30 mm in diameter in the fornix (Figure 1). As the tumor located in the fornix where EUS-FNA was unable to puncture its needle due to maximum bended endoscope position and STB was also difficult to create submucosal tunnel under maximum bended endoscope position, it was difficult to obtain sufficient tissue sample of this tumor (Figures 1 and 2A). A 5-10 mm straight incision was made on the top of the SMT by Dual knife (KD-650L, OLYMPUS Co., Tokyo, Japan) (Figures 2B and 3). After a 5-mm ring-shaped thread was delivered by grasping forceps and clipped on the left side mucosa of the incision edge (Figure 2C), second clip was hooked the ring-shaped thread (Figure 2D) and moved to be tied up the left gastric wall.

The same procedures were performed on the right side of the incision mucosa (Figure 4) making a straight incision like an oval-shaped incision (Figure 5). With more insufflation, both ring threads expanded the oval incision to a round-shaped incision from which the tumor capsule was clearly recognized (Figure 6). An approximately 5 mm incision of the tumor capsule by Dual knife made it possible to confirm the tumor itself which had abundant tumor vessels (Figures 2E and 6). A 5-mm piece of tumor tissue was obtained by cutting the tumor surface with a Dual knife. After both sides of the ring threads were detached, the opened mucosa was closed by hemoclips to restore it back to the original mucosa (Figures 2F and 7). The total procedure time was only 10 min, and there were no complications, such as bleeding or perforation. The histological result was gastrointestinal stromal tumor. Three weeks after this new bloc biopsy, the incised mucosa was completely recovered with a linear scar. Laparoscopy and endoscopy cooperative

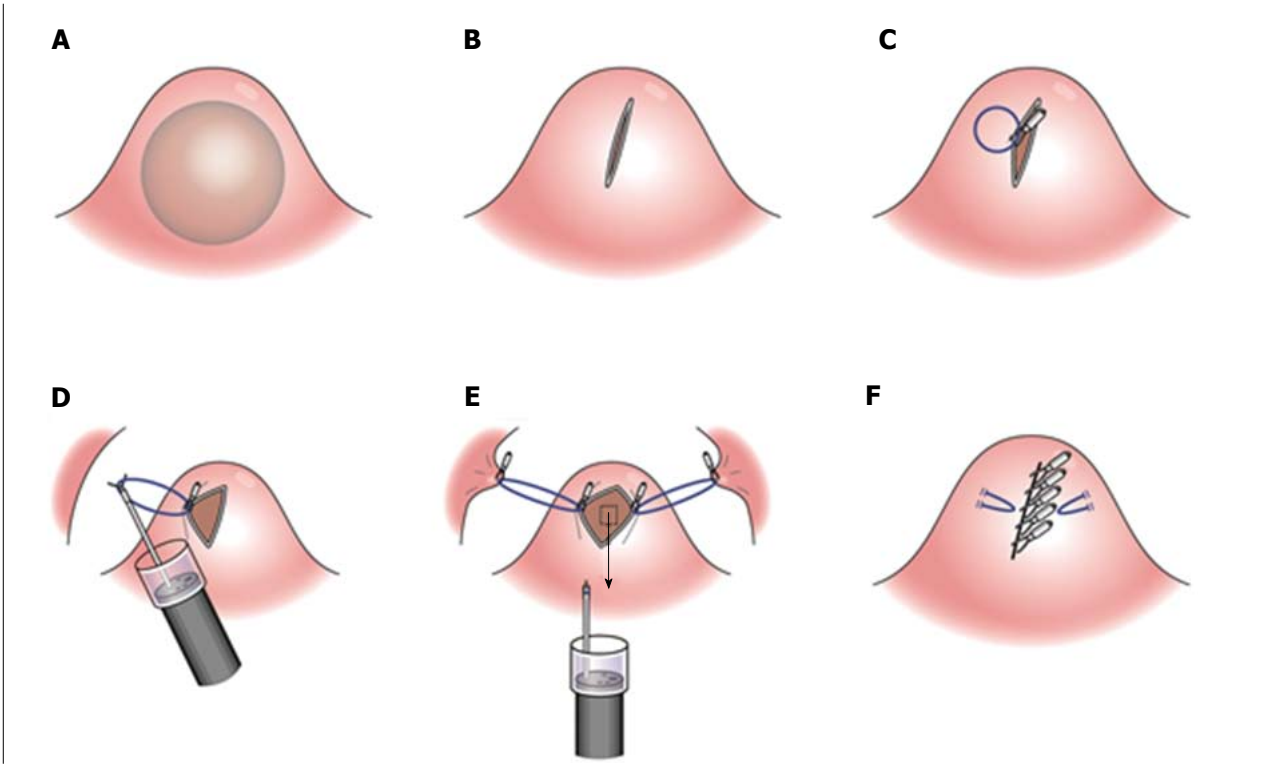


Figure 2 Oval mucosal opening bloc biopsy after incision and widening by ring thread traction. A: A gastric submucosal tumor (SMT) (30 mm in diameter) is shown in the fornix of the stomach; B: A 5-10 mm incision on the top of SMT was made; C: After a 5-mm ring-shaped thread was delivered by grasping forceps; D: Second clip was hooked the ring-shaped thread and moved to be tied up the left gastric wall; E: The same procedures were performed on the right side of the incision mucosa and made a straight incision like an oval-shaped incision; F: After both sides of the ring threads were detached, the opened mucosa was closed by hemoclips to restore it back to the original mucosa.

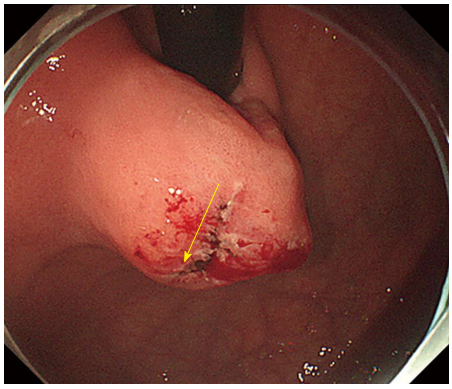


Figure 3 Incision at the top of the submucosal tumor. As endoscopic ultrasound sound fine needle aspiration and submucosal tunneling bloc biopsy were impossible due to the tumor's location, a 5-10 mm incision on the top of submucosal tumor was made (yellow arrow).

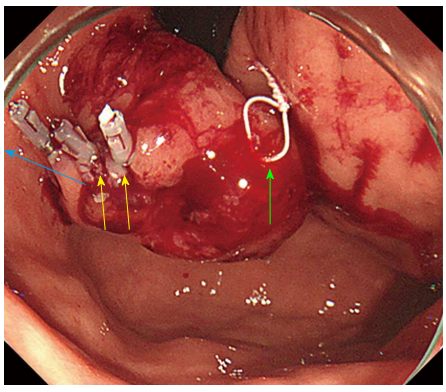


Figure 4 Ring- shaped thread counter traction. After clipping the 5-mm ring-shaped thread on the left side mucosa of the incision edge (yellow arrows), the other side of this ring thread was hooked and pulled to the posterior wall of the stomach (blue arrow). A 2nd white ring thread was placed on the other side of the incision edge (green arrow).

surgery (LECS) was successfully performed, and the histological finding of the GIST was low risk in accordance with Fletcher's classification. An endoscopic image revealed that straight incision on the top of the SMT was completely scarred and closed (yellow ring) (Figure 8) when laparoscopy and endoscopy cooperative surgery (LECS) was performed six week after oval mucosal opening bloc biopsy.

DISCUSSION

The natural history of 2-5 cm GISTs is unknown. In the Japanese Guidelines of GIST, accurate diagnosis, including the histological grade based on a sufficient

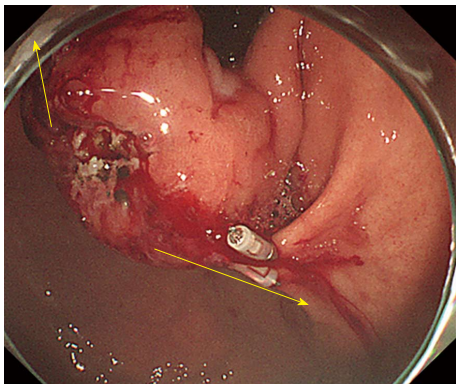


Figure 5 Oval mucosal opening after incision and widening by ring thread traction. The same procedures were performed on both sides of the incision mucosa with a straight incision to an oval shaped incision (yellow arrows).

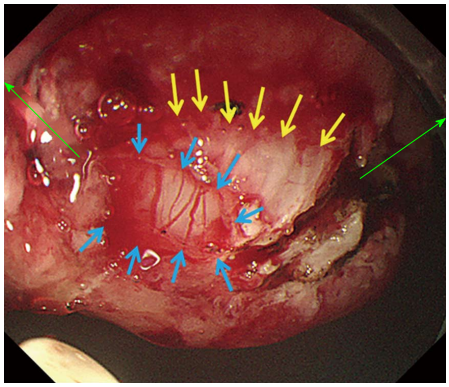


Figure 6 Direct view of capsule and abundant vessels of gastrointestinal stromal tumors. With more insufflation, both ring threads expanded the oval incision to a round shaped incision (green arrows) from which the tumor capsule was clearly recognized. An approximately 7-mm cut of the tumor capsule (yellow arrows) by Dual knife made it possible to confirm the tumor (blue arrows) with abundant tumor vessels.

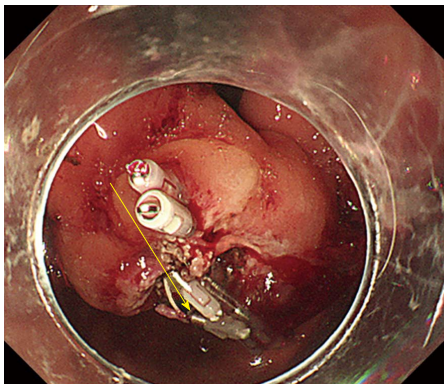


Figure 7 Reversible mucosa closure by hemoclips. After both sides of the ring threads were detached, the opened mucosa was closed by hemoclips to restore it back to the original mucosa (yellow arrow).

tissue sample, is recommended for GIST less than 2 cm, which is growing rapidly, or 2-5 cm GIST rather than endoscopic observation alone[8].

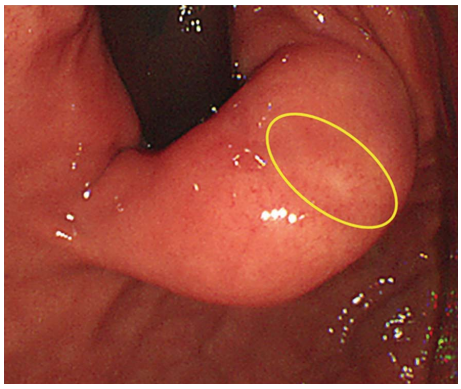


Figure 8 A mucosal incision six week after oval mucosal opening bloc biopsy. An endoscopic image revealed that straight incision on the top of the submucosal tumor was completely scarred and closed (yellow ring) when laparoscopy and endoscopy cooperative surgery was performed six week after oval mucosal opening bloc biopsy.

EUS-FNA is very useful for accurate diagnosis for SMTs since it was reported in 1992[9]. Its diagnostic sensitivity for GIST is very high at approximately 70% and the specificity is approximately 85%[10]. On the other hand, EUS-FNA does not always obtain sufficient tissue by needle sample for one of the grading factors of malignancy, such as the mitotic count under a 50 high power microscope field. The diagnostic rate for EUS-FNA was approximately 60% as the obtained samples were too small to pathologically diagnose the mitotic counts[11]. The combination of bite biopsy and endoscopic mucosal resection (CB-EMR) using a snare to cut the top of SMTs enabled us to obtain a large bloc specimen. However, the bleeding rate was very high at approximately 50%-60% from the snare resection site[12]. Bleeding after snare resection occurred due to a large mucosal defect at approximately 15-20 mm in diameter. Compared to CB-EMR, OMOB enable us to perform en bloc large tissue sampling without complications, such as bleeding, for GIST with rich vessels. OMOB consists of a 1-cm linear incision to round shaped excision using ring threads that expand with insufflation. After obtaining large bloc tissue, coagulation of bleeding vessels is performed followed by closure of the opening mucosa. Closure and recovery of mucosal incision is an important point of OMOB. STB using the ESD technique is another way to obtain a large tissue sample of GIST. As STB was safely performed using flexible endoscopic knives, only ESD experts could perform STB. It is difficult for ordinary endoscopists to perform STB[13], because making appropriate size and location of mucosal incision suitable for creating submucosal tunnel was very difficult for ESD beginner. And creating submucosal tunnel to correct direction and adjusting correct depth of submucosal dissection within the submucosal tunnel were more difficult than conventional gastric

ESD. Another disadvantage of STB is the creation of a submucosal tunnel that leaves an extra 1-cm tunnel scar outside of the GIST. This extra linear scar makes the surgical margin of LECS larger than that of OMOB.

In conclusion, OMOB was simple and enabled us to obtain a large sample under the direct procedure view; it also allowed us to restore to the original mucosa.

COMMENTS

Case characteristics

A forty-seven-year-old woman was diagnosed with a gastric submucosal tumor (SMT) that was 30 mm in diameter in the fornix.

Clinical diagnosis

The tumor located in the fornix was considered as gastric submucosal tumor.

Differential diagnosis

Gastrointestinal stromal tumor (GIST), leiomyoma, schwannoma, leiomyosarcoma, malignant lymphoma, ectopic pancreas and lipoma.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

Esophagogastroduodenoscopy showed gastric SMT 30 mm in diameter in the fornix .

Pathological diagnosis

The histopathological finding of the SMT was low risk GIST in accordance with Fletcher's classification.

Treatment

Complete surgical excision of lesion.

Related reports

Several reports have been published on tissue sampling of SMTs, such as with endoscopic ultrasound sound fine needle (EUS-FNA) aspiration, submucosal tunneling bloc biopsy, and the combination of bite biopsy and endoscopic mucosal resection.

Term explanation

Oval mucosal opening bloc biopsy by ring thread traction for submucosal tumor is new method for diagnosis of gastric SMT.

Experiences and lessons

Development of oval mucosal opening bloc biopsy after incision and widening by ring thread traction for submucosal tumor (OMOB) approach was useful for simpler, more accurate method for appropriate treatment of gastric SMT.

Peer-review

This case report presented a new biopsy method for GIST of the stomach. The authors demonstrate clearly that "reversible hinged double doors method" is useful to obtain large tissue sample. This method may certainly be of use for tough case even if we use EUS-FNA. This manuscript is well-written in terms of language and seems to be informative to the readers.

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CASE REPORT

Evidence from a familial case suggests maternal inheritance of primary biliary cholangitis

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Author contributions: Shin S wrote the paper; Moh IH collected the patients' clinical data; Woo YS, Jung SW, Kim JB, Park JW, Suk KT, Kim HS and Lee MS provided the resources and advice; Hong M performed pathologic diagnosis and reviewed the manuscript; Park SH designed the report and edited the manuscript.

Institutional review board statement: This study was approved by the Institutional Review Board at Kangnam Sacred Heart Hospital.

Informed consent statement: Informed consent was obtained from the patients for this case report.

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

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Abstract

Primary biliary cholangitis (PBC) is an idiopathic autoimmune liver disease characterized by chronic cholestasis and destruction of the intrahepatic bile ducts. Similar to other autoimmune diseases, the pathogenesis of PBC is considered to be a complex etiologic phenomenon involving the interaction of genetic and environmental factors. Although a number of common variants associated with PBC have been reported from genome-wide association studies, a precise genetic mechanism underlying PBC has yet to be identified. Here, we describe a family with four sisters who were

diagnosed with PBC. After the diagnosis of the index patient who was in an advanced stage of PBC, one sister presented with acute hepatitis, and two sisters were subsequently diagnosed with PBC. Notably, one half-sister with a different mother exhibited no evidence of PBC following clinical investigation. Our report suggests the possibility of a maternal inheritance of PBC susceptibility. Moreover, judging from the high-penetrance of the disease observed in this family, we inferred that a pathogenic genetic variant might be the cause of PBC development. We describe a family that exhibited diverse clinical presentations of PBC that included asymptomatic stages with mildly increased liver enzyme levels and symptomatic stages with acute hepatitis or advanced liver fibrosis. Additional studies are needed to investigate the role of genetic factors in the pathogenesis of this rare autoimmune disease.

Key words: Primary biliary cholangitis; Family history; Genetic susceptibility

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Core tip: The precise genetic mechanism underlying primary biliary cholangitis (PBC) has yet to be identified. Here, we describe a family with four siblings who were diagnosed with primary biliary cholangitis. This is the first case report to provide evidence of a maternal inheritance mechanism for PBC based on the identification of a non-PBC half-sibling. This report also highlights the occurrence of all clinical presentations of PBC in one family. Identification of a causal variant is important for a better understanding of the mechanism underlying PBC pathogenesis.

Shin S, Moh IH, Woo YS, Jung SW, Kim JB, Park JW, Suk KT, Kim HS, Hong M, Park SH, Lee MS. Evidence from a familial case suggests maternal inheritance of primary biliary cholangitis. *World J Gastroenterol* 2017; 23(39): 7191-7197 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7191.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7191

INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic autoimmune cholestatic liver disease that predominantly affects middle-aged women. PBC is characterized by immune-mediated destruction of the intrahepatic bile ducts that gradually leads to fibrosis, cirrhosis, and eventually liver failure. Most patients are diagnosed when asymptomatic with an elevation of alkaline phosphatase (ALP) or with pruritus and mild elevations on liver biochemical tests. PBC can be diagnosed if two of the three following criteria are met: the presence of anti-mitochondrial antibody (AMA); cholestatic biochemical test results, such as an elevated ALP level; and histologic evidence of non-suppurative cholangitis with destruction of the interlobular bile ducts[1,2].

The current hypothesis regarding the etiology of PBC is that it is a multifactorial disease that occurs due to a combination of genetic, immunologic, and environmental factors[3]. The influence of genetic factors is evidenced by familial clusters and twin studies[4]. Epidemiological studies have demonstrated that family members of patients with PBC are at a higher risk of developing PBC[5-7]. Associations between a number of genetic loci and PBC have been reported in genome-wide association studies (GWAS), which is similar to the cases of many other autoimmune diseases[8-10]. However, no precise genetic mechanism underlying PBC is known.

Herein, we report a family with four sisters who were diagnosed with PBC. The possible implications of a monogenic etiology of PBC are discussed.

CASE REPORT

A 56-year-old woman (Table 1, patient 1) was referred to Hallym University Kangnam Sacred Heart Hospital with abnormal liver function test results. Her past history was unremarkable, and she had no history of alcohol or drug abuse. Here, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, gamma-glutamyltranspeptidase (GGT), and immunoglobulin (Ig) M were elevated, and she was positive for AMA. A liver biopsy revealed non-suppurative destructive cholangitis and granulomatous inflammation with fibrosis (Figure 1A). The patient was diagnosed with PBC, and her family members, including three sisters, one brother, and one half-sister were evaluated (Figure 2).

Patient 2 had no symptoms but exhibited a mild elevation of ALP. She also exhibited positivity for AMA and non-suppurative cholangitis on a liver biopsy (Figure 1B) and was diagnosed with early-stage PBC.

Patient 3 was admitted to an outside university hospital with acute hepatitis of undetermined etiology. Here, liver enzymes were markedly elevated at the time of admission, *i.e.*, her AST was 744 IU/L, and her ALT was 1273 IU/L. Viral markers of acute hepatitis pathogens, including hepatitis A, hepatitis E, herpes simplex virus, Epstein-Barr virus, and cytomegalovirus, were negative. She then presented to our hospital, where laboratory tests revealed AMA positivity, an elevated IgM level, and liver histologic findings consistent with PBC (Figure 1C).

Patient 4 had no symptoms but exhibited mild elevations of the serum levels of AST, ALT, and GGT. She was AMA-positive, and her IgG and IgM levels were elevated. A subsequent liver biopsy revealed advanced histologic findings consistent with PBC, including portal inflammation, non-suppurative chol-

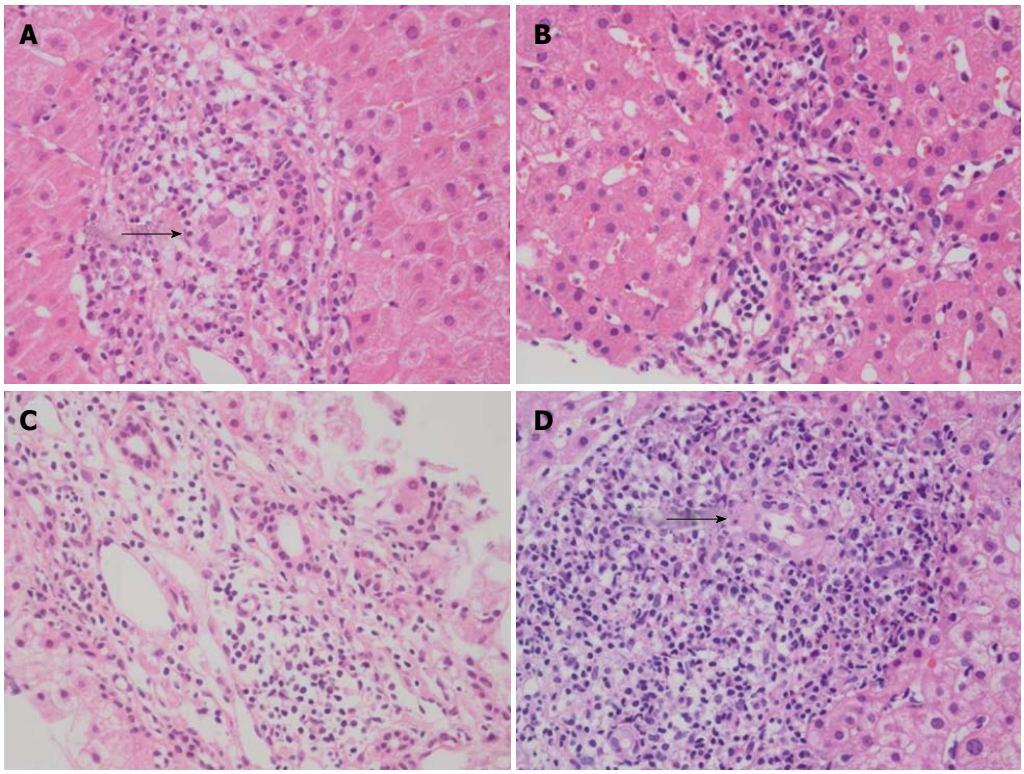


Figure 1 Microscopic features of the patients' liver biopsies (H&E stain, × 400). A: Patient 1: A non-necrotizing granuloma is surrounded by lymphoid infiltrates (arrow); B: Patient 2: Aggregates of lymphocytes indicate the early phase of the disease; C: Patient 3: Mild portal inflammation without bile duct damage or granuloma is present; D: Patient 4: A damaged bile duct is identified in a background of lymphocytes and plasma cells (arrow).

Table 1 Characteristics and initial laboratory findings from the family members

Characteristics (reference ranges)	Patient 1	Patient 2	Patient 3	Patient 4	Brother	Half-sister
Sex, age	F, 56	F, 54	F, 44	F, 38	M, 52	F, 48
Symptom	Fatigue, pruritus	(-)	Fatigue, nausea	(-)	(-)	(-)
Underlying disease	(-)	Thyroid cancer	(-)	(-)	(-)	(-)
AST, IU/L (9-39)	127	29	311	63	16	17
ALT, IU/L (6-45)	160	24	509	90	11	13
Total/direct bilirubin, mg/dL (0.4-1.3/0.1-0.4)	1.0 / 0.3	0.8 /0.2	1.3 / 0.7	0.6 / 0.2	0.5 / 0.2	0.9 / 0.3
ALP, IU/L (35-104)	132	122	122	101	83	58
GGT, IU/L (8-35)	169	63	188	112	14	15
HBs antigen/antibody	(-/+)	(-/+)	(-/-)	(-/+)	(-/+)	Not done
Anti-HCV antibody	(-)	(-)	(-)	(-)	(-)	Not done
Anti-mitochondrial antibody, titer	(+, > 1:1280)	(+, 1:640)	(+, > 1:1280)	(+, 1:640)	(-)	(-)
Anti-smooth muscle antibody	(-)	(-)	(-)	(-)	(-)	(-)
Anti-liver-kidney microsome antibody	(-)	(-)	(-)	(-)	Not done	Not done
Antinuclear antibody	(-)	(-)	(-)	(-)	(-)	(-)
IgG, mg/dL (700-1600)	1630	1150	1790	2070	1130	1210
IgA, mg/dL (70-400)	316	226	362	268	287	177
IgM, mg/dL (40-230)	421	289	542	542	247	94.4

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; HBs: Hepatitis B surface; HCV: Hepatitis C virus; Ig: Immunoglobulin.

angitis, granuloma, lymphoplasmacytic infiltrates, and peri-portal fibrosis (Figure 1D).

One brother (52 years old) with the same mother and one half-sister (48 years old) with a different mother were clinically evaluated for PBC; however, they both exhibited normal liver biochemistry and negative AMA results (Table 1).

The four patients were treated with ursodeoxycholic acid (UDCA; 15 mg/kg·d), and liver biochemistry results indicated good responses (Figure 3). The patients have been followed and maintained on UDCA therapy without complications.

DISCUSSION

Here, we report a family that displayed evidence of maternal inheritance and exhibited all possible clinical presentations of PBC. The index patient (patient 1) was

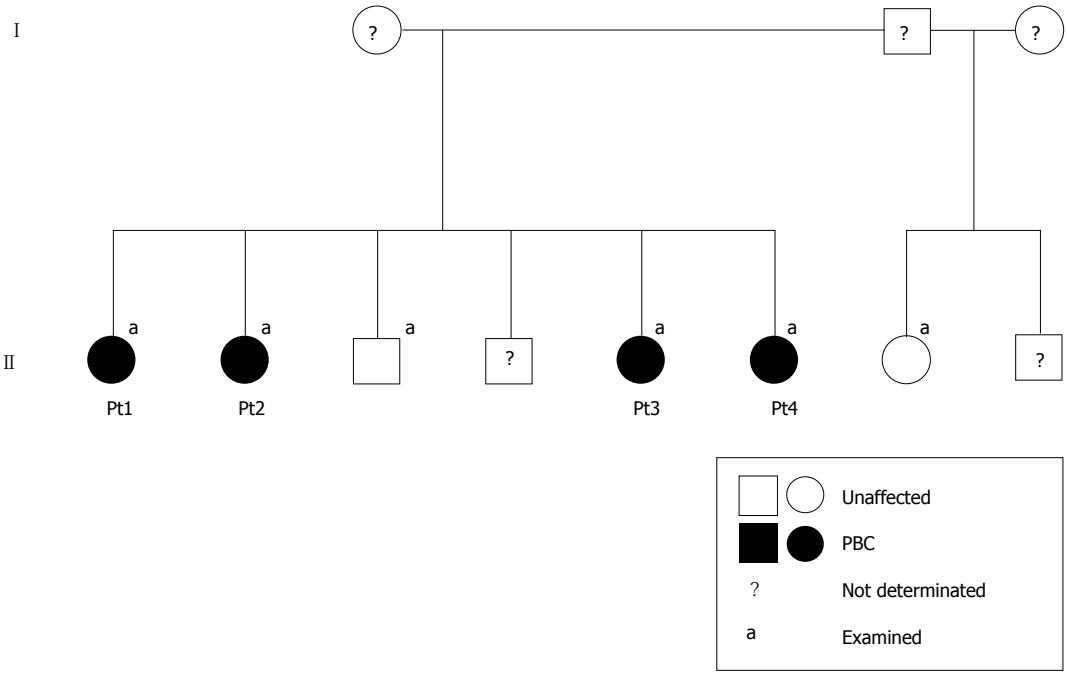


Figure 2 Pedigree of a family with four sisters with diagnoses of primary biliary cholangitis. The black symbols indicate clinically affected family members, and the white symbols indicate non-affected members. No phenotypically relevant information was available for the members with question marks. Pt: Patient; PBC: Primary biliary cholangitis.

diagnosed in a fairly advanced stage of the disease and with early cirrhosis. In contrast, two patients were diagnosed in asymptomatic stages with mildly increased liver biochemistry results (patients 2 and 4). Interestingly, one patient (patient 3) presented with an acute hepatitis-like condition with markedly elevated serum aminotransferases. In PBC, acute presentations have very rarely been reported[11]. Because acute presentations of an overlapping syndrome, *i.e.*, autoimmune hepatitis, are common, it has previously been suggested that autoimmune hepatitis might exhibit overlap with the early stage of PBC in PBC patients with acute presentations[12]. However, we were unable to find any relevant evidence of autoimmune hepatitis in patient 3, including in examinations of liver histology, auto-antibody studies, the serum IgG level, and the treatment response to UDCA[13,14].

There are some factors that are known to trigger PBC symptoms or signs that include adverse drug reactions, pregnancy, and delivery[12]. The mechanisms by which these factors affect disease state are thought to be related to an immunological influence[12]. However, our patient with the acute presentation was not pregnant and had no history of medication use. Our findings suggest that PBC should be considered in the differential diagnosis of acute hepatitis of unknown etiology. Further study is needed to identify possible undiagnosed cases and to investigate the mechanisms that trigger the acute phase of PBC.

Previous GWA studies have contributed to our understanding of the genetic architecture of PBC[8-10]. A number of susceptibility loci are located in genes with known immunologic functions, such as human leukocyte antigen (*HLA*), interleukin 12 receptor subunit beta 2 (*IL12RB2*), interleukin 12A (*IL12A*), C-X-C motif chemokine receptor 2 (*CXCR2*), and the CD80 molecule (*CD80*)[8-10]. However, the identified polymorphisms are also found in the general population at high frequencies and could partially explain the disease heritability. Based on the existence of familial cases with many affected members, as we report here[5,15-17], we infer the contribution of a rare, disease-causing variant to the development of PBC. Although the previous literature also reports a high prevalence of PBC in siblings of patients (four or more cases in 6-10 siblings)[5,15,16], the unique and interesting feature in our case is that PBC was diagnosed in all four sisters but not in one half-sister with a different mother. This finding suggests the possibility of maternal inheritance mechanism and a genetic pattern, such as X chromosome-linked or mitochondrial inheritance.

With rapid progression of next-generation sequencing (NGS) technology, the cost and time required to sequence data have substantially declined[18]. Using high-throughput NGS technology, it is technically feasible to quickly and efficiently investigate rare variants that cannot be identified by GWAS[18]. Therefore, we plan to detect the causal genetic variant in this family using whole-exome sequencing. The identification of a rare pathogenic variant is important for a better understanding of the mechanism underlying the pathogenesis of PBC and for identifying novel

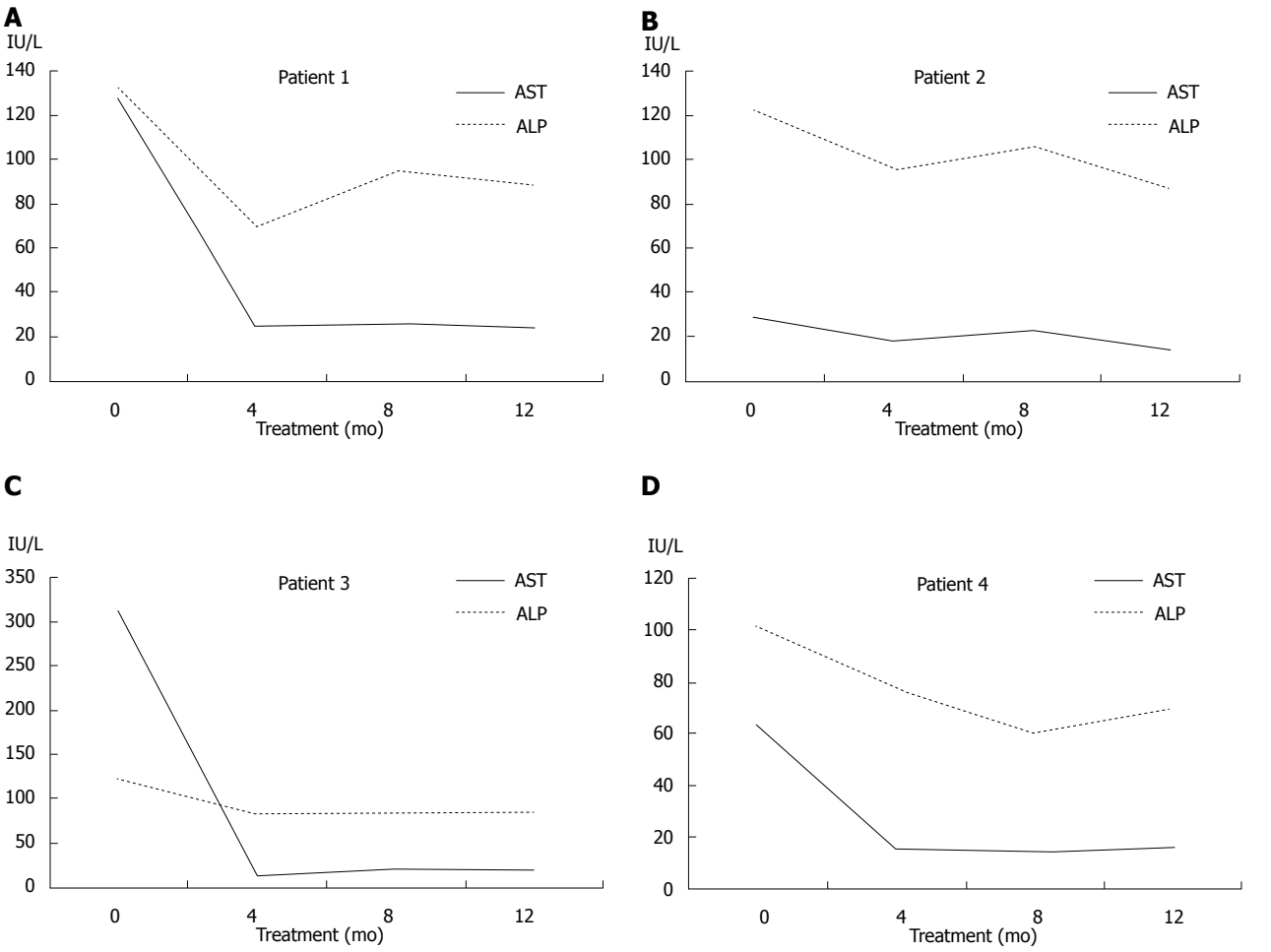


Figure 3 Biochemical responses to ursodeoxycholic acid treatment. A-D: Serum activities of aspartate aminotransferase (AST; solid lines) and alkaline phosphatase (ALP; dotted lines) showing good responses to ursodeoxycholic acid in patients 1-4.

therapeutic targets.

The early detection of PBC is important because UDCA treatment before the development of late-stage disease may normalize the life expectancy[19-21]. Long-term observational studies have demonstrated the benefits of UDCA on serum liver tests, histologic features, and improved survival[22,23]. The efficacy of a novel bile acid analogue, obeticholic acid, has also recently been demonstrated in patients who exhibit inadequate responses to UDCA[24,25]. Luckily, all of our patients responded well to UDCA therapy.

From our case and prior evidence indicating familial clustering of PBC, if one patient is diagnosed with PBC, screening with AMA and liver function tests should be recommended to other family members for the early detection and management of this condition, especially for female relatives.

The genetic etiology of PBC remains elusive despite much effort. To the best of our knowledge, this is the first case to provide evidence of a maternal inheritance mechanism for PBC based on the identification of a non-PBC half-sibling. This report also highlights the occurrence of all clinical presentations of PBC in one family. Additional studies are needed to identify a causal genetic variant in this family and the exact genetic mechanism that leads to the development of PBC.

COMMENTS

Case characteristics

Two patients presented with fatigue and nausea, and the other two patients exhibited no symptoms.

Clinical diagnosis

Four sisters in a family were diagnosed with primary biliary cholangitis (PBC), although one brother with the same mother and one half-sister with a different mother showed no evidence of PBC.

Differential diagnosis

Drug-induced cholestasis (history for medication), bile duct obstruction (ultrasound for gallstones or malignancy), autoimmune hepatitis [liver histology, auto-antibodies studies, serum immunoglobulin G level, and treatment response to ursodeoxycholic acid (UDCA)].

Laboratory diagnosis

All four patients showed the presence of anti-mitochondrial antibodies at high

titers (≥ 1:640) and elevated serum liver biochemistry results including those for alkaline phosphatase and aspartate aminotransferase.

Imaging diagnosis

For all patients, ultrasounds revealed no evidence of biliary obstruction due to gallstones or malignancy.

Pathological diagnosis

For all patients, microscopic observations of liver biopsy tissue revealed histologic findings consistent with PBC.

Treatment

The four patients were treated with UDCA.

Related reports

To date, many PBC candidate loci have been reported in genome-wide association studies. However, these loci are very heterogeneous, and the exact genetic cause of PBC remains elusive.

Explanations of terms

Whole-exome sequencing: also called WES or exome sequencing, is a technique for the sequencing of all human protein-coding exons.

Experiences and lessons

From the observations of the presentations of the four siblings diagnosed with PBC, we recommend that PBC occurrence should be considered in family members of any identified patients.

Peer-review

This is an interesting clinical observation that suggests the possibility of a maternal inheritance pattern of PBC. The presented data may provide an incentive for further research.

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LETTERS TO THE EDITOR

Duplicate publication bias weakens the validity of meta-analysis of immunosuppression after transplantation

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Author contributions: Fairfield CJ wrote this letter; Harrison EM and Wigmore SJ revised the letter.

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Abstract

Duplicate publication can introduce significant bias into a meta-analysis if studies are inadvertently included more than once. Many studies are published in more than one journal to maximize readership and impact of the study findings. Inclusion of multiple publications of the same study within a meta-analysis affords inappropriate weight to the duplicated data if reports of the same study are not linked together. As studies which have positive findings are more likely to be published in multiple journals this leads to a potential overestimate of the benefits of an intervention. Recent advances in immunosuppression strategies following liver transplantation have led to many studies investigating immunosuppressive regimes including immunosuppression monotherapy. In this letter we focus on a recently published meta-analysis by Lan *et al* investigating studies assessing immunosuppression monotherapy for liver transplantation. The authors claim to have identified fourteen separate randomised studies investigating immunosuppression monotherapy. Seven of the references appear to relate to only three studies which have been subject to duplicate publication. Several similarities can be identified in each of the duplicate publications including similar authorship, identical immunosuppression regimes, identical dates of enrolment and citation of the original publication in the subsequent manuscripts. We discuss the evidence of the duplicate publication inclusion in the meta-analysis.

Key words: Liver transplantation; Immunosuppression; Meta-analysis; Duplicate publication; Bias

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Core tip: The purpose of this letter to the editor is to comment on the potential inclusion of duplicate publications within the meta-analysis titled: "Efficacy of immunosuppression monotherapy after liver transplantation: A meta-analysis".

Fairfield CJ, Harrison EM, Wigmore SJ. Duplicate publication bias weakens the validity of meta-analysis of immunosuppression

after transplantation. *World J Gastroenterol* 2017; 23(39): 7198-7200 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i39/7198.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i39.7198

TO THE EDITOR

We read with interest the article titled "Efficacy of immunosuppression monotherapy after liver transplantation: A meta-analysis" by Lan *et al*[1]. The authors have performed a meta-analysis assessing the use of immunosuppression monotherapy after liver transplantation. The authors claim to have included fourteen randomised studies comparing monotherapy *vs* combination immunosuppression for liver transplanted patients and conclude that calcineurin inhibitor monotherapy is both effective and leads to fewer adverse events than combination therapy. The authors state that the review is the first meta-analysis to include multiple studies assessing the effect of immunosuppression with or without steroids on graft rejection after liver transplantation. Finally, the authors state that the strengths of their review include duplicate study elimination. For the following reasons, we do not agree with their results or their conclusions.

The authors claim to have included fourteen separate randomised studies. On closer inspection, the authors have included seven references relating to only three randomised studies and have not made adequate efforts to eliminate duplicate studies[2-8].

The first of these studies was performed in the United Kingdom and both publications share the same start date, protocol, several co-authors and the same recruitment centres[2,3]. The earlier publication appears to record preliminary results[2]. Manousou *et al*[3] appear to have included these preliminary results as a separate study. Furthermore, the main publication relating to this study clearly states that the findings are "similar to those in our preliminary report". The citation in support of this statement is identical to that included as a separate study in the meta-analysis by Lan *et al*[1].

The second of these studies was performed in Germany and both publications share the same enrolment dates, protocol, several co-authors, recruitment centre and numbers of patients allocated to each intervention arm[4,5]. Furthermore, the publication recording long-term follow-up for patients in this study explicitly states that the authors have previously published their study and that in the publication in 2010 they "present the results of a re-evaluation of our study patients"[5].

The third of these studies was performed in Italy and all three publications share the same enrolment dates, several co-authors, recruitment centre and protocol[6-8]. Furthermore, both duplicate studies with later publication dates explicitly state that the earlier publications are interim reports relating to the same study[6,7].

The authors also claim to have published the first meta-analysis assessing steroid-free immunosuppression in liver transplanted patients. Three meta-analyses[9-11] were published prior to the date of submission by Lan *et al*[1]. Two further meta-analyses have been published since this date[12,13]. In each case where any of the three studies discussed have been included in another meta-analyses the authors have concluded that the studies have been subject to duplicate publication.

The problem with inclusion of duplicated data in meta-analyses is that it creates bias with inappropriate weight being afforded to the duplicate data. The failure in Lan 2014 to adequately avoid duplicate publication bias may mean that the results of this meta-analysis are invalid.

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MINIREVIEWS

Non-celiac gluten sensitivity: All wheat attack is not celiac

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Abstract

Currently, 1% of the United States population holds a diagnosis for celiac disease (CD), however, a more recently recognized and possibly related condition, "non-celiac gluten sensitivity" (NCGS) has been suggested to affect up to 6% of the United States public. While reliable clinical tests for CD exist, diagnosing individuals affected by NCGS is still complicated by the lack of reliable biomarkers and reliance upon a broad set of intestinal and extra intestinal symptoms possibly provoked by gluten. NCGS has been proposed to exhibit an innate immune response activated by gluten and several other wheat proteins. At present, an enormous food industry has developed to supply gluten-free products (GFP) with GFP sales in 2014 approaching $1 billion, with estimations projecting sales to reach $2 billion in the year 2020. The enormous demand for GFP also reflects a popular misconception among consumers that gluten avoidance is part of a healthy lifestyle choice. Features of NCGS and other gluten related disorders (*e.g.*, irritable bowel syndrome) call for a review of current distinctive diagnostic criteria that distinguish each, and identification of biomarkers selective or specific for NCGS. The aim of this paper is to review our current understanding of NCGS, highlighting the remaining challenges and questions which may improve its diagnosis and treatment.

Key words: Non-celiac gluten sensitivity; Celiac disease; Gluten; Wheat; Gluten related disorder; Gluten free diet

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Core tip: Non-celiac gluten sensitivity, a less known clinical entity has been estimated to have a prevalence of up to 6% in the United States. This review identifies the pathophysiology of the disease delineating clearly the important components of wheat which play a role in its innate immune response. The updated guidelines on the diagnosis of this disease is discussed here with a bridge to other management strategies apart from the gluten free diet that are now being investigated.

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INTRODUCTION

In the past decade, the consumption of gluten free food has become increasingly popular in the Western world. A Gallup poll conducted in July 2015 showed that 20% of Americans opt for a gluten free diet (GFD) while 17% say they avoid gluten free foods[1]. Estimates by the nutrition industry indicate that sales of gluten-free products (GFPs) had a compound annual growth rate of 34% over the five year period ending in 2014, with annual sales totaling close to $1 billion[2]. Current market projections predict sales will again soar to approach $2 billion in the year 2020[3]. Market research by the Mintel group gives a liberal estimation of the sales of products in the gluten-free industry to be $11.6 billion in the year ending 2015, an estimated 136% increase from 2013[4]. The significant increase in the retail sales of GFPs is alarming. However, currently only approxiately 1% of the United States population is diagnosed with celiac disease[5]. Despite this, gluten-free food consumption is clearly on an exponential rise. This enormous allocation of resources reflects self-diagnosis for gluten sensitivity and may reflect an additional condition described as non-celiac gluten sensitivity (NCGS).

The first comprehensively documented case of "gluten sensitivity" (in a non-celiac disease/wheat allergy patient) was reported in 1980[6]. That report described eight adult women suffering from incapacitating abdominal pain and chronic diarrhea which rapidly remitted when a gluten-free diet was initiated, with symptomatic relapse after re-exposure to a gluten containing diet (GCD). Jejunal biopsies performed on these patients, however, failed to identify CD-like histopathology, leading to a consensus description of these patients as having "gluten-sensitive diarrhea without evidence of celiac disease". This was the beginning of the current era of what we now consider NCGS. Since then, many subsequent studies have attempted more extensive descriptions of NCGS to create effective diagnostic criteria and management strategies.

In 2011, an international consensus on NCGS reached an agreement on the definition of NCGS, defining it as a "non-allergic and non-autoimmune condition in which the consumption of gluten can lead to symptoms similar to those seen in CD." NCGS is defined as gluten sensitivity because symptoms are relieved by gluten withdrawal, and re-appear upon introduction to gluten[7]. At least part of the difficulty in handling NCGS patients is that essentially, it is often a diagnosis by exclusion. First, CD and WA need to be excluded as possible diagnoses. Beyond this, NCGS often carries an extensive and relatively broad set of symptoms which affects diverse organ systems[8]. Symptoms of NCGS could be very disabling presenting as gastrointestinal and/or extra-intestinal symptoms[9]. The onset of NCGS symptoms after gluten consumption can also range widely, appearing hours to even days following exposure to a GCD; the timing of resolution in NCGS symptoms may also vary widely.

Given that NCGS presentation could resemble CD or WA, further research into highlighting their hallmark findings is necessary. It is essential now that individuals with gluten-related disorders are identified accurately and managed appropriately. This is important in order to curtail the significant rise in the retail sales of GFPs in the food industry as millions of dollars could be salvaged given the appropriate diagnosis amongst other important reasons. This current review discusses our present understanding of NCGS, its differences and similarities to CD and other gluten-related disorders, etiopathogenesis and management strategies. It is important to recognize that NCGS is a distinct disorder from CD and it is imperative that it is accurately distinguished.

EPIDEMIOLOGY

Despite becoming a more common diagnosis, there is currently a paucity of information on NCGS especially regarding its actual prevalence in the general population. This lack of information reflects the decision of many patients to start GFD after self-diagnosis without any formal clinical testing or management recommendation by their physician. As a result of this ambiguity, the prevalence of NCGS has been reported to vary enormously from 0.6%-6% in Western populations[10-12]. It is unclear how much of the rise in consumption of gluten free foods actually reflect a higher prevalence of NCGS in the population or the choice to adhere to a GFD based on patient's preferences.

Despite the lack of accurate epidemiologic measurements of the actual US prevalence of NCGS, data in 2009-2010 from the National Health and Nutrition Examination Survey (NHANES) showed that of the

7762 NHANES participants who were free of celiac disease, 49 individuals reported a strict adherence to a GFD. This reflects a weighted prevalence of NCGS of only 0.55%[11]. Another more recent evaluation of the NHANES participants from 2009-2012 separated patients into those with CD and those without CD consuming a GFD. This study estimated a prevalence of NCGS of 0.8% in patients without CD who were consuming a GFD[10]. The prevalence in these NHANES studies were estimated based on the assessment that the individuals without CD avoiding gluten fall under the classification of NCGS.

During the period 2004 and 2010, 5896 patients were seen at the Center for Celiac Research at the University of Maryland. Of these, 347 participants met the criteria for diagnosis of "Gluten Sensitivity" reflecting about 6% of the patients seen[12]. These gluten triggered symptoms included abdominal pain (68%), eczema and/or rash (40%); headache (35%); "foggy mind" or difficulty focusing (34%); fatigue (33%); diarrhea (33%); depression (22%); anemia (20%); numbness in the legs, arms or fingers (20%); and joint pain (11%)[12]. The proportion of patients with NCGS in this study extrapolated to the general population estimated an enormous prevalence of NCGS compared with that previously seen in prior studies. Based on our review, the estimation of the prevalence of NCGS ranges widely from 0.6% to 6%, a ten-fold difference. As identified, the prevalence of NCGS in the general population may exceed that of CD - now estimated at 1%[5]. However this appears to be a high estimate based solely on symptomatology.

As NCGS is a relatively "new" entity compared to CD, it is also unclear whether or to what extent genetic or environmental risks might predispose individuals to this disorder. Studies have suggested gender contributions with the female to male prevalence ratio between 3:1[13,14] to 5.4:1[15], indicating a female predominance in NCGS. This may however reflect the observation that female patients are more prone to reporting gluten-related symptoms thus leading to referrals for further work-up as seen in a United Kingdom study in 2014[16]. There has also been a heightened interest in the association of NCGS with other medical conditions. An overlap between irritable bowel syndrome (IBS) and NCGS has been suggested since most of the gastrointestinal symptoms in NCGS resemble IBS (similar Rome III criteria), including abdominal pain/discomfort, bloating, diarrhea and constipation[15]. There is also a debate as to whether a GFD can help symptom resolution in IBS after excluding CD, as clinical trials have shown that GFD can reduce symptoms in patients with diarrhea-predominant IBS (IBS-D)[17]. Although recent research has suggested that a low fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) diet, regardless of gluten content, improves symptoms in IBS[18]. Given the close symptomatic resemblance between NCGS and IBS, prevalence estimates may be unclear as patients with NCGS could be mislabeled as IBS.

PATHOGENESIS

The pathobiology of NCGS is poorly understood, hotly debated and under intense research (Figure 1). NCGS potentially involves many triggers as are seen in CD and IBS. The initiating event in NCGS mainly involves exposure of gut epithelium to gluten containing foods leading to immune-mediated and/or non-immune mediated responses. Whereas CD shows increased gut permeability, NCGS shows a decreased gut solute permeability with increased epithelial barrier and elevated expression of the tight junctional component claudin-4[19-21]. Due to the lack of evidence for T-cell involvement and the apparent contribution from toll-like receptors (*e.g.,* TLR-2, TLR-1)[21], NCGS may be more of an innate rather than adaptive immune response. Since specific triggers are not clear in NCGS, this could make NCGS a relatively different pathobiological entity from other gluten related conditions like CD and WA[20,22]. Changes in the gut microbiome (dysbiosis) produced by gluten consumption[23] may also influence NCGS. This predominantly innate TLR based systemic response potentially incited by changes in microbiota was further supported by the increased levels of LBP (lipopolysaccharide binding protein) and soluble CD14 proteins[24]. Additionally there is evidence for NCGS developing in individuals with some genetic predispositions (*e.g.,* HLA-DR4, HLA-DR2)[21], which is higher than general population but lower than patients with CD who have a strong genetic component. However, currently this association does not appear to be as clear[21,22].

Gluten, the main inciting agent in CD also has a significant role in NCGS pathology[25]. In addition to gluten, several other food-derived stimuli have been shown to also be important in the etiopathogenesis of NCGS include alpha amylase/trypsin inhibitors (ATIs)[26], FODMAPS[18] and other short chain fructans. Gliadin is the alcohol soluble fraction of gluten, and contains the bulk of the "immunogenic" components[27]. Gliadin has the ability to promote an inflammatory response primarily in the upper gastrointestinal tract. Gliadin has shown to trigger both innate and adaptive response via recruitment of CD4+ T cells and increasing expression of interleukin-15 by enterocytes[28]. The effects of gliadin-induced enteric responses are not however completely seen in NCGS with innate immune response apparently playing a bigger role than adaptive response[21,29]. The heterogeneous behavior of NCGS in comparison to CD can be explained by different motifs of gliadin protein exerting different responses with alpha gliadin peptides having a significant role in innate adaptive response, and probably expressed more in NCGS individuals[30]. NCGS individuals also have increased levels of TLR2 and to a lesser extent TLR1 expression, increased

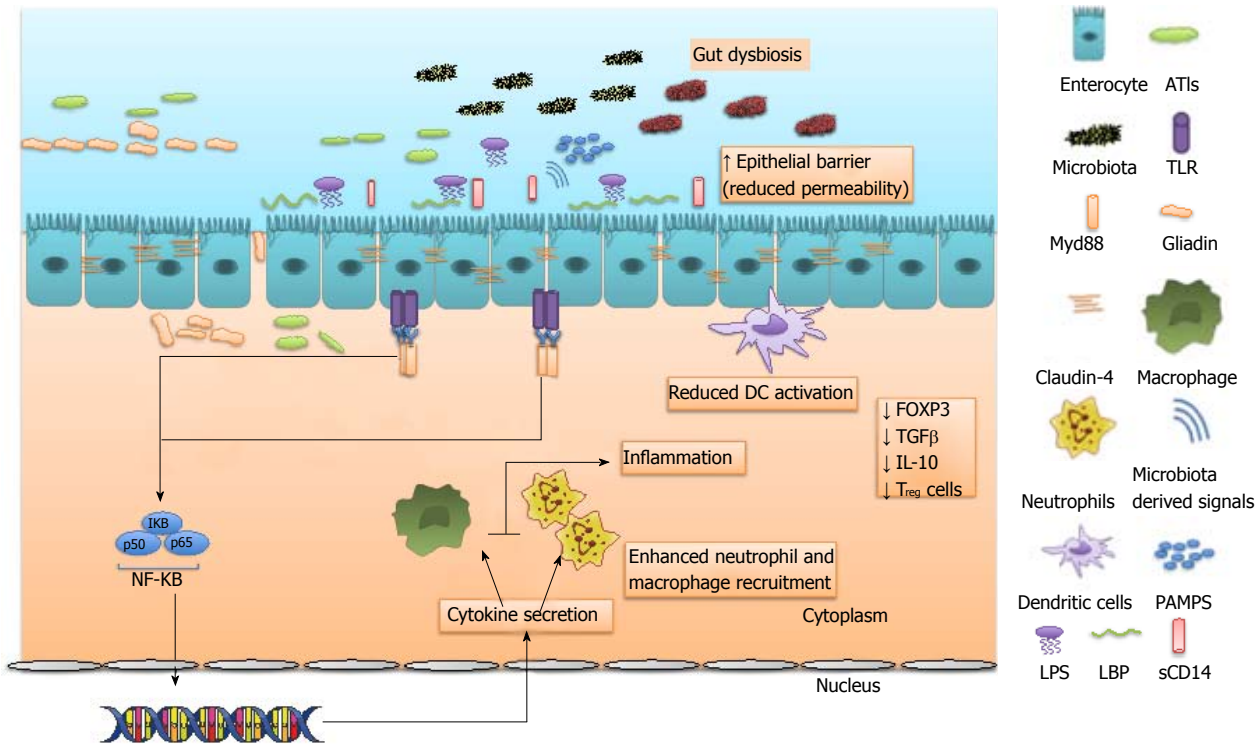


Figure 1 Toll like receptors act as primary sensors to gliadin and non-gluten proteins like alpha amylase/trypsin inhibitors, and microbiota derived signals in non-celiac gluten sensitivity. Dysregulated microbiota also release lipopolysaccharide (LPS) in circulation which result in production of LPS-binding protein (LBP) from gastrointestinal and hepatic epithelial cells and soluble CD14 (sCD14) from monocytes/macrophages. This leads to activation of transcription factor nuclear factor-kappaB (NF-κB), which controls the expression of an array of inflammatory cytokine genes resulting in recruitment of neutrophils and macrophages. In contrast to CD, there is limited dendritic cell activation, resulting in reduced expression of Treg cells, reduced FOXP3, TGFβ, IL-10. All these factors with increased expression of claudin-4 culminate in reduced intestinal permeability and increased epithelial barrier. Gut dysbiosis can also promote inflammation through expansion of pro-inflammatory pathobionts. TLRs: Toll like receptors.

number of α and β intraepithelial lymphocytes, and reduced number of T-regulatory cells[21]. Solute barrier function remains largely unchanged in NCGS, which is consistent with fairly normal adaptive immune response, normal intestinal permeability and expression of tight junction proteins[14,21]. Gluten's opioid like effect on the intestinal transit time and nocebo effect has also been studied and may have an important role on the overall pathology and clinical presentation of the disease[31].

In the recent years, other non-gluten components of wheat have also been shown to play a major role. ATIs in particular, have been implicated in NCGS pathology. The role of ATIs in mounting an immunological response has been shown in animal and human research models and is believed to be an important oral antigen both in CD as well as in NCGS[26]. This predominantly innate immune response involves macrophages, neutrophils and intestinal dendritic cells *via* activation of the toll like receptor complexes[21,26]. Their less tendency to affect the overall gut morphology falls well in line with NCGS pathology.

The gut microbiota has been shown to play a significant role in the pathogenesis of CD, in which gut dysbiosis precedes the activation of inflammatory and immune mediated pathways[32]. The role of gut dysbiosis has also recently been emphasized in NCGS pathobiology and might help to explain both intestinal and non-intestinal manifestations in the disease by upregulating gut and systemic inflammation[33].

CLINICAL PRESENTATION

The symptoms of NCGS can occur within hours to days following exposure to gluten-containing diet and can then dissipate upon withdrawal of gluten. Most of the presentation resembles CD, the main similarities and differences are presented in Table 1. Frequent symptoms reported in NCGS include bloating (87%), abdominal pain (83%), epigastric pain (52%), diarrhea (50%), and constipation (24%)[8]. Extra-intestinal manifestations are also reported which include; lack of well-being (68%), tiredness (64%), headache (54%), anxiety (39%), "foggy mind" or difficulty focusing (38%)[8].

Some of the other less commonly reported symptoms include weight loss, depression and skin rash[8]. Unlike CD, nutrient deficiencies such as iron, vitamin D and vitamin B 12 deficiencies are not significantly seen in NCGS[34]. NCGS also has a lesser association with autoimmune disorders when compared with CD[14,35].

Table 1 Comparison of gluten related disorders[34,51-72]

	NCGS	CD	IBS	WA
Colonic manifestations	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting	Diarrhea Abdominal pain Bloating Constipation Mucous Discharge Dyspepsia Early satiety	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting
Extra-colonic manifestations	Headache Migraine Foggy mind Fatigue Eczema like rash Myositis Numbness Psychological changes	Anemia Osteoporosis Neurological disturbances Pubertal delay Dermatitis herpetiformis Foggy mind Lymphoma	Major depression Anxiety Somatoform disorder Fibromyalgia Temporomandibular disorder Dyspareunia	Hives Angioedema Asthma Cough Post Nasal Drip Eczema
Symptom Onset	Hours to Days	Hours to months	Unclear relation to gluten ingestion	Minutes to Hours
Cytomorphology	Small bowel intraepithelial lymphocytosis (Marsh 0-1)	Villous atrophy with crypt hyperplasia	Normal	Normal
Biomarkers	IgG-AGA Zonulin LBP sCD14	IL-17(A) TCR-γδ IELs IgA tTGA IgA EMA CD3 + IELS Zonulin	TNF- α IL-6 IL-8	IgE antibodies to wheat protein
Immunophenotype	HLA- DQ2 and DQ8 genotypes in 50% patients	HLA- DQ2 and DQ8 genotypes in 80% patients	Increase in: B cells expressing IgG or co-stimulatory molecules CD80 or CD86 T cells expressing b7 + HLADR+ and CD69+	Transforming growth factor-b (TGFb) mutations have been associated with higher rates of allergic disease
Diagnosis	See figure 2	*Serologic testing followed by small bowel biopsy	Rome III diagnostic criteria	Skin prick test Presence of serum IGE antibodies to wheat protein
Management	GFD Probiotics AN-PEP	GFD	Symptomatic treatment and elimination of stressors	GFD Subcutaneous epinephrine for any acute episodes
ICD codes	K90.41	K90.0	K58	K52.29

*4 out of 5 of the following: Typical symptoms of CD, Serum CD IgA class auto antibodies at high titer, HLA-DQ2 and/or HLA-DQ8 genotypes, Celiac enteropathy found on small bowel biopsy, Response to a GFD(14).

DIAGNOSIS

NCGS should be considered in patients with the history and physical examination indicative of a gluten related disorder. The clinical presentation of NCGS, as outlined earlier, should include the remission of symptoms upon withdrawal of gluten. The proposed diagnostic work-up includes 3 vital steps.

The 1st step in an NCGS diagnosis is the exclusion of CD and WA. The patient needs to be on a GCD for a period of 6-wk. Several tests should be performed during this period to exclude WA; wheat specific IgE and skin prick test, and CD; IgA-tTG, IgG-DGP and IgA-EMA. If highly suspicious of CD, the physician can proceed with upper endoscopy for duodenal biopsy, although this should not be routine testing for every patient. If the biopsy results indicate less CD probability (Marsh 0-1) then the clinician can proceed to the next step[5]. Of note, these tests can be tailored by the clinician based on the patients' presenting symptoms. The physician can bypass testing of CD or WA and proceed with the work-up for NCGS if patients' history and physical is not suggestive of the condition.

The 2nd step consists of starting the patient on a GFD for a period of at least 6 wk and monitoring for symptom response. This symptom response is evaluated using the gastrointestinal symptom rating scale (GSRS) and a numerical rating scale (NRS). GSRS is used to identify the symptoms while the NRS is used to quantify the symptoms, weekly from week 0 (baseline) till week 6[36]. A symptomatic response to the GFD is defined as a decrease in 30% of the baseline score with no worsening of other symptoms in at least 50% of the observation time (3 wk)[36]. If the patient fails to show an improvement in symptoms in 6 wk upon resumption of GFD, the diagnosis of NCGS is ruled out and other diagnoses such as IBS and other functional bowel disorders need to be explored.

The 3rd step required to confirm the diagnosis

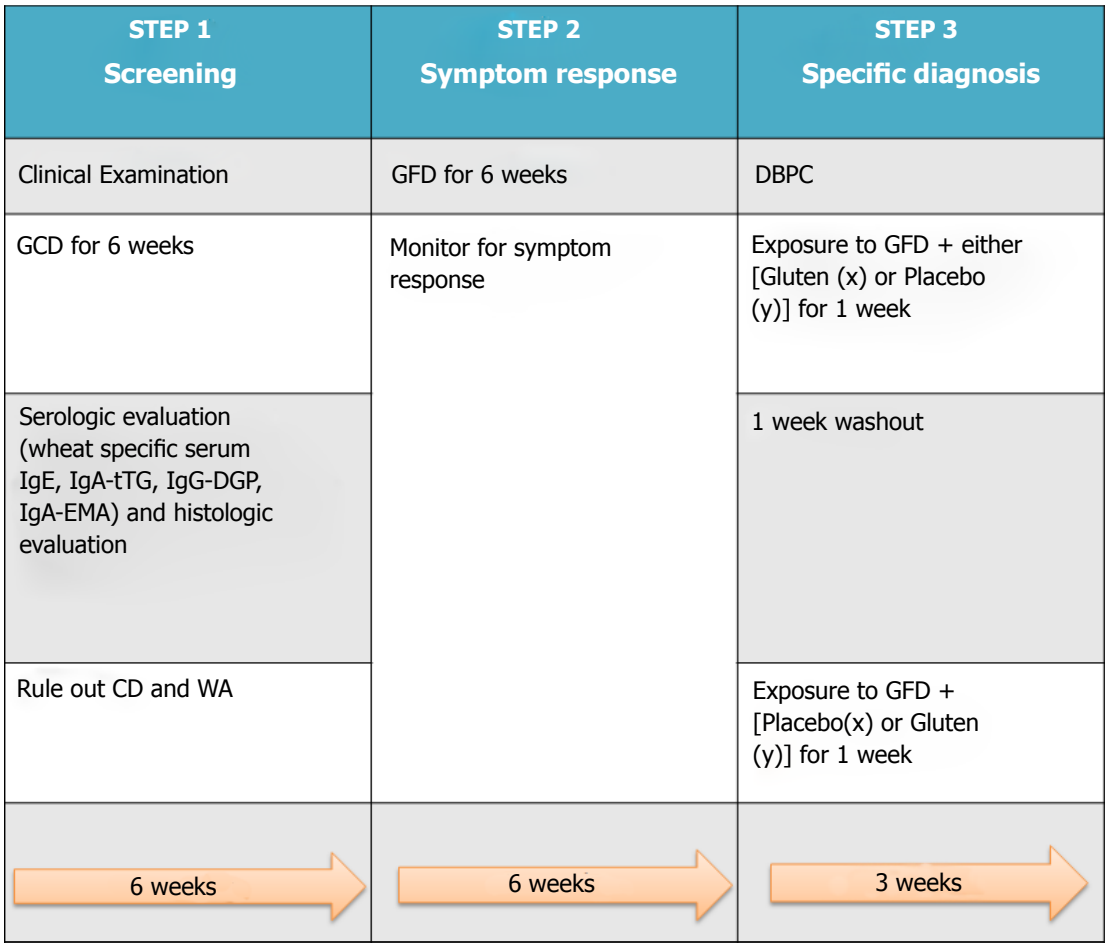


Figure 2 Proposed diagnostic algorithm for non-celiac gluten sensitivity. GCD: Gluten containing diet; GFD: Gluten free diet; CD: Celiac disease.

of NCGS in patients who respond to treatment with the GFD involves the re-introduction of the GCD. This is performed because a potential nocebo effect (test subjects believe there is a side effect and they experience them as a result of this) secondary to the exposure to gluten in step 1 cannot be ruled out[37]. In this step, the patient is randomly assigned into test group x or y. The patient is exposed to either GFD + placebo (x) or GFD + gluten (y) for a week. A 1-week washout period of strict GFD is observed, followed by the crossover to GFD + gluten (x) or GFD + placebo (y)[36]. A variation of a 30% symptomatic improvement on introduction of the diet free from gluten in test group y or a variation of 30% symptomatic appearance on introduction of the GCD in test group x (assessed by the modified GSRS and NRS scales) indicates a positive result. Below this 30% value is considered a negative result[36]. This 30% mark is not a scientific estimate but is rather a fair estimate of an appropriate response[36]. This threshold although used in the Salerno experts' criteria needs scientific validation.

In step 3, the placebo added should be gluten-free and must look very similar to the gluten content so that both the clinician and the patient are unaware of the difference between the gluten and the placebo. The dose of gluten to be used for the challenge should be close to the average daily intake of gluten (10-15 g)[38]. The dose should also be easy to mix with the gluten carrying package. The package carrying gluten can be in any form; bread, muffin. This package should also consist of ATIs as well. The suggested prepared package should contain 10-15 *g* of gluten and at least 0.3 g of ATIs. The package must also be free of FODMAPs[36]. The proposed diagnostic algorithm for NCGS is shown in Figure 2.

MANAGEMENT

Currently, the management of NCGS involves a multi-disciplinary approach. The patient is started on a GFD with the help of a registered dietician[14]. A gluten free diet usually helps resolve the intestinal and extra-intestinal symptoms of NCGS. The recommendation is to continue adherence to GFD for lifetime. There have been few if any studies showing whether or not reintroduction of gluten into the diet after a prolonged period of being asymptomatic will cause reversion into previous symptoms[39].

The importance of gluten has been outlined in recent research. Gluten consumption has been associated with a decreased risk of developing type 2 diabetes Mellitus and also a decreased risk of coronary heart disease[40,41]. Understanding these benefits of gluten beyond nutrition reflects the need for caution in the use of GFDs in patients without a proven diagnosis of NCGS. Although GFD seems to be the most important management strategy, it should be suggested only after careful examination and a definite diagnosis of NCGS.

Several ongoing clinical trials are now examining other possible treatment methods for NCGS besides the GFD. One study looks at the use of probiotics for alleviation of NCGS symptoms while maintaining a gluten free diet with introduction of gluten in a controlled environment. This randomized DBPC study evaluates NCGS patients' response to a gluten source (two slices of bread each day for 7 d) whilst getting probiotics (Bifidobacterium longum ES1 - a patented probiotic bacterial strain) or a placebo during that time[42]. Theoretically the use of probiotics could result in resolving gut dysbiosis by reintroducing gut flora, with reduction in both gut and systemic inflammation but this needs to be clinically examined. Probiotic use has long been a source of debate amongst the medical community regarding its exact role as far as therapeutic option, it's essential to see if it can be proven to have an ultimate benefit.

Another treatment option under investigation is the use of the enzyme Aspergillus niger prolyl endoprotease (AN-PEP). It has been reported in previous studies that the AN-PEP enzyme significantly enhanced gluten digestion in the stomach of healthy volunteers[43]. A randomized placebo controlled clinical trial is ongoing looking at the effect of AN-PEP on gluten degradation in our target population; gluten sensitive individuals[44]. If we can apply AN-PEP as a means of degrading gluten before it can affect the human gut, it would achieve a major goal as patients can enjoy foods without worrying about diet restriction, worrisome symptoms or soaring food costs.

The use of ancient diploid wheat species (*e.g.,* Triticum monococcum ssp.) as compared with common wheat as a new treatment strategy is gaining ground[45]. Gianfrani *et al*[45]. demonstrated the low toxicity of these wheat proteins in celiac disease patients following in vitro gastrointestinal digestion. Newer studies have shown the distinction between these older wheat variants and modern wheat[46]. Older wheat variants were shown to have lower bioactivity and a lower concentration of ATIs in comparison with modern wheat[46]. Mechanistically, the modern wheats were observed to have high levels of TLR-4-activating ATIs which are highly resistant to intestinal proteolysis[46]. The application of these studies to favor the use of ancient wheat variants in the NCGS population would be a major step in the advancement of treatment strategies in this disease.

Mayer and Tillisch highlighted the role of the brain-gut axis in abdominal pain syndromes[47]. Recent literature has attempted to further describe how various clinical manifestations seen in IBS and also syndromes like NCGS could be viewed as a dysregulation in the signaling pathway that involves the gut, the enteric nervous system and the central nervous system[33,47,48]. Newer therapeutic options currently being hypothesized in NCGS include the use of vagus nerve stimulation and corticotropin releasing factor (CRF) antagonists to normalize any possible gut brain dysregulation[33,48]. The use of vagus nerve stimulation is based on its positive effects on many inflammatory diseases of vagus-innervated organs (including the GI tract) which thus suggest a case for its application in NCGS[33]. The use of CRF antagonists (seen to induce significant signal reductions in emotional control centers of the brain[23]) is based on the theory that NCGS often has a chronic anxiety or stress related component associated with the anticipation of abdominal pain. Consequently controlling the emotional impact of NCGS may improve its symptoms. The several on-going studies/ theories in this area hopefully can shed light on the neuropsychiatric therapeutic components of NCGS.

With the release of the updated ICD 10 coding system, NCGS, along with WA, IBS and CD each have their own codes despite lingering ambiguities about NCGS diagnosis/management. NCGS's code is K90.41 and it falls under the categories "enteropathy" (code K63.9) and gluten sensitivity (Code K90.41). Although treatment strategies in NCGS are still evolving, it is important to note that NCGS is a specific and billable diagnosis for reimbursement purposes[49].

CONCLUSION

A recent study involving gastroenterologists showed that although most of these specialist physicians were able to "identify" NCGS and prescribe GFD for its treatment, 44% of them were unable to define or agree on a uniform definition[50]. This highlights the difficulty in making the diagnosis of NCGS based on a specific clinical criterion. NCGS warrants further investigation and updated diagnostic and therapeutic strategies.

As we currently stand, the NCGS diagnostic process is arduous for the patient, requiring them to be on a GCD diet through several parts of the process. This could cause distress by eliciting unpleasant symptoms. Thus, an alternative diagnostic approach without subjecting the patient to the stressor would be a significant clinical advancement. Some biomarkers identified to aid in the diagnosis of NCGS are IgG-AGA[15] and Zonulin[51]. However these markers are evidently increased in CD as well[15,51]. Establishing NCGS specific biomarkers is imminent to also monitor for both the resolution of the disease and compliance

of the patient to the GFD. Whether NCGS is a transient disease or a chronic disease warranting lifetime diet restriction still needs to be investigated.

The lack of information with regards to the prevalence of NCGS in the developing world is outstanding. Most of the studies being done on this condition are focused on the population, which includes the affluent communities of Europe and North America. Adhering to the GFD is expensive and not easily affordable, the likelihood of a low-income patient self-diagnosing as NCGS and going on a GFD is very minimal. This warrants the question as to if NCGS is really a condition more prevalent in the affluent as they are much more conscious of their diet intake as well as informed.

New and evolving science around gluten related disorders has led to better understanding of the disease process, making it easier for the clinicians to set specific management guidelines. Prospective clinical trials in a diverse population is warranted to better investigate the etiology and progression of this disease. There is still a role of further research to better understand NCGS as a medical condition.

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ORIGINAL ARTICLE

Basic Study

Glucagon-like peptide-2 modulates the nitrergic neurotransmission in strips from the mouse gastric fundus

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Abstract

AIM

To investigate whether glucagon-like peptide-2 (GLP-2) influences the neurally-induced responses in gastric strips from mice, since no data are available.

METHODS

For functional experiments, gastric fundal strips were mounted in organ baths containing Krebs-Henseleit solution. Mechanical responses were recorded *via* force-displacement transducers, which were coupled to a polygraph for continuous recording of isometric tension. Electrical field stimulation (EFS) was applied via two platinum wire rings through which the preparation

was threaded. The effects of GLP-2 (2 and 20 nmol/L) were evaluated on the neurally-induced contractile and relaxant responses elicited by EFS. Neuronal nitric oxide synthase (nNOS) enzyme was evaluated by immunohistochemistry.

RESULTS

In the functional experiments, electrical field stimulation (EFS, 4-16 Hz) induced tetrodotoxin (TTX)-sensitive contractile responses, which were reduced in amplitude by GLP-2 ($P < 0.05$). In the presence of the nitric oxide (NO) synthesis inhibitor L-NNA, GLP-2 no longer influenced the neurally-evoked contractile responses ($P > 0.05$). The direct smooth muscle response to methacholine was not influenced by GLP-2 ($P > 0.05$). In the presence of guanethidine and carbachol, the addition of GLP-2 to the bath medium evoked TTX-sensitive relaxant responses that were unaffected by L-NNA ($P > 0.05$). EFS induced a fast NO-mediated relaxation, whose amplitude was enhanced in the presence of the hormone ($P < 0.05$). Immunohistochemical experiments showed a significant increase ($P < 0.05$) in nNOS immunoreactivity in the nerve structures after GLP-2 exposure.

CONCLUSION

The results demonstrate that in gastric fundal strips, GLP-2 influences the amplitude of neurally-induced responses through the modulation of the nitrergic neurotransmission and increases nNOS expression.

Key words: Immunohistochemistry; Gastric motility; Glucagon-like peptide-2; Neuronal nitric oxide synthase; Non-adrenergic non-cholinergic neurotransmission

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Core tip: The results of the present study demonstrate for the first time that, in strips from the mouse gastric fundus, glucagon-like peptide-2 (GLP-2) depresses the amplitude of the neurally-induced contractile responses and enhances the amplitude of the relaxant ones through the modulation of the nitrergic neurotransmission. GLP-2 also increases neuronal nitric oxide synthase immunoreactivity in the nerve structures. All these inhibitory effects might contribute to gastric relaxation, thus increasing the organ capacity. Since gastric distension represents a peripheral satiety signal from a physiological point of view, it could be speculated that the relaxant effects of GLP-2 might concur to suppress feeding behavior in rodents.

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INTRODUCTION

Glucagon-like peptide-2 (GLP-2), produced by enteroendocrine "L" cells[1,2], plays a role in the regulation of metabolism and energy homeostasis[3,4], and has been reported to influence many gastrointestinal functions[5,6]. GLP-2 acts on G protein-coupled receptors (GLP-2r)[7-9] largely distributed in the gastrointestinal tract[2,10]. In the gut, GLP-2r has been revealed in several cell types, including excitatory and inhibitory neurons[10-13]. In this view, GLP-2 has been demonstrated to exert a neuromodulatory action either by inhibiting intestinal transit *in vivo*[14] *or* by reducing colonic and duodenal cholinergic muscle contractions *in vitro*[10,11].

Concerning gastric motility, contrasting results have been reported in humans regarding the ability of GLP-2 to slow emptying[15-17]. *In vivo* experiments in pigs have shown that the hormone reduced antral motility through central nervous system mechanisms[18]. In addition to the central actions, experiments carried out *in vitro* have demonstrated that GLP-2 exerts peripheral effects on either gastric strips or isolated whole stomach from mice[19]. Particularly, the hormone has been shown to relax gastric smooth muscle acting indirectly through the stimulation of vasoactive intestinal polypeptide (VIP) release from myenteric neurons with site-related effects, exerting its action in the fundal region only[19]. However, to our knowledge, no data have been reported about the influence of GLP-2 on the neurally-induced gastric responses elicited by electrical stimulation in *in vitro* preparations. For the above reason, we presently investigated whether GLP-2 influences the neurally-induced contractile and relaxant responses in gastric fundal strips from mice. Along with VIP[20], nitric oxide (NO)[21] is considered one of the major non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitters responsible for the proximal stomach relaxation; thus, we also evaluated the effects of GLP-2 on both the nitrergic neurotransmission and the neuronal nitric oxide (nNOS) expression.

MATERIALS AND METHODS

Animals

Experiments were performed on 8- to 12-week-old female mice (CD1 Swiss strain; Envigo, Udine, Italy). The animals were kept under the following conditions: 12-h light/12-h dark photoperiod, constant temperature (21 ± 1 ℃), and standard laboratory feed. The mice were killed by prompt cervical dislocation to minimize animal suffering.

Mechanical experiments

As formerly reported[22-25], the stomach was promptly removed, and two full-thickness longitudinal strips (2 mm × 10 mm) were cut from each gastric fundal region. One end of each strip was tied to a platinum

rod, while the other was connected to a force displacement transducer (Grass model FT03, Quincy, MA, United States) by a silk thread for continuous recording of isometric tension. The transducer was coupled to a polygraph (Grass model 7K, Quincy, MA, United States). Preparations were mounted in double-jacketed organ baths (5 mL) containing Krebs-Henseleit solution, gassed with a 95% O_2-5% CO_2 mixture, of the following composition (mmol/L): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 10 (pH 7.4). The temperature of the Krebs-Henseleit solution in the organ baths was maintained at 37 ± 0.5 ℃.

Electrical field stimulation (EFS) was applied by means of two platinum wire rings (2 mm diameter, 5 mm apart) through which the strip was threaded. Electrical pulses (rectangular waves, 80 V, 4-16 Hz, 0.5 ms, for 15 s) were delivered by a Grass model S8 stimulator. Preparations were allowed to equilibrate for 1 h under an initial load of 0.8 g: during this period, prolonged washes with Krebs-Henseleit solution were performed to avoid the accumulation of metabolites in the organ baths.

Experimental protocol

The influence of GLP-2 (2 nmol/L and 20 nmol/L) on either neurally-induced or direct smooth muscle contractile responses was firstly investigated. EFS (4-16Hz) was also performed in the presence of 1 μmol/L tetrodotoxin (TTX) or 200 μmol/L N^G-nitro-L-arginine (L-NNA). Elapsed time between two subsequent electrical stimulations depended on the time taken by the strip tension to regain baseline. The interval between two subsequent direct muscular responses to methacholine (2 μmol/L) was at least 30 min. During this period prolonged washes with Krebs-Henseleit solution were performed. To verify the viability of the strips, contractions to methacholine (2 μmol/L) were evaluated at the beginning and at the end of each experiment.

In a second series of experiments, the effects of GLP-2 (2 nmol/L and 20 nmol/L) on the EFS-induced NANC relaxant responses were investigated. For this purpose, functional experiments were carried out in the presence of 1 μmol/L guanethidine sulphate and 1 μmol/L carbachol (CCh) to rule out adrenergic and cholinergic influences, respectively. Elapsed time between two subsequent additions of CCh was at least 15 min, during which prolonged washes with Krebs-Henseleit solution were performed. When a stable plateau phase of the contraction evoked by CCh was reached, EFS or drugs were applied. The response to GLP-2 (2 nmol/L and 20 nmol/L) was also tested 20 min following the addition of TTX or the NO synthesis inhibitor L-NNA to the bath medium. Immunohistochemical experiments were performed in parallel.

Tissue sampling for morphological studies

Two gastric fundus specimens per animal (6 mice) were taken, which were prepared as reported above and handled as follows. The specimens were stabilized in 5 mL organ baths containing Krebs-Henseleit solution; at the end of the steadying period, one half of the specimens was exposed to GLP-2 for 30 min, which was added directly to the bath reaching 20 nmol/L concentration. The second half was maintained in Krebs solution and used as a control. At the end of the exposure time, the specimens were transferred into a solution of paraformaldehyde (4%) dissolved in phosphate-buffered saline (0.1 mol/L, PBS, pH 7.4), maintained overnight (ON) at 4 ℃. The day after, the specimens were dehydrated, cleared in xylene and embedded in paraffin. The rotary microtome (MR2, Boeckeler Instruments Inc. Tucson, Arizona, United States) allowed to cut 5 μm thick sections, which were collected on slides suitable to histological and immunofluorescent staining.

Histological and immunofluorescent staining

The sections were deparaffinized through consecutive passages in xylene and in decreasing ethanol concentration solutions up to the final step in distilled water. Some sections were H&E stained to visualize the integrity of the muscle wall. Some others underwent the immunofluorescent procedure. For antigen retrieval the sections were transferred for 20 min in a EDTA 1 mmol/L, pH 9.0 + tris buffer 10 mmol/L, maintained at the temperature of 90-92 ℃; then they were allowed to cool in the same solution. After few washes in 0.1 mol/L PBS, the section were first incubated for 20 min with bovine serum albumin (1.5%, Sigma Aldrich, Milan, Italy) diluted in PBS and then ON at 4 ℃ in the presence of the nNOS antibody (rabbit polyclonal; 1:1500; Chemicon, Temecula, CA, United States). After several washes, the appropriate fluorochrome-conjugated secondary antibody (goat anti-rabbit, AlexaFluor 488; 1:333; Jackson ImmunoResearch, West Grove, PA, United States) diluted in PBS were applied for 2 h at RT. Finally, the sections were counterstained with Bisbenzimide Hoechst Trihydrochloride (BHT; Sigma Aldrich srl, Milan, Italy) to visualize the cell nuclei, and set in the aqueous medium (Immu-Mount, Thermo Scientific, Waltham, MA, United States). To verify the specificity of the primary antibody, the procedure has been performed omitting the antibody. An epifluorescence microscope (Zeiss Axioskop microscope, Oberkochen, Germany) allowed to visualize the fluorescent labelling. A digital camera (Leica DFC310 FX 1.4-megapixel camera, Leica Microsystems, Mannheim, Germany) coupled to an image acquisition software (LAS V3.8, Leica Microsystems, Germany) allowed to capture and archive the related images.

Quantitative analysis

The quantitative analysis of the labelled structures was done acquiring digitized images for the whole length of the section (1 section/animal; 6 animals/group) using 20 x objective and the acquisition of overlapping images was avoided. The total number of images obtained was comparable between control and GLP-2 treated sections as confirmed by the similar length of the fundal specimens. ImageJ (NIH, Bethesda, MD, United States) was used to analyze the fluorescent labeling in the acquired images. The area of the labelled structures in the muscle wall was quantified and expressed as fraction of the total image area x 100. The labelling was converted to a binary image; the threshold value was set in control images and maintained in treated ones. Two researchers counted in blind the myenteric labelled neurons in each section (1 section/mouse; 6 mice/group) and the result was proposed as neuron number per mm.

Drugs

The following drugs were used: guanethidine sulphate, CCh, GLP-2, methacholine, L-NNA, TTX. All of the above drugs were purchased from Sigma Chemical (St. Louis, MO, United States) with the exception of GLP-2 that was obtained from Tocris Bioscience (Bristol, United Kingdom). Solutions were freshly prepared, except for TTX, for which a stock solution was stored at -20 ℃. Drug concentrations are referred as final bath concentrations and are in the range of those previously reported to be effective in *in vitro* gastric preparations[26-28]. Particularly, the chosen doses of GLP-2 employed are those reported able to cause gastric relaxation in mice[19].

Data analysis and statistical tests

Amplitude of contractions is given as percentage of the muscular contraction elicited by methacholine (2 μmol/L) taken as 100% or as absolute values (g). Amplitude of responses to methacholine was measured 30 s after a stable plateau phase was reached. Relaxant responses are calculated as percentage decrease relative to the muscular tension induced by CCh (1 μmol/L) just before obtaining relaxations. With respect to pre-stimulus level, amplitude values of EFS-induced fast relaxations refer to the maximal peak obtained during the stimulation period and amplitude values of EFS-induced sustained relaxations refer to the maximal peak, obtained following the stimulation period. The statistical significance was evaluated by paired or unpaired Student *t*-test to compare two experimental groups or one-way ANOVA followed by Newman-Keuls posttest when more than two groups were compared (Prism 3.0; GraphPad Software, San Diego, CA, United States). Differences were considered significant when $P \leq 0.05$. Results are mean ± SE. The number of muscle strip preparations is indicated by *n* in the results.

RESULTS

Functional experiments

At basal tension ($n = 28$), EFS (4-16 Hz) induced contractile responses whose amplitude increased by increasing the stimulation frequency (Figure 1A and B). These responses were abolished by 1 μmol/L TTX ($n = 4$) indicating their nervous nature. The addition of GLP-2 (2 nmol/L or 20 nmol/L) to the bath medium ($n = 16$), other than causing a long-lasting decay of the basal tension (0.15 ± 0.02 and 0.20 ± 0.03 g at 2 nmol/L and 20 nmol/L, respectively), decreased the amplitude of the excitatory responses elicited by EFS (Figure 1A and B).

In the presence of the NO synthesis inhibitor L-NNA (200 μmol/L) the amplitude of the EFS-induced contractions was enhanced (Figure 2A and B). The effects of L-NNA, already appreciable 10 min after its addition to the bath medium, persisted up to 1 h (longer time not observed). In the presence of L-NNA (200 μmol/L), GLP-2 (2 nmol/L or 20 nmol/L) still evoked the decay of the basal tension (Figure 2A and B) but no longer depressed the amplitude of the EFS-induced contractile responses even at the highest concentration employed (Figure 2A and B).

Methacholine (2 μmol/L) caused a sustained contraction that was TTX-insensitive and reached a plateau phase (mean amplitude 0.98 ± 0.3 g) that persisted until washout. GLP-2 (2 nmol/L or 20 nmol/L) did not influence the response to methacholine (mean amplitude 1.02 ± 0.2 g, $P > 0.05$). No significant differences ($P > 0.05$) in amplitude of the contractile response to methacholine (2 μmol/L) were observed between that obtained at the end or at the beginning of the experiments, thus indicating that the viability of the preparations was not compromised.

The effects of GLP-2 were also tested on the neurally-induced relaxant responses. In the presence of guanethidine, addition of CCh (1 μmol/L) to the bath medium ($n = 28$) caused a rapidly arising contraction (mean amplitude 1.2 ± 0.2 g), which persisted until washout. In CCh precontracted strips, GLP-2 (2 nmol/L or 20 nmol/L) elicited ($n = 12$) dose-dependent slow and long-lasting relaxations (Figure 3) that were abolished by 1 μmol/L TTX ($n = 4$; data not shown) and unaffected ($P > 0.05$) by L-NNA (200 μmol/L) ($n = 4$) (mean amplitude 27 ± 1.5% *vs* 25 ± 1.9% and 47 ± 1.7% *vs* 50 ± 1.6% at 2 nmol/L and 20 nmol/L, respectively).

In CCh precontracted strips, EFS (4-16 Hz) elicited ($n = 16$) a fast relaxant response followed, at the higher stimulation frequencies, by a slow relaxation (Figure 4A). As previously observed in the mouse gastric fundus[23], the fast component of the response was abolished by 200 μmol/L L-NNA ($n = 4$), indicating its nitrergic nature. In the presence of GLP-2 (2 nmol/L or 20 nmol/L), the amplitude of the EFS-induced fast nitrergic relaxation was increased ($P < 0.05$; Figure

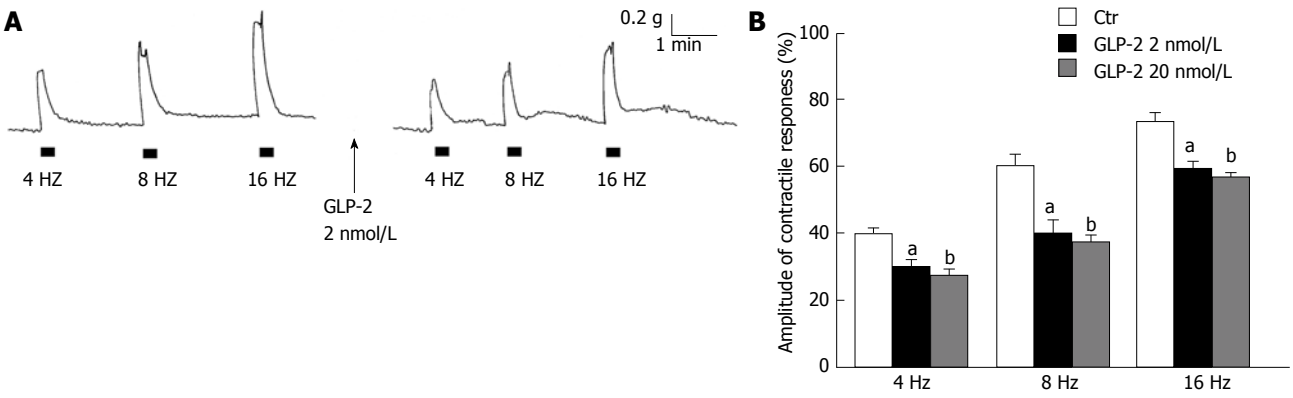


Figure 1 Influence of glucagon-like peptide-2 on the neurally-induced contractile responses. A: Typical tracing showing the electrical field stimulation (EFS)-induced contractile responses at different stimulation frequencies (left panel). Ten min after the addition of 2 nmol/L glucagon-like peptide-2 (GLP-2) to the bath medium (right panel), the amplitude of the EFS-induced excitatory responses is decreased in the whole range of stimulation frequencies employed; B: Bar chart of the effects of GLP-2 (2 and 20 nmol/L) on the mean amplitude of the EFS-induced contractile responses elicited at different stimulation frequencies. Excitatory responses are expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, taken as 100%. All values are mean ± SE of six preparations. [a]*P* < 0.05 *vs* the ctr [b]*P* < 0.05 and *P* > 0.05 *vs* the ctr and *vs* 2 nmol/L GLP-2, respectively, ANOVA and Newman-Keuls post-test).

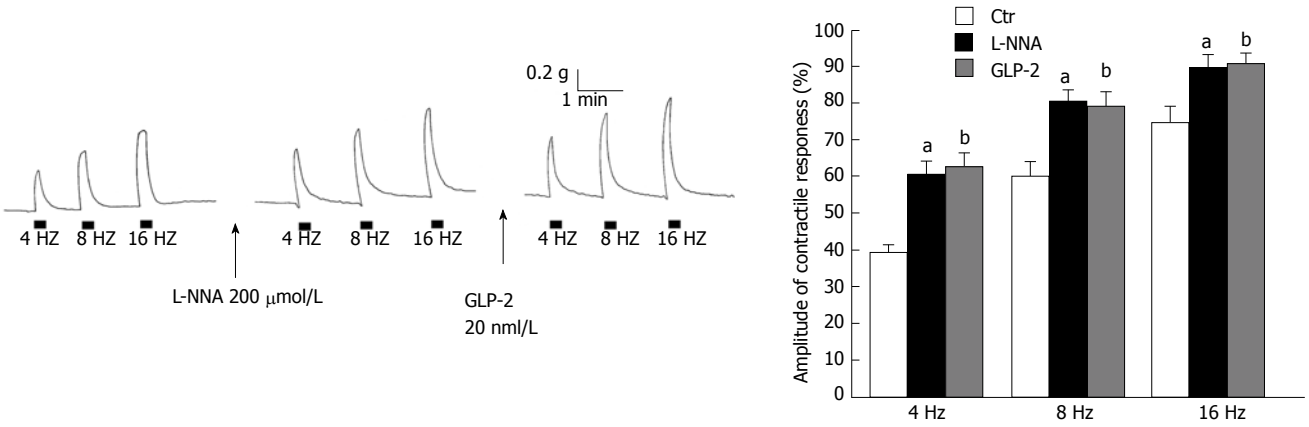


Figure 2 Lack of effects of glucagon-like peptide-2 on the neurally-induced contractile responses in the presence of the nitric oxide synthesis inhibitor L-NNA. A: Typical tracing showing the electrical field stimulation (EFS)-induced contractile responses at different stimulation frequencies (left panel). Following the addition of L-NNA (200 μmol/L) to the bath medium, the amplitude of the EFS-induced excitatory responses is enhanced (middle panel). In the presence of L-NNA, glucagon-like peptide-2 (GLP-2, 20 nmol/L) no longer depresses the amplitude of the neurally-induced contractile responses (right panel); B: Bar chart of the effects of GLP-2 (20 nmol/L) on the mean amplitude of the EFS-induced contractile responses in the presence of L-NNA (200 μmol/L). Excitatory responses are expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, taken as 100%. All values are mean ± SE of six preparations. [a]*P* < 0.05 *vs* the ctr [b]*P* < 0.05 and *P* > 0.05 *vs* the ctr and *vs* GLP-2, respectively (ANOVA and Newman-Keuls post-test).

4A and B), whereas that of the slow relaxation was reduced (6.1 ± 0.6% *vs* 12.75 ± 2.3% and 19.68 ± 1.6% *vs* 30.46 ± 2.2%, at 8 and 16 Hz, respectively; *P* < 0.05) (Figure 4A) or even abolished at the highest dose employed.

Morphological experiments

Anatomical and histological evaluation: At the end of the tissue sampling procedure, the control and GLP-2 treated specimens appeared anatomically intact and the H&E staining confirmed the integrity of the muscle wall (Figure 5).

Immunofluorescence: In the gastric fundus of both control (Figure 6A-C) and GLP-2 (20 nmol/L) treated specimens (Figure 6D-F), the nNOS-immunoreactivity (IR) was observed in the soma of the myenteric neurons and in the fibers located in the longitudinal and circular muscle layers. No IR was detected when the primary antibody was omitted (Figure 6G-H). Quantitation of the IR showed a statistically significant increase in nNOS positive neuron number and nerve fibers (Figure 6I). The length of gastric fundal strips subjected to quantitative analysis was not different between control and GLP-2 treated specimens (4.17 ± 0.60 mm controls *vs* 4.07 ± 0.48 mm GLP-2 treated).

DISCUSSION

The present study demonstrates for the first time that GLP-2 influences the neurally-induced contractile and relaxant responses in mouse fundal strips through a modulatory action on nitrergic neurotransmission and increases nNOS expression. Particularly, the functional

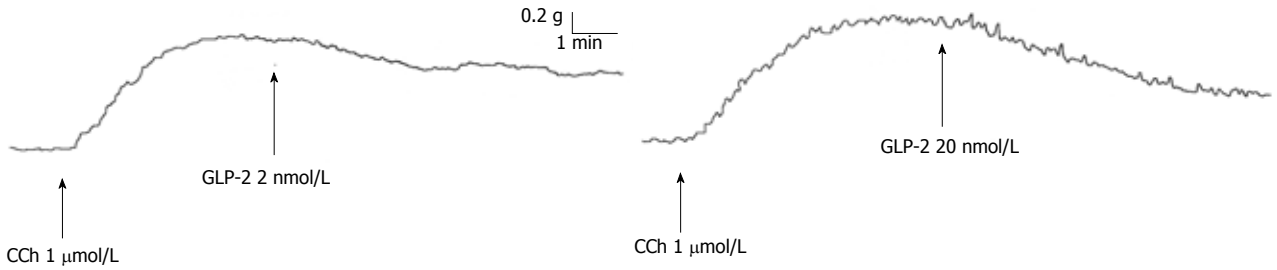
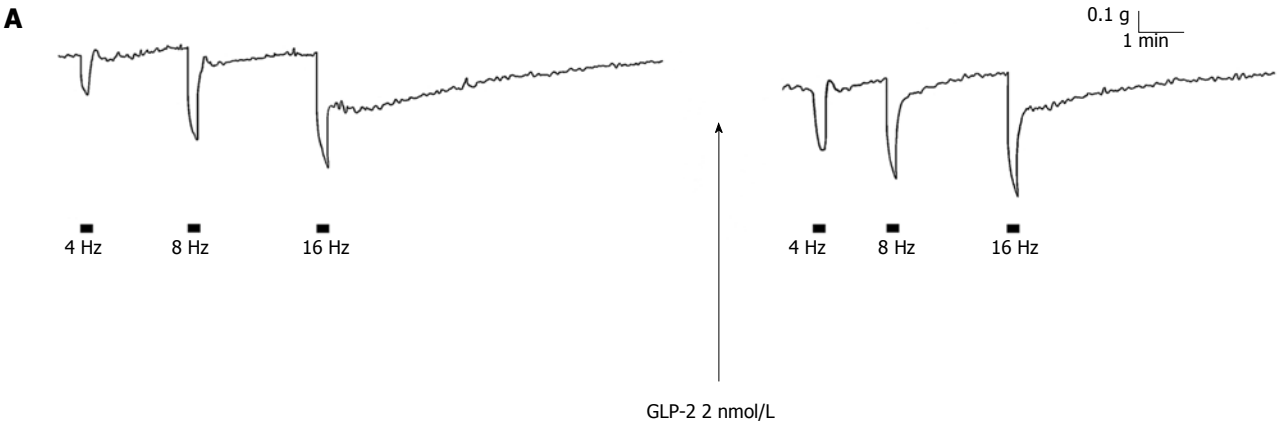


Figure 3 Effects of glucagon-like peptide-2 on gastric strips precontracted with carbachol. Typical tracings showing the effect of the addition of glucagon-like peptide-2 (GLP-2, 2 and 20 nmol/L) to the bath medium. GLP-2 causes a dose-dependent slow and long-lasting relaxation. CCh: Carbachol .



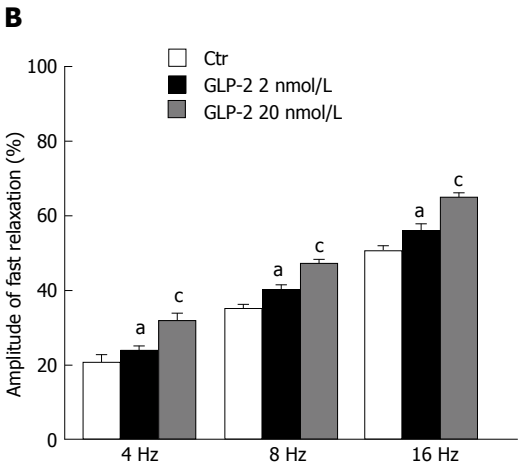


Figure 4 Influence of glucagon-like peptide-2 on the neurally-induced relaxant responses. A: Typical tracing showing the electrical field stimulation (EFS)-induced relaxant responses at different stimulation frequencies (left panel). EFS evokes fast relaxant responses, followed by a slow component at the higher stimulation frequencies (≥ 8 Hz). GLP-2 at 2 nmol/L causes (right panel) an increase in amplitude of the EFS-induced fast relaxation in whole range of stimulation frequencies employed and a decrease of the slow one; B: Bar chart of the effects of GLP-2 (glucagon-like peptide-2, 2 and 20 nmol/L) on the mean amplitude of the EFS-induced fast relaxation. Note that GLP-2 increases the amplitude of the EFS-induced fast relaxant responses in a dose-related manner. Relaxant responses are expressed as percentage decrease relative to the muscular tension induced by 1 μmol/L CCh taken as 100%. Amplitude values of EFS-induced fast relaxations refer to the maximal peak obtained during the stimulation period. All values are mean ± SE of six preparations. $^{a}P < 0.05$ *vs* the ctr; $^{c}P < 0.05$ *vs* the ctr and *vs* 2 nmol/L GLP-2 (ANOVA and Newman-Keuls post-test).

experiments show that GLP-2 is able to depress the amplitude of the EFS-induced contractile responses at each stimulation frequency applied.

It is known that gastric motor responses are a balance between nervous excitatory (mainly cholinergic) and inhibitory (NANC) influences exerted on the smooth muscle. Since during EFS both excitatory and inhibitory nervous fibers are simultaneously activated, the reduction in amplitude of the neurally-induced contractions caused by GLP-2 might be ascribable either to a minor activation of the excitatory component or to a major nervous inhibitory influence exerted on the smooth muscle. In this view, the increase in amplitude of the EFS-induced contractile responses by the NO synthesis inhibitor L-NNA indicates the removal of a nitrergic inhibitory nervous influence. Notably, in

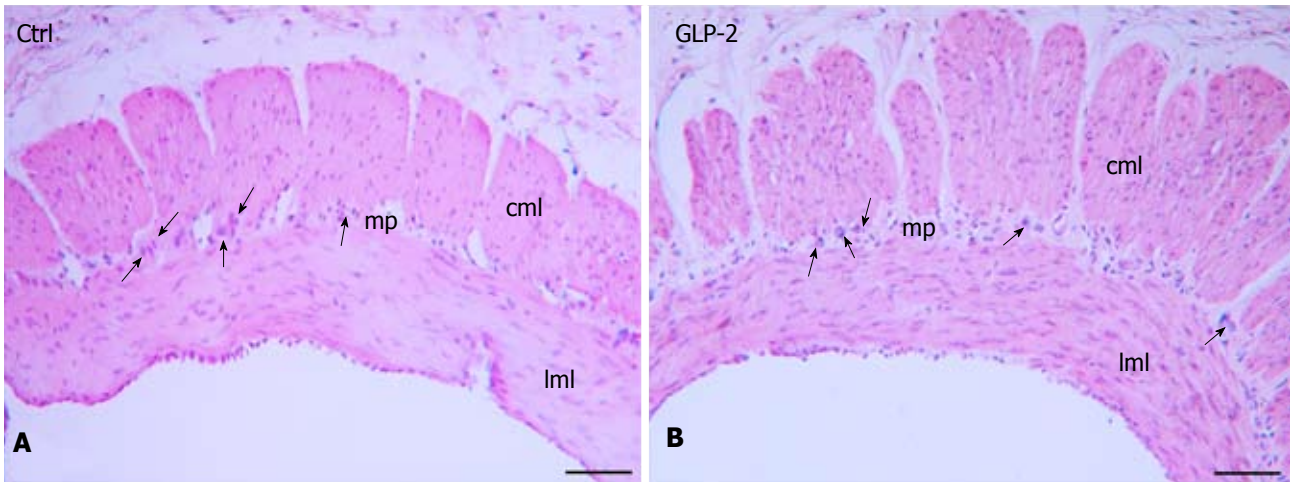


Figure 5 H&E staining of gastric fundus strip. The longitudinal and circular muscle layers (lml, cml) and the myenteric plexus (mp) are shown both in control (A) and in GLP-2 treated (B) specimens. The arrows indicated some myenteric neurons. Bar: A, B = 10 μm.

the presence of L-NNA, GLP-2 no longer depressed the amplitude of the neurally-induced excitatory responses. These data demonstrate that the hormone effects on the EFS-induced contractions occur through the modulation of the nitrergic neurotransmission and, in addition, exclude its inhibitory action on the cholinergic neurotransmission.

The lack of effects of GLP-2 on the direct smooth muscle responses elicited by methacholine further supports its neuromodulatory action. This observation also proves that the depressant actions of GLP-2 on the EFS-induced contractile responses are not ascribable to aspecific effects such as the decay of the basal tension. Moreover, the similar amplitude of the contraction to methacholine at the beginning and at the end of the experiments demonstrates that muscle responsiveness is not compromised.

In keeping with the above observations, the results of the experiments performed in the presence of guanethidine and CCh demonstrate, for the first time, that the hormone influences the amplitude of the neurally-induced relaxant responses in gastric fundal strips through a modulatory action on the nitrergic neurotransmission. As previously observed in strips from the mouse gastric fundus in the presence of guanethidine and CCh[23,26,28], EFS induced a fast NO-mediated relaxation, followed by a slow and sustained relaxant response at the highest stimulation frequencies. In the present experiments, the observation that GLP-2 increased the amplitude of the EFS-induced fast nitrergic relaxation in the whole range of stimulation frequencies in a dose-related manner further supports the involvement of the NO pathway in the hormone effects. To ascertain whether the effects of GLP-2 on both the neurally-induced contractile and relaxant responses were ascribable to an increased NO production, nNOS expression was evaluated in gastric preparations by immunohistochemistry. The results show that GLP-2 induces an increase in the nNOS labeling in the nerve structures of the gastric fundus muscle coat. Accordingly, an increased NO production in neurons induced by exogenous GLP-2 has been suggested to occur in the mouse duodenum in which the hormone inhibited the spontaneous mechanical activity[11]. In the present experiments, the increased nNOS expression in the nerve structures of the muscle wall fits well with the progressive decrease in amplitude of the EFS-induced contractile responses as well as with the increase of the fast relaxant responses caused by GLP-2. Thus, these hormone effects occur only during EFS, *i.e.*, when the nitrergic pathway is engaged, also explaining the ineffectiveness of L-NNA to influence the relaxant response induced by the addition of GLP-2 to bath medium. Indeed, gastric relaxation to GLP-2 in mice has been reported to occur through the release of VIP from myenteric neurons[19]. In this view, immunohistochemical studies in mouse, human and porcine intestine reported the presence of the GLP-2 receptor in the enteric neurons[10-12,29] and showed that some of them co-localized with VIP[11,12]. We previously observed[26] that, in strips from the mouse gastric fundus, the EFS-induced slow relaxation was abolished following VIP receptors desensitization, suggesting the involvement of the peptide in this kind of response. On this basis, it could be speculated that the reduction/abolition of the neurally-induced slow relaxation by GLP-2 observed in the present experiments occurred because VIP, released earlier from the nerve terminal by the hormone, has already occupied the muscular receptors engaged in the EFS-induced vipergic relaxant response. However, even if the validation of this hypothesis was beyond the aim of the present work, the observation that GLP-2 influences in an opposite manner the two components of the neurally-induced relaxant responses excludes an aspecific effect.

The present results demonstrate that the hormone depresses the amplitude of the neurally-induced

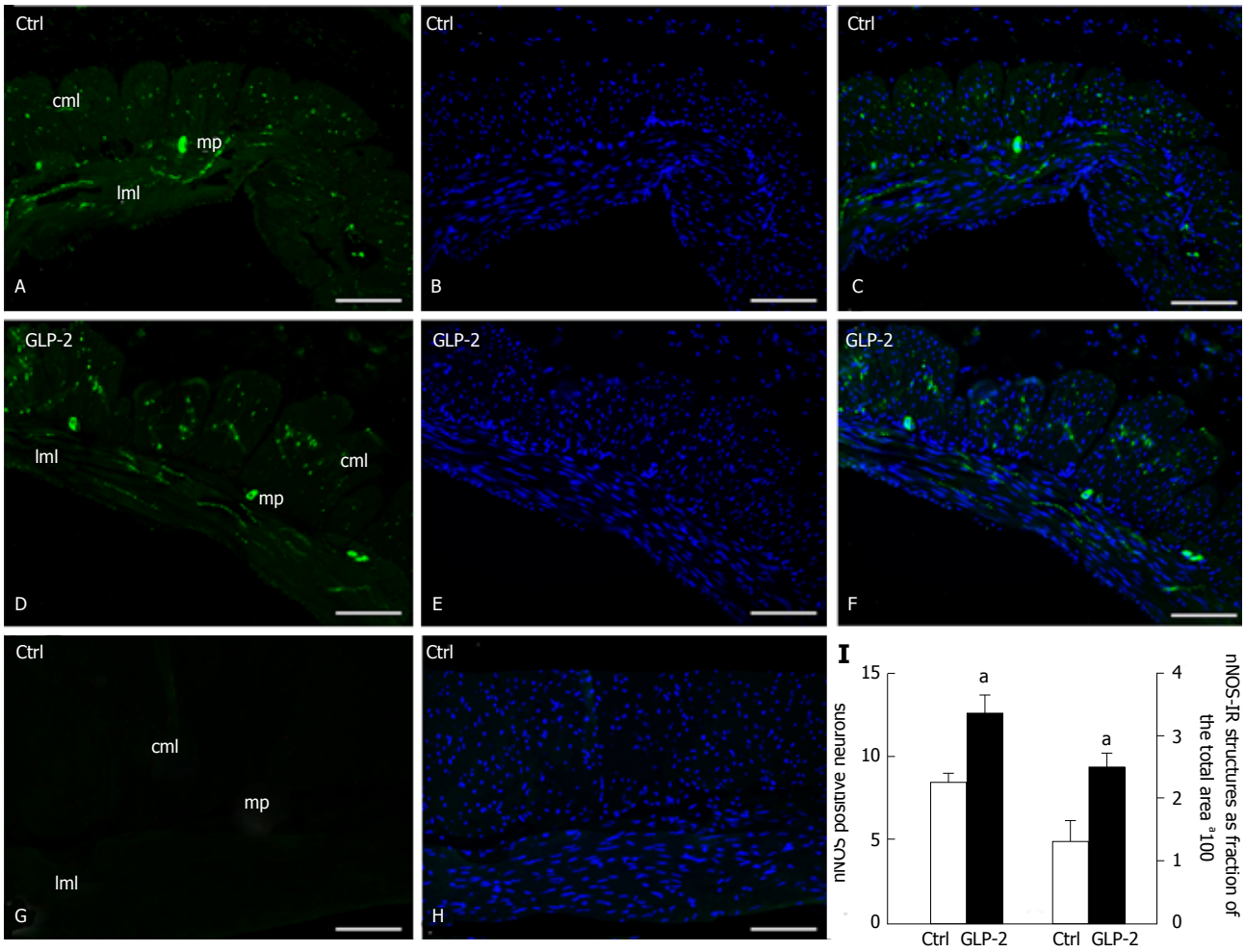


Figure 6 Neuronal nitric oxide synthase-immunoreactivity in control and glucagon-like peptide-2 treated specimens. In control (A) and glucagon-like peptide-2 (GLP-2) treated (D) specimens, neuronal nitric oxide synthase-immunoreactivity (nNOS-IR) (green) is present in the soma of some myenteric neurons and in several nerve fibers. DAPI fluorescent staining for DNA (blue) identified the nuclei of both smooth muscle and neural cells (B-E). In C and F, merged images of nNOS and Bisbenzimide Hoechst Trihydrochloride (BHT) fluorescent staining are shown. When the primary antibody is omitted (G), only the BHT labelling is observed (H). The quantitative analysis showed a significant increase (I) in nNOS-IR myenteric neurons (left side) and in IR fibers in the entire muscle coat (right side). $^{a}P < 0.05$ *vs* controls (unpaired Student's t-test). mp: Myenteric plexus; cml: Circular muscle layer; lml: Longitudinal muscle layer. Bar: A-H = 10 μm.

contractile responses, enhances the amplitude of the NO-mediated fast relaxant responses and increases the nNOS expression in the nerve fibers. All of these effects of GLP-2 might contribute to gastric relaxation, thus increasing the organ capacity. Since gastric distension represents a peripheral satiety signal[30] from a physiological point of view, it could be hypothesized that the relaxant effects of GLP-2 might contribute to suppress feeding behavior in rodents[31,32].

In conclusion, this study shows for the first time that GLP-2 influences the neurally-induced responses in gastric strips from mice through a modulatory action on the nitrergic neurotransmission and increases nNOS expression.

COMMENTS

Background

Glucagon-like peptide-2 (GLP-2) has been shown to relax gastric smooth muscle acting on myenteric neurons. However, to our knowledge, no data are present in the literature on the effects of GLP-2 on the neurally-induced gastric responses elicited by electrical stimulation in *in vitro* preparations. For the above reason, they presently investigated whether GLP-2 influences the neurally-induced contractile and relaxant responses in strips from the mouse gastric fundus. Moreover, since nitric oxide (NO) is considered one of the major NANC inhibitory neurotransmitters responsible for the proximal stomach relaxation, they also evaluated the effects of GLP-2 on both the nitrergic neurotransmission and the neuronal nitric oxide (nNOS) expression.

Research frontiers

Previous studies have indicated that GLP-2 might contribute to suppress feeding behavior in rodents. Thus, the relaxant effects of GLP-2 on gastric preparations, leading to organ distension, might represent peripheral satiety signals.

Innovations and breakthroughs

This is the first study showing that GLP-2 is able to influence the neurally-induced responses in gastric strips from mice through a modulatory action on nitrergic neurotransmission and to increase nNOS expression.

Applications

The evidence that GLP-2 is able to induce gastric relaxation, through the modulation of the nitrergic neurotransmission, contributes to the knowledge of

its peripheral effects. The results highlight an additional site of action that might be implicated in the hormone control of feeding behavior.

Terminology

Gastric motor responses are the result of a balance between nervous excitatory, mainly cholinergic, and NANC inhibitory influences exerted on smooth muscle. During EFS, both excitatory and NANC inhibitory nervous fibers are simultaneously activated. The release of neurotransmitters from enteric neurons may be modulated by a variety of substances, including hormones.

Peer-review

This is a very interesting study investigating the influence of GLP2 on neurally-induced responses in gastric fundal strips.

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ORIGINAL ARTICLE

Basic Study

Hypothermic machine perfusion with metformin-University of Wisconsin solution for *ex vivo* preservation of standard and marginal liver grafts in a rat model

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Abstract

AIM

To compare the effect of University of Wisconsin (UW) solution with or without metformin, an AMP-activated

protein kinase (AMPK) activator, for preserving standard and marginal liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP).

METHODS

Eighteen young (4 mo old) and 18 aged (17 mo old) healthy male SD rats were selected and randomly divided into three groups: control group, UW solution perfusion group (UWP), and UW solution with metformin perfusion group (MUWP). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), interleukin-18 (IL-18), and tumor necrosis factor-alpha (TNF-α) in the perfused liquid were tested. The expression levels of AMPK and endothelial nitric oxide synthase (eNOS) in liver sinusoidal endothelial cells were also examined. Additionally, microscopic evaluation of the harvested perfused liver tissue samples was done.

RESULTS

AST, ALT, LDH, IL-18 and TNF-α levels in the young and aged liver-perfused liquid were, respectively, significantly lower in the MUWP group than in the UWP group ($P < 0.05$), but no significant differences were found between the young and aged MUWP groups. Metformin increased the expression of AMPK and eNOS protein levels, and promoted the extracellular release of nitric oxide through activation of the AMPK-eNOS mediated pathway. Histological examination revealed that in the MUWP group, the extent of liver cells and tissue damage was significantly reduced compared with the UWP group.

CONCLUSION

The addition of metformin to the UW preservative solution for *ex vivo* HMP can reduce rat liver injury during cold ischemia, with significant protective effects on livers, especially of aged rats.

Key words: Metformin; AMP-activated protein kinase; Cold ischemia injury; Hypothermic machine perfusion; Liver Grafts

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Core tip: Metformin can activate the AMP-activated protein kinase pathway that could enhance the activity of endothelial nitric oxide synthase and finally increase the generation of nitric oxide, which plays an important role in the protection of liver sinusoidal endothelial cells. Hence, our study was designed to evaluate the protective effect of University of Wisconsin storage solution with metformin for preserving standard and marginal liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP). According to the results, HMP with metformin plays a significant protective role for liver grafts during cold ischemia, with significant effects especially for aged-marginal donors.

Chai YC, Dang GX, He HQ, Shi JH, Zhang HK, Zhang RT, Wang B, Hu LS, Lv Y. Hypothermic machine perfusion with metformin-University of Wisconsin solution for *ex vivo* preservation of standard and marginal liver grafts in a rat model. *World J Gastroenterol* 2017; 23(40): 7221-7231 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7221.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7221

INTRODUCTION

Currently, liver transplantation is the only effective therapy for end-stage liver disease[1]. Both preservation of donor organs and post-transplant ischemic reperfusion injury (IRI) are important factors affecting the prognosis of transplantation[2]. At present, due to the shortage of liver donation, marginal donation, which includes aged donation, adipo-hepatic donation, and donation after cardiac death (DCD), increases the risk for more severe IRI because of suboptimal function and long-term warm and cold ischemia[3-5]. Cold ischemia injury plays an important role in the IRI mechanism after revascularization of transplants. In this period, the liver sinusoidal endothelial cells are the first to be injured in a donor liver, causing damage of the stable hepatic microenvironment, hepatic microcirculation disturbance, and exacerbation of IRI[6]. Therefore, there is a current pressing need to explore and improve methods of organ preservation and minimize IRI of donor livers during transplantation[7,8].

In recent years, machine perfusion (MP) has been explored as an alternate method of organ preservation to static cold storage. Clinically, hypothermic machine perfusion (HMP, 4-6 ℃) has been effective for kidney transplantation, but MP methods have not been widely used in liver transplantation. According to the latest research, MP has been meaningful for the preservation and repair of marginal liver donation[9], but this still needs further clinical verification[10,11].

Another important direction of research on donor liver cold preservation is the auxiliary protective intervention of donor livers against IRI factors of microcirculation[12] and hepatocyte metabolism[13] through drugs. Activation of adenosine 5′-monophosphate-activated protein kinase (AMPK) signaling pathways increases the activity of endothelial nitric oxide synthase (eNOS) to generate nitric oxide. This provides a cytoprotective effect to the hepatic sinusoidal endothelium of the donor liver and has been considered for preconditioning of the donor liver to reduce IRI. In addition, AMPK signaling is also known to regulate glucose metabolism and prevent cell death, thus extending the cytoprotective effect on hepatocytes[14]. Therefore, it plays an important role in protecting hepatic sinusoidal endothelium and reducing injury of donor livers[15]. As an agonist of AMPK, metformin additionally lowers the blood glucose by reducing hepatic gluconeogenesis and strengthening

glucose uptake of peripheral tissue[16].

Hence, we hypothesized that liver sinusoidal endothelial cells can be protected from injury by activating AMPK signaling pathways with the addition of metformin perfused *in vitro*, which could ultimately lead to an improvement of liver donor organ preservation. Consequently, in this study, we added metformin to University of Wisconsin (UW) solution, *ex vivo*, in HMP models of livers of young and aged rats and investigated the effects on biochemical indicators and sinusoidal cell morphology.

MATERIALS AND METHODS

A total of 18 young healthy male SD rats (4 mo old, weighing 250–300 g) and 18 aged (17 mo old, weighing 600–630 g) SD rats were randomly selected for the study. The experimental animals were provided by Animal Experiment Center of Xi'an Jiaotong University.

LongerPump DG-2-B/D Precise Miniature Peristaltic Pump (Longer Precision Pump Co., Ltd., Baoding, China) with a rotation speed of 0-100 rpm and a flow rate of 0-48 mL/min; SPS-1 Static Preservation Solution (UW solution, Organ Recovery Systems, Inc.); and metformin (1,1-dimethylbiguanide hydrochloride, CAS # 1115-70-4, Biomol GmbH) were used. A 165 mg/L stock solution of metformin was prepared by adding 2.5 mL of metformin solution at a concentration of 1 g/10 mL to 1000 mL of sterile water, stirring until dissolved, and then adding this (0.66 mL) to 1000 mL of UW solution. A final metformin concentration of 0.165 mg/L or 1 mmol/L was used. Serum interleukin-18 (IL-18) and tumor necrosis-alpha (TNF-α) levels were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Rat Interleukin 18 ELISA kit, CLOUD-CLONE CORP., TX, United States; Rat TNF-alpha ELISA kit, MultiSciences Biotech Co., Ltd, Shanghai, China).

Study design

Young and aged rats were respectively randomly divided into three groups, with six rats in each group. The groups were: control groups A (young rats) and D (aged rats), UW solution perfusion (UWP) groups B (young rats) and E (aged rats), and experimental groups C (young rats) and F (aged rats) that were perfused with metformin-UW solution (MUWP).

Step one - Establishing the experimental model

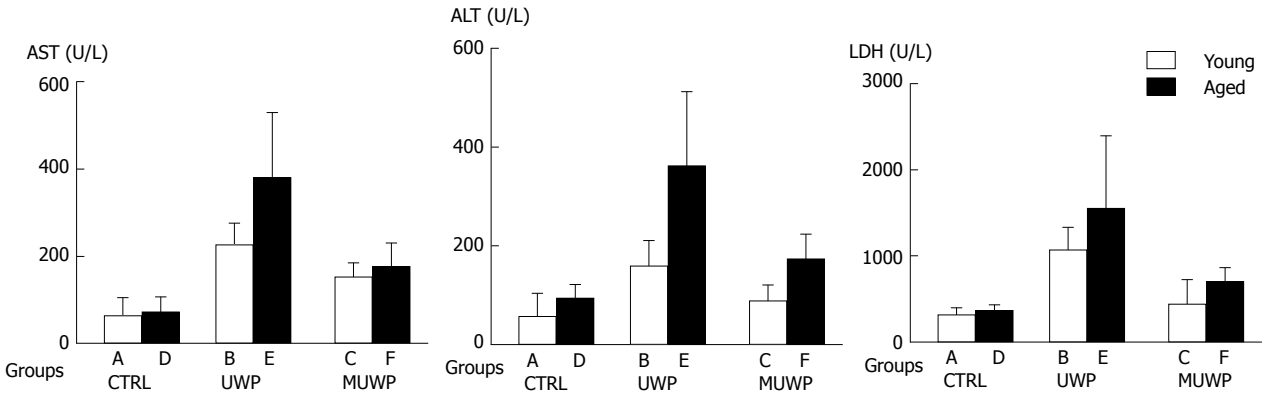
Rats were fasted for 8 h and general anesthesia was induced in all rats by intraperitoneal injection of 5% pentobarbital sodium at 20 mg/kg. Fixation, skin preparation, and disinfection were completed, and then a large median and transverse abdominal incision for laparotomy was made. The liver was isolated, heparinized, perfused *in situ* with cold UW solution until the liver turned into a khaki color, and rapidly harvested at room temperature. The *ex vivo* liver was then placed into a basin filled with cold UW solution and made to lie in the basin on an ice pad. All *ex vivo* livers were grouped and underwent HMP with circulating UW solution at 4 ℃ at a flow rate of 4 mL/min maintained at 80 mL of the total circulation volume with the help of a peristaltic pump. Groups A and D did not require extended period of HMP (only 2 h); groups B and E were perfused with UW solution for 12 h; and groups C and F were perfused mechanically with UW solution with 0.165 mg/L of metformin for 12 h. After HMP, 6 mL of the perfused liquid was collected from every group and stored at -20 ℃.

Step two - Examination of the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in liver sinusoidal endothelial cells of young rats

Extraction of liver sinusoidal endothelial cells from young rats: After step one, the *ex vivo* livers of every group were perfused with Gey's balanced salt solution (150 mL free of Ca^{2+} and Mg^{2+}, mixed with pronase 400 mg and collagenase 40 mg) for 7 min at 37 ℃ at a flow rate of 20 mL/min. Then, the resulting mixture was centrifuged at 2500 rpm with Krebs-Henseleit solution (with 10% fetal calf serum and 0.002% DNase I) perfused at a flow rate of 10 mL/min. Cell pellet was then resuspended in DMEM supplemented with 20% fetal calf serum and 10 mL of 100 U/L penicillin G and loaded carefully in the centrifuge for 3 min at a flow perfusion rate of 15 mL/min, and accelerated at 18 mL/min for 1 min. The input flow rate was increased again by 20 mL/min, while 50 mL of the cell suspension of the effluent was collected. Once more, a 50 mL cell suspension was collected by 25 mL/min. The 100 mL homogenate was centrifuged at 2000 rpm for 5 min. The supernatant liquid was extracted out, and the liver sinusoidal endothelial cell-debris pellet was collected in the bottom of collection tube.

Extraction of total protein and examination of the expression levels of target proteins: The cell debris was lysed in Cell Lysis Buffer (Cell Signaling Technology; Beverly, MA, United States; carefully chilling 1 × stock buffer on ice and add 1 mmol/L PMSF immediately just prior to use) for 30 min on ice, and then centrifuged at 12000 rpm for 5 min at 4 ℃. The supernatant with total cellular proteins was collected from every group, determined, transferred to a sterile tube (1.5 mL), and stored at -20 ℃. Then, the two-dimensional electrophoresis (2-DE) maps of target proteins were obtained by Western blot to examine the expression levels, and the amounts of expression of AMPK and eNOS were analyzed with gray scale from the maps.

Tests included: (1) Biochemical indicator tests: the levels of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH),



Groups	A / D	B / E	C / F
AST (U/L)	66.40 ± 40.59 / 75.47 ± 31.11	223.50 ± 55.07 / 378.15 ± 150.13	152.53 ± 35.58 / 176.93 ± 55.10
ALT (U/L)	59.97 ± 46.58 / 95.78 ± 30.62	161.20 ± 45.53 / 363.25 ± 150.12	88.25 ± 36.22 / 173.6 ± 51.72
LDH (U/L)	322.13 ± 93.48 / 385.17 ± 45.28	1039.99 ± 308.78 / 1543.05 ± 855.93	450.72 ± 287.96 / 709.3 ± 164.20

Figure 1 Analysis of biochemical indicators in the perfused liquid. Aspartate aminotransferase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) levels (mean ± SD) in the perfused liquid of the ex vivo livers of the three groups: CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F). Data are presented as mean ± SD (n = 6) and compared by two-way analysis of variance and the Sidak's multiple comparisons test.

IL-8, and TNF-α in the perfused liquid were examined; (2) Histo-morphological by light microscopy: the liver tissues after perfusion were fixed with 10% formalin and immersed in the wax for sections and hematoxylin-eosin (HE) dye. A scoring system was used to grade the degree of histological damage quantitatively (by blinding) at the Department of Pathology, based on the following histological features: hydropic degeneration of hepatocytes and liver sinusoidal endothelial cells, stenosis of the hepatic sinusoid, and number of Kupffer cells. Each feature was graded as absent, mild, moderate, or massive, with a score of 0-3, respectively; and (3) Electron microscopic examination: The specimens of perfused hepatic tissue were fixed to observe by transmission electron microscopy.

Statistical analysis

All calculations were performed with SPSS 22.0 software. The grayscale of 2-DE maps of target proteins was analyzed with ImageProPlus 6.0 software. Quantitative data are expressed as mean ± SD and assessed by one- or two-way analysis of variance, and multiple comparisons between groups were performed using Student-Newman-Keuls method. Scoring data were analyzed by Kruskal-Wallis one-way analysis of variance on ranks and compared between any two groups using Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

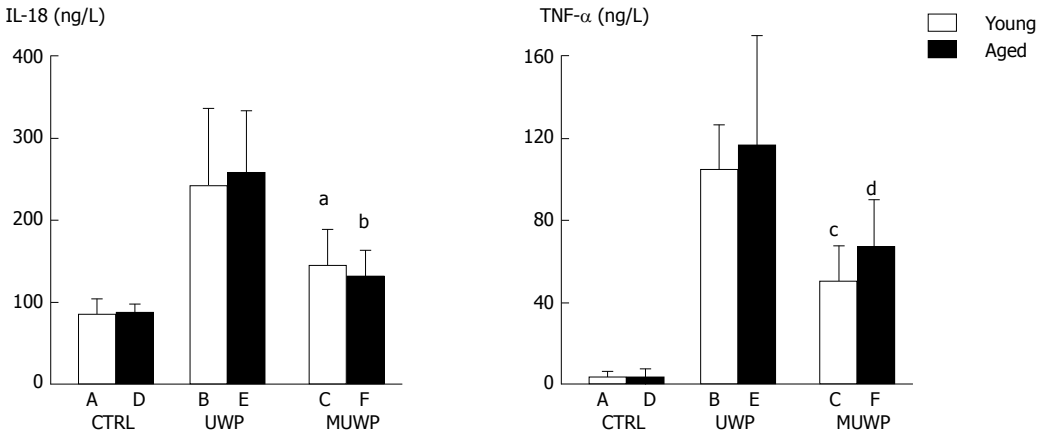
RESULTS

Analysis of biochemical indicators

The control group (groups A and D) was subject to HMP for only 2 h, while the UWP (groups B and E) and MUWP groups (groups C and F) underwent HMP for 12 h.

After completing the perfusion of each group, AST, ALT, and LDH levels in the perfused liquid were determined (Figure 1). There were no significant differences in AST, ALT, or LDH between the young rat control group (group A) and the aged rat control group (group D). The AST, ALT, and LDH in the UWP group (groups B and E) were significantly higher than those in groups A and D ($P < 0.05$), Besides, the AST and ALT in the aged group E were significantly higher than those in the young group B ($P < 0.05$). The AST, ALT, and LDH in the MUWP group, which consisted of the young group C and aged group F, were, respectively, significantly lower than those in the young group B and aged group E of UWP ($P < 0.05$), although no significant differences between the young and the aged group were found.

IL-18 and TNF-α levels in the perfused liquid were measured by ELISA (Figure 2). There were no significant differences in the levels of IL-18 or TNF-α between the young rat control group (group A) and the aged rat control group (group D). IL-18 and TNF-α levels in the UWP group were significantly higher than those in the control group ($P < 0.05$). Nevertheless, there were no significant differences between the young group B and the aged group E of UWP. IL-18 and TNF-α levels in the young group C and the aged group F of MUWP were, respectively, appreciably lower than those in the young group B and the aged group E of UWP ($P < 0.05$), but no significant differences between the young group C and the aged group F of MUWP were found.



Groups	A / D	B / E	C / F
IL-18 (ng/L)	87.9 ± 14.97 / 88.5 ± 8.63	243.50 ± 91.50 / 260.37 ± 72.73	145.05 ± 44.42 / 131.38 ± 32.53
TNF-a (ng/L)	3.60 ± 2.80 / 3.76 ± 3.70	91.65 ± 18.63 / 101.43 ± 47.37	43.70 ± 15.28 / 57.80 ± 20.77

Figure 2 Analysis of inflammatory factors in the perfused liquid. The IL-18 and TNF-α levels in the perfused liquid (mean ± SD) of the *ex vivo* livers of the three groups: CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F). Data are presented as mean ± SD (n = 6) and compared by two-way analysis of variance and the Tukey's and Sidak's multiple comparisons tests. [a]P < 0.05 *vs* group B; [b]P < 0.05 *vs* group E; [c]P < 0.05 *vs* group B; [d]P < 0.05 *vs* group E.

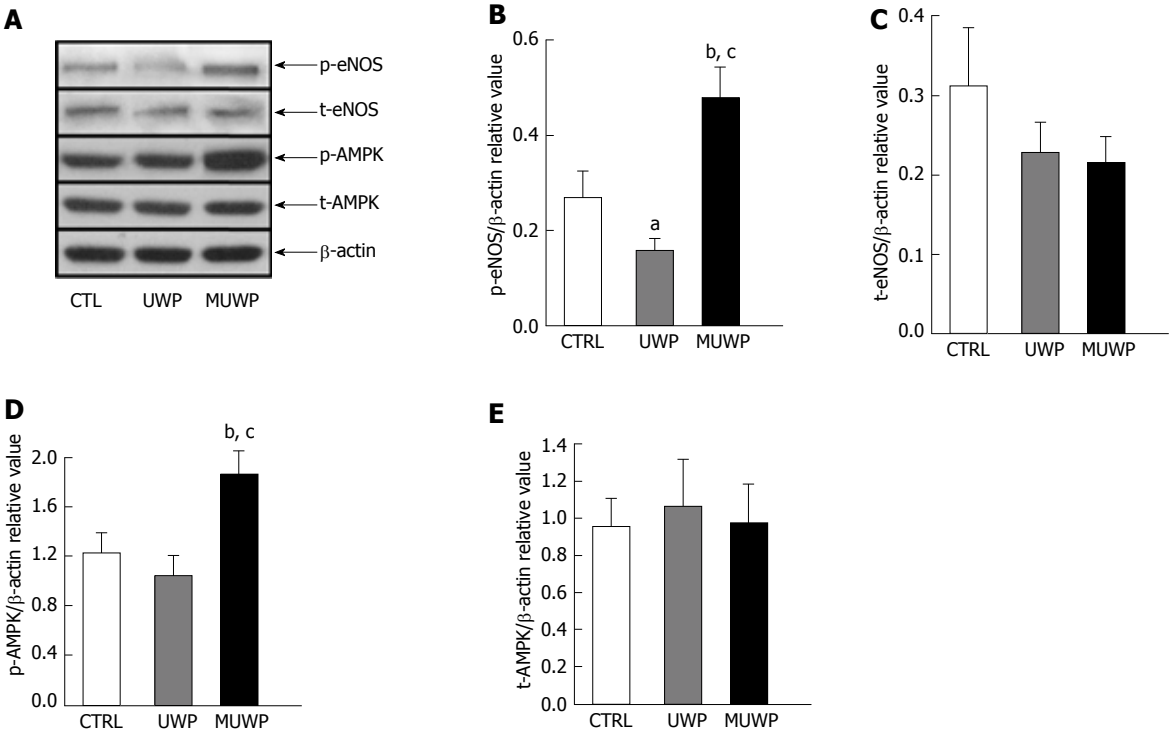


Figure 3 Histograms of relative gray values of the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in the liver sinusoidal endothelial cells of young rats. AMPK/eNOS pathway was activated *ex vivo* by metformin. A: Phosphorylation of AMPK and eNOS was increased in the MUWP group at 12 h after HMP; B-E: Quantitative analysis of total AMPK, total NOS, phosphorylated AMPK, and eNOS expression in CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F) groups. Data are presented as mean ± SD (n = 3) and compared by ordinary one-way analysis of variance and the Dunnett's multiple comparisons test. [a]P < 0.05 *vs* CTRL group; [b]P < 0.05 *vs* CTRL group; [c]P < 0.05 *vs* UWP group.

Expression levels of target proteins

Discriminant analysis of relative gray values was used to determine the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in liver sinusoidal endothelial cells of young rats (Figure 3). The expression level of p-eNOS in the MUWP group was significantly higher (80.1%) than that in the control group (P = 0.036) and 205.88% higher than that in the UWP group (P = 0.008), which was significantly (41.1%) lower than that in the control group (P = 0.045) (Figure 3B). The expression level of t-eNOS in the control group exhibited no significant difference compared with the

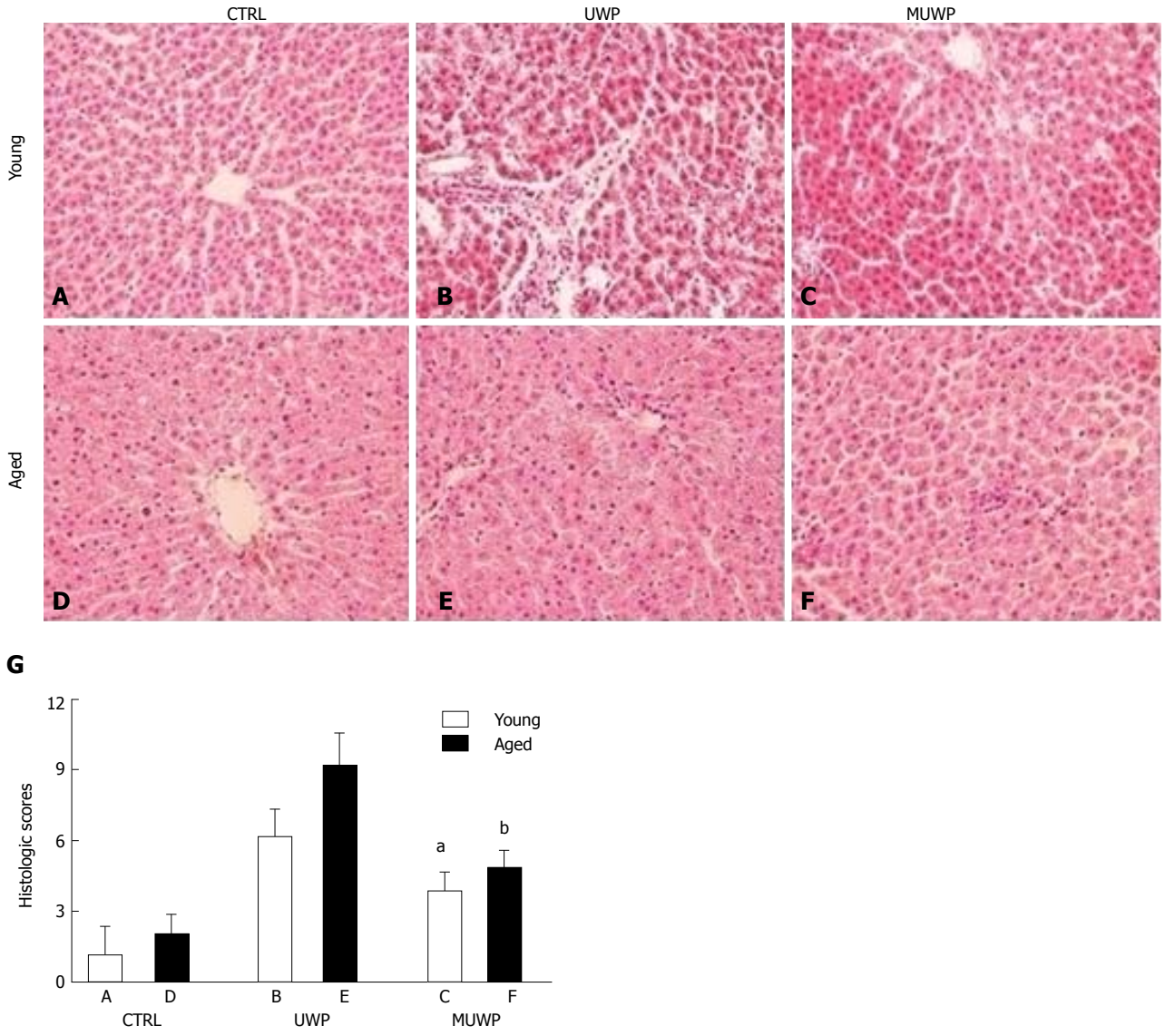


Figure 4 Staining of the slices of *ex vivo* rat liver tissue. A and D: The young control group A and the aged control group D, respectively; B and E: The young group B and aged group E of UWP, respectively; C and F: The young group C and the aged group F of MUWP, respectively; G: Histopathologic scoring of structural injury in each group. Data are presented as mean ± SD (n = 6) and compared by Kruskal-Wallis one-way analysis of variance on ranks and Mann-Whitney U test. [a]P < 0.05 *vs* group B of UWP; [b]P < 0.05 *vs* group E of UWP. Original magnification (A-F): × 400. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution.

UWP and MUWP groups (P = 0.251) (Figure 3C). The expression level of p-AMPK in the MUWP group was significantly higher (51.7%) than that in the control group (P = 0.038) and 78.5% higher than that in the UWP group (P = 0.018), even though there were no significant differences between the control and UWP groups (P = 0.217) (Figure 3D). The expression level of t-AMPK in the control group demonstrated no significant differences compared with the UWP and the MUWP groups (P = 0.868) (Figure 3E).

Histo-morphological examination

The HE-stained sections were observed under a microscope. The hepatocytes in the young rat control group (group A) and the aged rat control group (group D) were shaped normally, with no stenosis observed in the hepatic sinusoid and no swelling in the sinusoidal endothelial cells. The size of hepatocytes in group D was slightly larger than that in group A (Figure 4A-D). Cellular swelling was obvious, accompanied by narrowing of the hepatic sinusoid, in the UWP group (groups B and E), particularly, more severe in the aged group E. The swelling of the sinusoidal endothelial cells, as well as Kupffer cells was observed in both groups B and E (Figure 4B-E). Hepatocytes in the MUWP group showed mild edema, which was slightly more severe in group F than in group C, although there were no obvious differences in the hepatic sinusoid between them. Swelling of sinusoidal endothelial cells was inconspicuous in both groups; however, a small number of Kupffer cells were observed (Figure 4C-F). There were no significant differences in the histologic

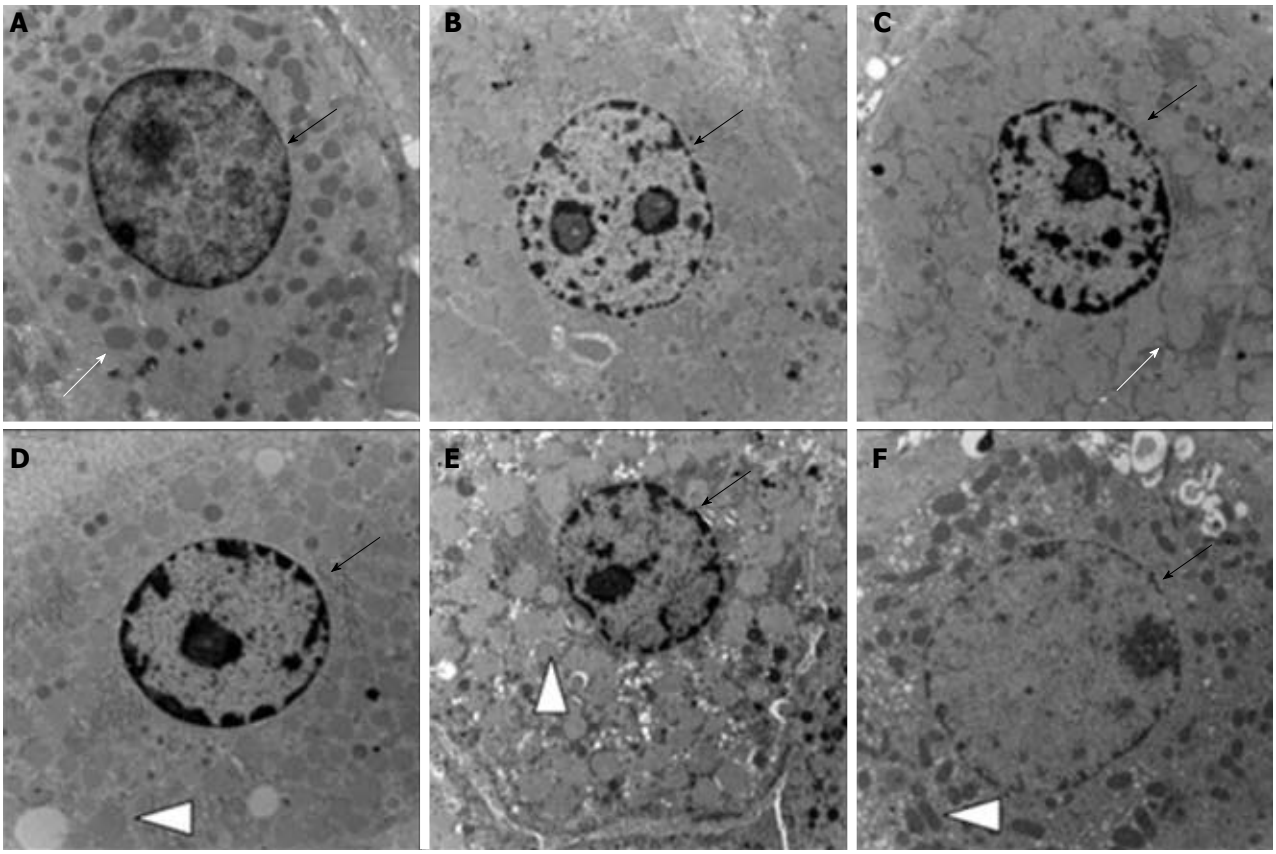


Figure 5 Ultrastructural changes of hepatocytes. A and D: Hepatocytes of the young control group A and the aged control group D, respectively; B and E: Hepatocytes of the young group B and aged group E of UWP without metformin, respectively; C and F: Hepatocytes of the young group C and aged group F of MUWP, respectively. The black arrows indicate the nuclei, the white arrows indicate the intracellular mitochondria of the young groups, and the white triangles indicate the intracellular mitochondria of the aged groups. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution. Original magnification (A-F): ×10000.

scores between the young rat control group (group A) and the aged rat control group (group D) ($P < 0.05$). The histologic scores of the young rat group and the aged rat group of UWP and MUWP were, respectively, appreciably higher than those of the young rat control group (group A) and the aged rat control group (group D), and that histologic scores of the young group C and the aged group F of MUWP were, respectively, appreciably lower than those of the young group B and the aged group E of UWP ($P < 0.05$). Besides, the histologic scores of aged group E were significantly higher than those of young group B of UWP ($P < 0.05$), but no significant differences were found between the young group C and the aged group F of MUWP (Figure 4G).

Histological observation by electron microscopy

The ultrastructural changes of hepatocytes were observed under an electron microscope. Structure of hepatocytes in the young control group (group A) and aged control group (group D) was generally normal with a round and clear nucleus located in the center of the cells, while lipid droplets in the cytoplasm increased in the aged control group (group D), and fat-storing cells were seen in the Disse's space with irregular shapes, and the cytoplasm in it contained a large amount of lipid droplets. In contrast, lipid droplets and fat-storing cells were not found in the young control group (group A), but the cells contained rich cytoplasmic organelles (Figure 5A-D). Hepatocyte swelling, nuclear chromatin condensation, extensive mitochondrial swelling, and crista fragmentation were observed in groups B and E of UWP, more severe in the aged group E. Likewise, fat-storing cells in the Disse's space increased in the aged group E, but not found in the young group E (Figure 5B-E). In contrast, hepatocytes of the MUWP group showed mild edema with round nucleus, slightly more evident in the young group C than in the aged group F, and also the mitochondrial swelling degree was slightly higher in the young group C. In addition, the increase in lipid droplets in the cytoplasm and the number of fat-storing cells, dilation of the smooth endoplasmic reticulum, and intercellular collagenous fibrosis were only seen in the aged group F (Figure 5C-F).

The ultrastructural changes of the hepatic sinusoid were also observed under an electron microscope. Structure of the hepatic sinusoidal endothelial cells in the young control group A and the aged control group D remained generally intact with protrusion into the sinusoid, being clear in the young group A. Kupffer cells

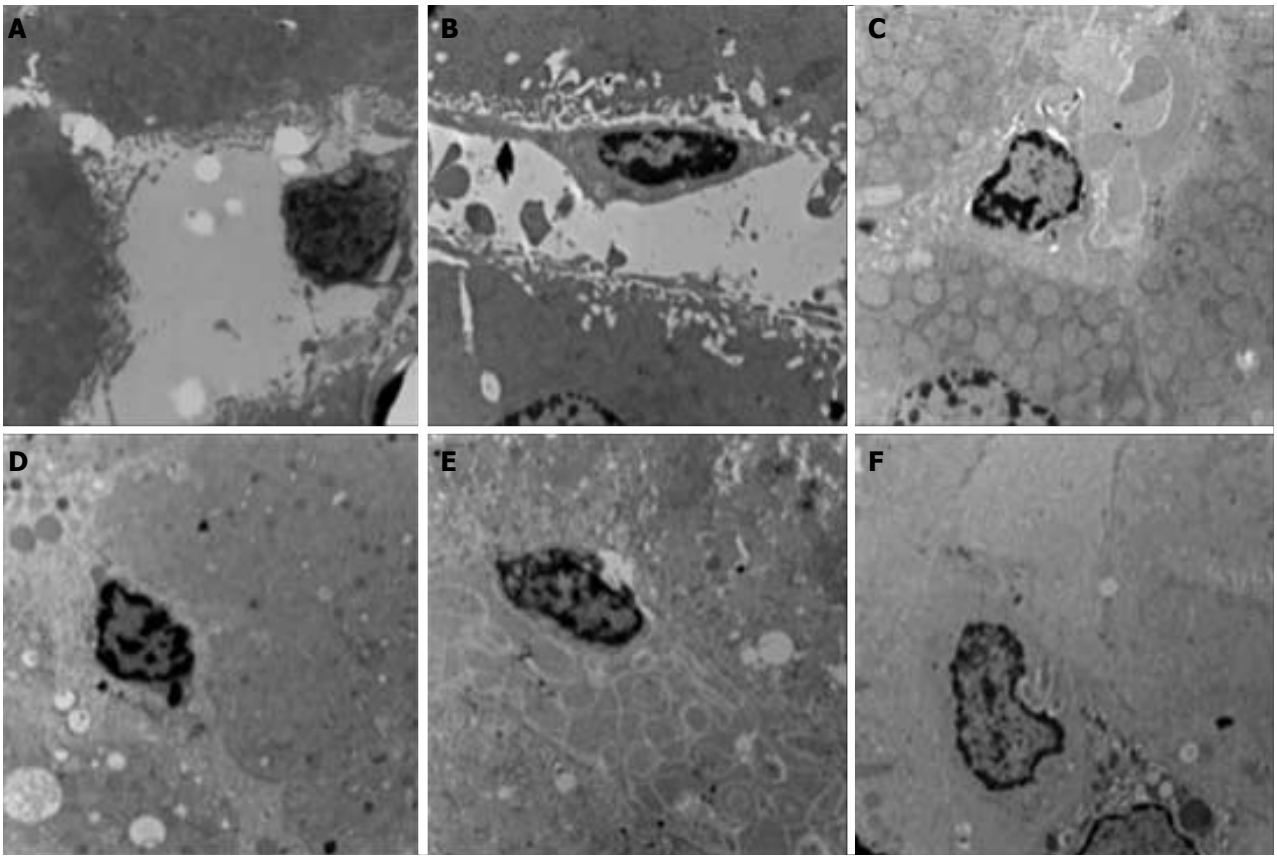


Figure 6 Ultrastructural changes of liver sinusoidal endothelial cells. A and D: Sinusoidal endothelial cells of the young control group A and the aged control group D, respectively; B and E: Sinusoidal endothelial cells of the young group B and aged group E of UWP, respectively; C and F: Sinusoidal endothelial cells of the young group C and aged group F of MUWP, respectively. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution. Original magnification (A–F): ×10000.

were seen in the aged group D, though (Figure 6A-D). Sinusoidal endothelial cells were kept meristematic and flat-shaped, with chromatin showing mild margination, and cell debris-like structure together with Kupffer cells in the sinusoids was increased in groups B and E of UWP, particularly, more severely in aged group E. Likewise, fat-storing cells increased in the aged group E, while they were not found in the young group E (Figure 6B-E). The structure of the hepatic sinusoidal endothelial cells in the MUWP group remained generally normal, while a slightly larger cell volume was seen in the aged group F, and cubic-shaped sinusoidal endothelial cells with protrusion into the sinusoid were seen in the young group C. In addition, the low-electron-density protein substance could be seen in the sinusoid in the aged group F.

DISCUSSION

An increasing number of patients with liver disease await liver transplantation; the acute shortage of donor livers is only likely to continue. It was believed that aged livers from donors above 60 years old were characterized by small sizes, atherosclerosis, steatosis, and decreased function of metabolism, and were not suitable for donation[17]. However, to cope with the demand for donor livers, marginal liver donation has been applied clinically after following strict screening protocols with good transplantation outcomes and breaking the myth for the age limit of liver donors[18,19]. Unlike hearts, lungs, and kidneys, livers are much less influenced by age with respect of pathophysiological changes. However, aged livers also show morphologic changes, such as hepatic fibrosis, hepatocyte swelling, trend for multinucleation, increase of lipofuscin, and reduction in the size of the hepatocyte. Compared with young donors, aged donors show reduced and shrunken fenestra of liver sinusoidal endothelial cells, thickened hepatic sinusoidal endothelium, and pseudo capillarization formed by discontinuous basement membranes, leading to potential microcirculatory disturbance and a risk of aggravating IRI[20]. Thus, easier apoptosis and detachment of liver sinusoidal endothelial cells of aged livers further aggravate microcirculatory disturbance, seriously influence post-transplant effects, and even cause failure of some liver transplantation[21]. According to the results of this study, aged groups appeared to be more sensitive to the possible protective effect of metformin. Furthermore, the histological observations by light and

electron microscopy also support this conclusion.

In order to prevent cold ischemia injury, auxiliary protective intervention by drugs with different mechanisms of action, including the MAPK agonist, is being explored[22]. Scientists from different academic institutions have published research articles in which they revealed the molecular mechanism by which metformin could activate AMPK and inhibit the mTORC1 signaling pathway in livers *via* the AXIN/LKB1-v-ATPase-Ragulator pathway[23,24], protect the integrity of epithelial cells in multiple stressed conditions (such as inflammation, infection, and anoxia), and even demonstrate an anticancer effect[25-27]. Before revealing this molecular mechanism, it was known that AMPK activation plays a critical role in reducing liver IRI. However, how AMPK and eNOS phosphorylation direct their effects on the endothelial function is still elusive[28], even if there is plenty of evidence to show that AMPK activation can enhance the activity of eNOS, resulting in generation of nitric oxide[15,29,30]. Extension of cold preservation by maintaining a supercooled state can make donor liver cells remain viable up to 96 h, and human livers showed improvement in endothelial function with 2 h of HMP[31]. In the current study, the AMPK activator metformin was added to e*x vivo* HMP models of rat livers in UW solution after HMP for 12 h. Our results showed that all the biochemical indexes and inflammatory factors in the UWP group were significantly higher than those in the control group, but all the indexes in both the young and aged groups of MUWP, respectively, were appreciably lower than those in the young and aged groups of UWP. Histological observation by light and electron microscopy showed that injury of the related microstructure was milder in the MUWP group than in the UWP group. To a certain extent, it can be deduced that metformin activated protective mechanisms. Detection of expression levels of eNOS and AMPK in the liver sinusoidal endothelial cells of young rats showed that phosphorylation of AMPK and eNOS was increased in the MUWP group at 12 h after HMP, and this can be inferred as the AMPK/eNOS pathway was activated *ex vivo* by metformin.

This study confirmed that the addition of metformin to organ preservation solution can activate the AMPK/eNOS pathway, and this can not only significantly decrease the inevitable injury to donor livers caused by long-term HMP, but can reduce the difference between aged and young livers after HMP, protecting livers of aged rats, which should probably improve the utilization of marginal liver donor tissues. However, whether metformin can sequentially improve hepatic injury during reperfusion-ischemia requires further investigation. But at least, we provided a novel idea, which is also a simple procedure for drug auxiliary intervention with HMP in *ex vivo* rat donor liver and deserves further research with a promising prospect.

In conclusion, this study confirmed that metformin can activate the AMPK/eNOS pathway and reduce injury to *ex vivo* rat liver during cold ischemia in MP. The combination of metformin with the organ preservation solution effectively enhanced the quality of donor livers, with significant protective effects on livers especially of aged rats, which could be used to improve the utilization of marginal livers.

ARTICLE HIGHLIGHTS

Background

Liver transplantation is the only effective therapy for end-stage liver disease. At present, due to the shortage of liver donation, marginal donation, which includes aged donation, adipo-hepatic donation, and donation after cardiac death, increases the risk for more severe ischemic reperfusion injury (IRI) because of suboptimal function and long-term warm and cold ischemia. Therefore, there is a current pressing need to explore and improve methods of organ preservation and minimize IRI of donor livers during transplantation.

Research frontiers

According to the latest research, machine perfusion has been meaningful for the preservation and repair of marginal liver donation. Another important direction of research on donor liver cold preservation is the auxiliary protective intervention of donor livers against IRI factors of microcirculation and hepatocyte metabolism through drugs. Activation of adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling pathways increases eNOS activity to generate nitric oxide (NO), which plays an important role in the protection of liver sinusoidal endothelial cells. Metformin is an agonist of AMPK. Hence, we assumed that liver sinusoidal endothelial cells can be protected from injury by activating AMPK signaling pathways with the addition of metformin perfused *in vitro*, which could ultimately cause an improvement of liver donor organ preservation.

Research objectives

In this study, we added metformin to University of Wisconsin (UW) solution, to compare the effect of UW solution with or without metformin, an AMPK activator, for preserving standard and marginal criteria liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP).

Research methods

Eighteen young (4-mo-old) and 18 aged (17-mo-old) healthy male SD rats were selected and randomly divided into three groups: control group, UW solution perfusion group (UWP), and UW solution with metformin perfusion group (MUWP). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), *lactate dehydrogenase* (LDH), interleukin-18 (IL-18), and tumor necrosis factor-alpha (TNF-α) in the perfused liquid were tested. The expression levels of AMPK and eNOS in liver sinusoidal endothelial cells were also examined. Additionally, microscopic evaluation of the harvested perfused liver tissue samples was done.

Research results

AST, ALT, LDH, IL-18 and TNF-α levels in the young and aged liver-perfused liquid in the MUWP group were, respectively, significantly lower than those in the UWP group ($P < 0.05$), but no significant differences between the young and aged MUWP groups were found. Metformin increased the expression of AMPK and eNOS protein levels, and promoted the extracellular release of nitric oxide through activation of the AMPK-eNOS mediated pathway. Histological examination revealed that in the MUWP group, the extent of liver cells and tissue damage was significantly reduced compared with the UWP group.

Research conclusions

This experiment confirmed that the addition of metformin to organ preservation solution can activate AMPK/eNOS pathway, which can not only reduce injury to *ex vivo* rat livers during cold ischemia, but can reduce the difference between aged and young livers after HMP, with especially significant effects of protecting livers of aged rats, which should probably improve the utilization of marginal liver donor tissues. However, whether metformin can sequentially improve hepatic injury during reperfusion-

ischemia requires further investigation. But at least, we provided a novel idea, which is also a simple procedure for drug auxiliary intervention with HMP in *ex vivo* rat donor livers and deserves further research with a promising prospect.

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ORIGINAL ARTICLE

Basic Study

Relationship between autophagy and perineural invasion, clinicopathological features, and prognosis in pancreatic cancer

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Abstract

AIM

To investigate the relationship between autophagy

and perineural invasion (PNI), clinical features, and prognosis in patients with pancreatic cancer.

METHODS

Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent radical resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. Expression levels of the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) and PNI marker ubiquitin carboxy-terminal hydrolase (UCH) in pancreatic cancer tissues were detected by immunohistochemistry. The correlations among LC3 expression, PNI, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed for further survival analysis.

RESULTS

In 109 cases of pancreatic cancer, 68.8% (75/109) had evidence of PNI and 61.5% (67/109) had high LC3 expression. PNI was associated with lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$). LC3 expression was related to lymph node metastasis ($P < 0.05$) and was positively correlated with neural invasion ($P < 0.05$, $r = 0.227$). Multivariate logistic regression analysis indicated that LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor for high LC3 expression. Univariate analysis showed that LC3 expression, neural invasion, and CA19-9 level were related to the overall survival of pancreatic cancer patients ($P < 0.05$). Multivariate COX regression analysis indicated that PNI and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer ($P < 0.05$).

CONCLUSION

PNI in patients with pancreatic cancer is positively related to autophagy. Neural invasion and LC3 expression are independent risk factors for pancreatic cancer with a poor prognosis.

Key words: Pancreatic cancer; Perineural invasion; Autophagy; Clinical pathological features; Prognosis

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Core tip: The relationship between autophagy and perineural invasion (PNI) was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to microtubule-associated protein 1A/1B-light chain 3 (LC3) expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, which contributes to pancreatic cancer progression. This study might provide a new insight for the mechanism of PNI in pancreatic cancer.

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INTRODUCTION

Pancreatic cancer, also known as "the king of cancer", is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. The incidence of pancreatic cancer is increasing yearly[1]. Surgical resection is the only possible cure of pancreatic cancer, although less than 20% of patients are eligible for radical surgery[2]. At the time of diagnosis, most pancreatic cancer patients have distant metastasis due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is up to 80%-100% and is an important factor leading to postoperative pancreatic cancer recurrence. Previous studies have shown a higher recurrence rate after surgery and shorter disease-free and overall survival rates in cases of pancreatic cancer with PNI compared with those of cases without PNI. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery[3,4]. However, the pathogenesis of PNI has not yet been defined.

Autophagy has a dual role in promoting and inhibiting tumor growth[5,6]. Autophagy, as a mechanism of avoidance of anoikis in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells[7]. In addition, a previous study also showed that pancreatic cancer cells with PNI have higher levels of autophagy[7].

No study has examined the relationship between autophagy and PNI in pancreatic cancer cells. However, it can be inferred that only those pancreatic cancer cells that can survive within nerve tissues can eventually develop into a clinically visible form of pancreatic cancer PNI. Autophagy is likely one of the mechanisms involved in cancer cell survival. Therefore, this study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features, and prognosis, with an aim to provide a clinical basis for further study of the autophagy mechanisms affecting

the pathogenesis of pancreatic cancer PNI.

MATERIALS AND METHODS

General data

Retrospective data were collected from 109 cases of pathologically confirmed pancreatic ductal adenocarcinoma patients who underwent radical surgery for pancreatic cancer from January 2011 to August 2016 at the First Affiliated Hospital of Zhengzhou University. The included pancreatic cancer patients were not treated with radiation or chemotherapy prior to surgery, but received postoperative adjuvant gemcitabine- or non-gemcitabine-based chemotherapy, and/or radiotherapy. Tissue specimens were fixed in formalin and paraffin-embedded for histological study. Clinical and pathological data were collected, and all cases were followed. The date of surgical resection was considered as the starting time, and August 2016 was the deadline. The primary endpoint was death due to pancreatic cancer. Eighty cases were followed for more than 12 mo. All patients provided informed consent for the collection of biological samples, and this study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (Scientific Research No. 5, 2015).

Out of the 109 patients studied, 61 were male, and 48 were female. Their ages ranged from 19 to 81 years, and the median age was 59 years. Tumor diameters ranged from 0.7-12 cm with a median value of 4.0 cm. The survival time of 80 patients who were followed for more than 12 mo was 1 to 54 mo, and the median survival time was 20 mo. By the end of the follow-up period, 38 patients had died. The clinical stages were classified according to the AJCC 2011 standard.

Immunohistochemistry

Expression levels of LC3 and the nerve fiber marker ubiquitin carboxy-terminal hydrolase (UCH) were detected by immunohistochemistry using a standardized streptavidin-peroxidase (SP) method and the SP immunohistochemical Kit (ZSGB-Bio, Beijing, China) according to the manufacturer's instructions. The pancreatic cancer tissues were routinely embedded in paraffin and sliced into 4-μm-thick continuous sections. The sections were then warmed in an autoclave to remove residual wax and were hydrated before antigen retrieval. Endogenous peroxidase activity was eliminated by incubating the tissue sections at room temperature with 3% H_2O_2 for 10 min. The tissue sections were washed three times with PBS continuously and incubated with a small amount of goat serum at room temperature in a closed chamber for 15 min. The goat serum was then poured off (not washed), and anti-LC3 (Proteintech Group, diluted 1:120) and anti-UCH antibodies (Proteintech, diluted 1:100) were added and incubated overnight at 4 ℃. The next day, the slides were thoroughly washed with PBS, and a biotinylated secondary antibody in a working liquid was added and incubated at room temperature for 15 min. After the slides were washed carefully with PBS again, horseradish peroxidase was added, and the slides were incubated for 5 min at room temperature. The slides were then rinsed with PBS three times, and a drop of DAB buffer was placed on the tissue slice and rinsed after 1 min to stop the color reaction. Then, the nuclei were stained with hematoxylin, and the samples were dehydrated in gradient alcohol, covered with transparent balata, and observed by microscopy. PBS was used in place of the primary antibody to serve as the negative control, and a known positive slice served as the positive control. The immunohistochemical results were evaluated in a double-blind manner by two pathologists. If the results were inconsistent, a third pathologist reviewed the data to reach a consensus.

LC 3 immunohistochemical score

LC3-positive cells detected by immunohistochemistry required the following characteristics: (1) clear cell structure; (2) accurate localization of the positive particle; and (3) obviously higher pigmentation than that of the background and clear contrast. Positive LC3 expression was mainly localized in pancreatic cancer cell cytoplasm. Five random power fields (400 × magnification) were observed for each case using an optical microscope. One hundred homogeneous cells were counted, and the staining intensity and the proportion of positive cells were observed. A semi-quantitative analysis was conducted using the product method. Dye intensity was scored as follows: no yellow, 0 points; light yellow, 1 point; yellow or deep yellow, 2 points; and brown or tan, 3 points. The expression range was scored as follows: <10%, 0 points; 10% to 25%, 1 point; 26% to 50%, 2 points; 51% to 75%, 3 points; and >75%, 4 points. The value from dye intensity was then multiplied by the value from the expression range to obtain the overall score: 0 points, negative (-); 1 to 3 points, weakly positive (+); 4 to 6 points, moderately positive (+ +); and 7 to 12 points, strongly positive (+ + +). More than 3 points indicated high expression, and 3 points or fewer indicated low expression.

PNI evaluation

UCH was expressed in the cytoplasm of all nerve fibers. The location of nerve fibers can be clearly defined by UCH to determine the invasion of cancer cells to the nerve tissue. According to previous reports, positive PNI status was determined as cancer cells being found in the perineural spaces, perineurium or nerve tract[8].

Survival analysis

The effects of clinicopathological factors such as LC3 expression and PNI on the overall survival in pancreatic cancer were analyzed in 80 patients with

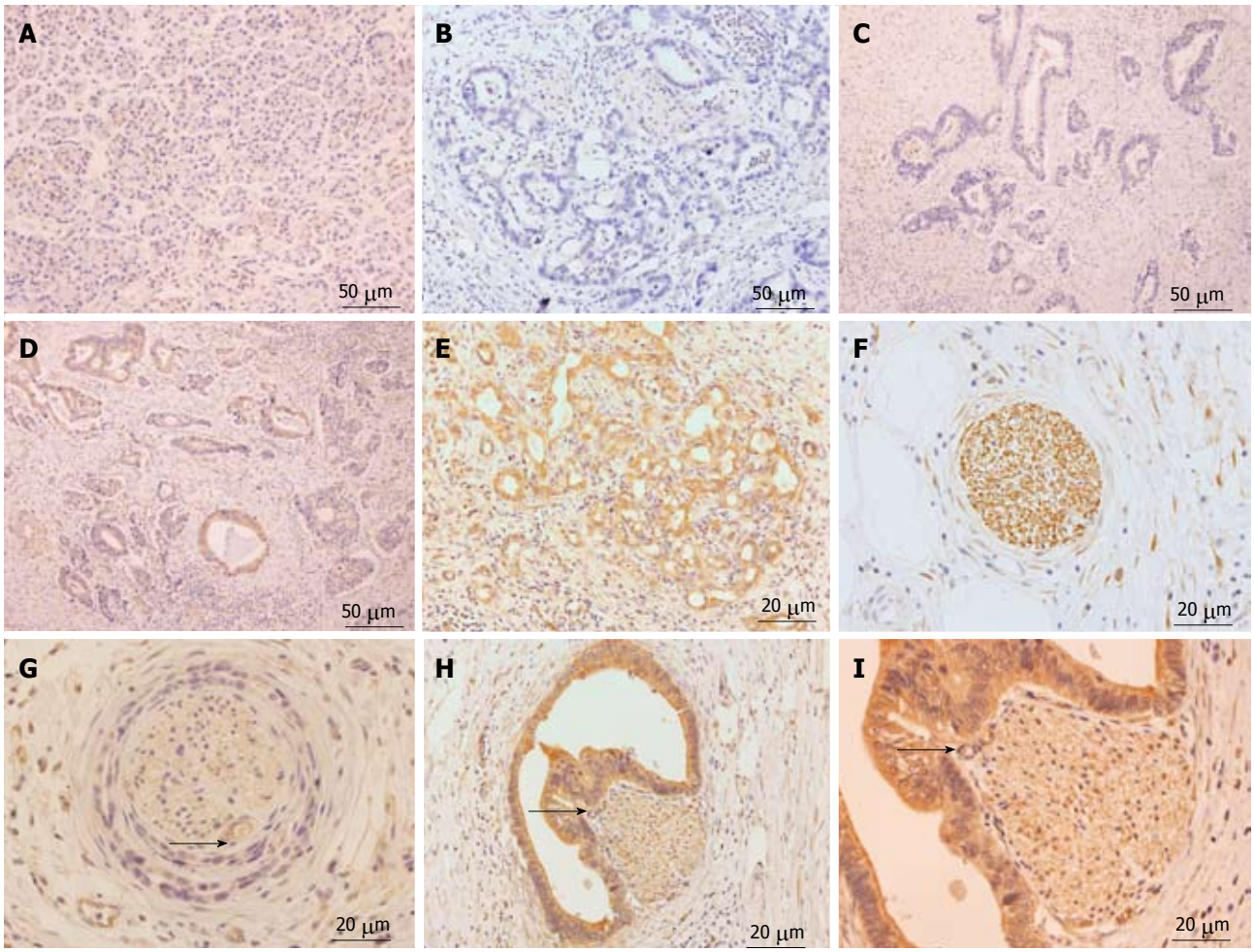


Figure 1 Representative immunohistochemical results of microtubule-associated protein 1A/1B-light chain 3 and perineural invasion. A: Negative expression of LC3 in normal paraneoplastic pancreatic tissue (× 200); B: Negative expression of LC3 in pancreatic cancer tissue (× 200); C: Weakly positive expression of LC3 in pancreatic cancer tissue (× 200); D: Moderately positive expression of LC3 in pancreatic cancer tissue (× 200); E: Strongly positive expression of LC3 in pancreatic cancer tissue (× 200); F and G: Perineural invasion in pancreatic cancer tissues (× 400, arrow represents cancer cells infiltrating into nerve tissue); H: Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); I: Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 400, arrow represents cancer cells infiltrating into nerve tissue). LC3: Microtubule-associated protein 1A/1B-light chain 3.

more than 12 mo of follow-up data. The following factors were included in the survival analysis: LC3 expression, age, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, pancreatitis, diabetes status, and preoperative CA19-9 level.

Data analysis

Statistical analyses were performed using SPSS 19.0 and GraphPad Prism 5.0 statistical software. Enumeration data were checked by the Chi-square test or the four-grid table Fisher exact probability method. Correlations between clinicopathological factors such as LC3 expression and PNI were analyzed using the Spearman correlation method. LC3 expression and the factors that independently influenced neural infiltration were analyzed using two categories and unconditional logistic regression. Univariate and multivariate analyses were performed on factors that might affect the prognosis according to a COX risk regression model. The survival curve was plotted according to the Kaplan-Meier method. Results were considered significant when $P < 0.05$.

RESULTS

Evaluation of LC3 expression and PNI in pancreatic cancer

LC3 expression was mainly localized to the cytoplasm. In contrast to normal paraneoplastic pancreatic tissues (Figure 1A), the expression of LC3 in pancreatic cancer tissues ranged from low to high in four grades: negative, weakly positive, moderately positive, and strongly positive (Figure 1B-E). The immunohistochemical results indicated that LC3 protein expression was observed in pancreatic ducts, acinar epithelial cells, islet cells, and pancreatic cancer tissues. There was significantly increased LC3 protein expression in pancreatic cancer tissues and peritumoral tissues. In 109 pancreatic cancer tissues, 42 had

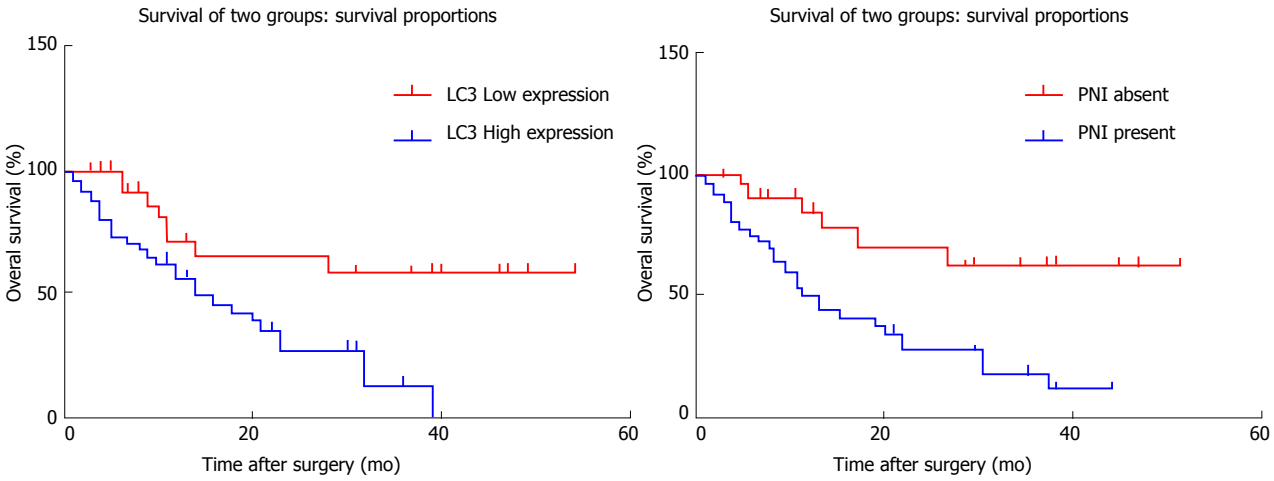


Figure 2 Kaplan-Meier estimates of overall survival in patients who underwent radical surgery. A: The overall survival rate of the LC3 low-expression group was better than that of the high-expression group ($P < 0.05$); B: The overall survival rate of the patients without nerve invasion group was better than that of those with nerve infiltration ($P < 0.05$).

Table 1 Relationship between microtubule-associated protein 1A/1B-light chain 3 expression and perineural invasion in pancreatic cancer

LC3	PNI		*P* value	*r*
	Absent	Present		
Low expression	18	22	0.018	0.227
High expression	16	53		

$P < 0.05$, PNI absent *vs* present. PNI: Perineural invasion

low LC3 expression (termed "low autophagy level"), including 6 (5.5%) negative cases and 36 (33%) weakly positive cases (Figure 1B and C); while 67 had high LC3 expression (termed "high autophagy level"), including 50 (45.9%) that were moderately positive and 17 (15.6%) that were strongly positive (Figure 1D and E). PNI by pancreatic cancer cells occurred mainly in the pancreatic cancer stroma. According to a previous study[9], four types of relationships exist between PNI and cancer nests: no PNI, perineurium invasion, perineural space invasion, and invasion to the nerve fiber tracts. In 109 cases of pancreatic cancer, 75 were positive (Figure 1F-I), and 34 were negative for nerve invasion. The positive rate of nerve invasion was 68.8%. High LC3 expression was also found in the nests surrounding the PNI (Figure 1I).

Relationship between LC3 expression and PNI in pancreatic cancer

The analysis of LC3 expression and PNI of pancreatic cancer demonstrated a significant positive correlation between these two parameters ($P = 0.018$, $r = 0.227$). LC3 expression in pancreatic cancer tissues with PNI was significantly higher than that in pancreatic cancer tissues without PNI (Table 1).

Relationship between pancreatic cancer cell autophagy and PNI and clinicopathological features

LC3 expression was associated with lymph node metastasis ($P < 0.05$). LC3 expression was not related to sex, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status. PNI was related to lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$) and was not related to sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status (Table 2).

Multivariate logistic regression analysis of LC3 expression and PNI

The clinicopathological factors possibly associated with LC3 expression and PNI were evaluated by multivariate analysis using a logistic regression model. The clinicopathological factors included LC3 expression and nerve infiltration, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, diabetes, pancreatitis, and preoperative CA19-9 level. The results showed that LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 level were the factors that influenced the occurrence of PNI, which was an independent factor affecting LC3 expression (Tables 3 and 4).

Survival analysis

According to LC3 expression, patients were divided into a high-expression or a low-expression group. The overall survival rate of the low-expression group was better than that of the high-expression group, and the risk of death was 2.78-times higher in the LC3 high-expression group than that in the low-expression group. This difference between the two groups was significant (Figure 2A). The patients were also

Table 2 Relationships of perineural invasion and microtubule-associated protein 1A/1B-light chain 3 expression with clinicopathological features

Parameter	*n*	PNI		*P* value	LC3		*P* value
		Absent	Present		Low expression	High expression	
Age (yr)							
≤ 58	54	19	35	0.373	23	31	0.206
> 58	55	15	40		17	38	
Gender							
Male	61	15	46	0.093	26	35	0.148
Female	48	19	29		14	34	
Tumor location							
Head	66	17	49	0.129	28	38	0.124
Body/tail	43	17	26		12	31	
Tumor size (cm)							
≤ 2	18	6	12	0.83	8	10	0.455
> 2	91	28	63		32	59	
Histologic grade							
Well or moderate	74	24	50	0.685	23	51	0.077
Poor	35	10	25		17	18	
Vascular invasion							
Negative	83	26	57	0.957	31	52	0.801
Positive	26	8	18		9	17	
Lymph node metastasis							
Negative	36	17	19	0.011[a]	18	18	0.043[a]
Positive	73	17	56		22	51	
AJCC stage							
I + II	69	22	47	0.838	25	44	0.895
III + IV	40	12	28		15	25	
Pancreatitis							
Negative	30	15	15	0.009[a]	12	18	0.659
Positive	79	19	60		28	51	
Diabetes							
Negative	89	27	62	0.684	33	56	0.862
Positive	20	7	13		7	13	
CA19-9 level (U/mL)							
≤ 37	37	19	19	0.001[a]	13	24	0.808
> 37	72	15	57		27	45	

[a]*P* < 0.05, LC3 low expression *vs* high expression; PNI absent *vs* present. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

Table 3 Multivariate logistic regression analysis of perineural invasion with clinicopathological features in pancreatic cancer

Parameter	Estimate, B	Standard error	Wald statistic	*P* value	Odds ratio	95%CI
Lymph node metastasis (positive *vs* negative)	1.068	0.499	4.581	0.032[a]	2.911	1.094-7.743
CA199 (> 37 U/mL *vs* ≤ 37 U/mL)	1.508	0.493	9.368	0.002[a]	4.520	1.720-11.874
Pancreatitis (present *vs* absent)	1.301	0.514	6.419	0.011[a]	3.673	1.343-10.049
LC3 (high *vs* low)	1.032	0.491	4.406	0.036[a]	2.806	1.071-7.351
Constant	-7.209	1.799	16.058	0	0.001	

[a]*P* < 0.05. CI: Confidence interval; LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

divided into a nerve-invasion group and a no-nerve-invasion group according to whether there was nerve infiltration. The overall survival rate of the patients without nerve invasion was better than that of the nerve-invasion group. The risk of death was 2.93-times greater in the PNI-positive group than in the PNI-negative group, and the difference between the two groups was significant (Figure 2B). Univariate analysis showed that CA19-9 level, PNI, and LC3 expression influenced the prognosis (Table 5). A factor of *P* < 0.2 was added into the COX risk regression model. Multivariate analysis was performed using the stepwise conditional method. The results showed that PNI and LC3 expression were independent prognostic factors that influenced pancreatic cancer (Table 6).

DISCUSSION

Pancreatic cancer has a poor treatment outcome because of a low resection rate, early invasion and metastasis, and insensitivity to radiotherapy and chemotherapy[10-13]. PNI is common in pancreatic cancer, and also found in breast, prostate, and rectal cancers[14]. Many studies have suggested that PNI is the main cause of abdominal pain in patients and is also one of the important causes of local recurrence of pancreatic

Table 4 Multivariate logistic regression analysis of microtubule-associated protein 1A/1B-light chain 3 expression with clinicopathological features in pancreatic cancer

Parameter	Estimate, B	Standard error	Wald statistic	*P* value	Odds ratio	95%CI
PNI (present *vs* absent)	0.997	0.427	5.451	0.02[a]	2.71	1.174-6.259
Constant	-1.115	0.732	2.316	0.128	0.328	

[a]$P < 0.05$. PNI: Perineural invasion.

Table 5 Univariate analysis of survival in patients who underwent radical surgery

Parameter	Hazard ratio	95%CI	*P* value
PNI (present *vs* absent)	3.701	1.539-8.903	0.003[a]
LC3 (high *vs* low)	3.196	1.433-7.126	0.005[a]
Sex (male *vs* female)	1.154	0.590-2.260	0.676
Age (> 58 yr *vs* ≤ 58 yr)	1.176	0.621-2.225	0.619
Tumor location (body/tail *vs* head)	1.102	0.570-2.131	0.773
Histologic grade (poorly *vs* well or moderate)	1.287	0.636-2.604	0.484
Tumor size (> 2 cm *vs* ≤ 2 cm)	0.940	0.444-1.991	0.871
Vascular invasion (positive *vs* negative)	1.821	0.883-3.755	0.105
Lymph node metastasis (positive *vs* negative)	0.871	0.449-1.688	0.682
AJCC stage (III + IV *vs* I + II)	1.473	0.752-2.889	0.259
Diabetes (present *vs* absent)	1.105	0.522-2.337	0.795
Pancreatitis (present *vs* absent)	1.075	0.520-2.222	0.845
CA19-9 level (> 37 U/mL *vs* ≤ 37 U/mL)	2.648	1.286-5.454	0.008[a]

[a]$P < 0.05$. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

cancer[15-19]. PNI is a continuous process involving multiple molecular factors and tumor microenvironment, but it is unclarified how cancer cells maintain their survival and proliferation from pancreatic cancer tissues to the external pancreatic plexus[20]. Autophagy is the process of degrading cytoplasmic proteins or organelles through lysosomes. Under physiological conditions, autophagy plays a major role in maintaining the intracellular environment stability[21,22]. Autophagy is an important mechanism of escaping apoptosis for tumor cells. Moreover, autophagy may mediate resistance to chemotherapy in pancreatic cancer[23-25]. Therefore, this study was designed and completed in a retrospective manner to evaluate the relationship between pancreatic cancer cell autophagy and PNI and patient survival.

This study found that high expression of LC3 was present in the cancer nests around the nerve infiltration, consistent with the discovery of Yang *et al*[7]. In histology terms, it has been suggested that high LC3 expression is related to PNI. Further analysis of immunohistochemical data showed that there was a significant positive correlation between LC3 expression and PNI in pancreatic cancer tissues. Therefore, we speculated that, in the PNI process, a high autophagy level may assist cancer cells in escaping apoptosis, avoiding the damage of adverse stress and providing energy for the invasion and metastasis of pancreatic cancer.

There is dissidence in the correlation between PNI and lymph node metastasis[26-28]. This study showed that PNI was associated with lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$), while it had no association with sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus. LC3 was related to lymph node metastasis but no other clinicopathological features. Multivariate logistic regression analysis also showed that LC3, lymph node metastasis, pancreatitis, and CA19-9 levels were the factors that influenced PNI, while PNI was an independent factor affecting LC3 expression. Tanaka *et al*[26] and Ozaki *et al*[28] believe that lymph node metastasis can promote PNI. The cancer cells in lymph node metastases can form a lymphatic satellite around the nerve and break through the nerve membrane; then, nerve invasion occurs. This study is consistent with results suggesting the need for regional lymph node dissection during surgical treatment[27]. Through this study, we can conclude that high LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 levels usually indicate the possibility of nerve invasion. Gender, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, and diabetes are not effective indicators of neural invasion and autophagy and cannot be used as a determinant of resection of the peripancreatic nerve

Table 6 Multivariable analysis of survival in patients who underwent radical surgery

Parameter	Hazard ratio	95%CI	*P* value
PNI (present *vs* absent)	2.962	1.212-7.238	0.017[a]
LC3 (high *vs* low)	2.491	1.107-5.608	0.027[a]

[a]$P < 0.05$. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

during surgery.

The overall survival rates of the LC3 high-expression group and the nerve-invasion group were significantly lower than those of the low-expression group and the no-nerve-invasion group. Univariate analysis showed that the level of CA19-9, PNI, and autophagy were associated with prognostic factors. Multivariate analysis showed that PNI and high LC3 expression were independent prognostic factors in pancreatic cancer patients. Autophagy is very complex and often plays an important role in tumor progression[6,29]. Interestingly, this study found that high autophagy level is closely related to PNI, while both of which are independent risk factors for pancreatic cancer with a poor prognosis. The autophagy associated with poor survival in pancreatic cancer could be explained by the properties of autophagy assisting cancer cells to evade stress-induced apoptosis in PNI environment and undoubtedly promote tumor cell survival[30,31]. Therefore, the high autophagy of cancer cells may promote the malignant progression of pancreatic cancer, resulting in PNI and the poor treatment outcome in patients with the disease.

In summary, autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. How to control the role of autophagy in PNI of pancreatic cancer and then improve cancer prognosis requires further studies into the molecular mechanisms involved.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. At the time of diagnosis, most pancreatic cancer patients have distant metastasis due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is 80%-100% and is an important factor leading to postoperative pancreatic cancer recurrence. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery. However, the pathogenesis of PNI has not yet been defined. Autophagy, as a mechanism of avoidance of anoikis in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells. In addition, a previous study showed that pancreatic cancer cells with PNI have higher levels of autophagy. No study has examined the relationship between autophagy and PNI in pancreatic cancer cells.

Research motivation

The relationship between autophagy and PNI was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to LC3 expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, which contributes to pancreatic cancer progression. This study might provide new insight into the mechanism of PNI in pancreatic cancer.

Research objectives

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features, and prognosis. The authors found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis, which requires further studies into the molecular mechanisms involved.

Research methods

Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent radical resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. Expression levels of the autophagy-related protein LC3 and perineural invasion marker ubiquitin carboxy-terminal hydrolase in pancreatic cancer tissues were detected by immunohistochemistry. The correlations among LC3 expression, perineural invasion, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed for further survival analysis.

Research results

In this study, they found that LC3, lymph node metastasis, pancreatitis, and CA199 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor of high LC3 expression. Perineural invasion and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer. There is a significant correlation between them.

Research conclusions

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features and prognosis. The authors found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there must be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis, which requires further studies into the molecular mechanisms involved.

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ORIGINAL ARTICLE

Basic Study

Dachaihu decoction ameliorates pancreatic fibrosis by inhibiting macrophage infiltration in chronic pancreatitis

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Author contributions: Zhang H designed the research; Duan LF and Xu XF contributed equally to the work, and both performed most of the experiments and wrote the paper; Zhu LJ, Wu N, Fan JW and Xin JQ performed the experiments; Zhang XQ and Liu F analyzed the data.

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Abstract

AIM

To explore the role of macrophages in chronic pancreatitis (CP) and the effect of Dachaihu decoction (DCHD) on pancreatic fibrosis in mice.

METHODS

KunMing mice were randomly divided into a control group, CP group, and DCHD group. In the CP and DCHD groups, mice were intraperitoneally injected with 20% L-arginine (3 g/kg twice 1 d/wk for 6 wk). Mice in the DCHD group were administered DCHD intragastrically at a dose of 14 g/kg/d 1 wk after CP induction. At 2 wk, 4 wk and 6 wk post-modeling, the morphology of the pancreas was observed using hematoxylin

and eosin, and Masson staining. Interleukin-6 (IL-6) serum levels were assayed using an enzyme-linked immunosorbent assay. Double immunofluorescence staining was performed to observe the co-expression of F4/80 and IL-6 in the pancreas. Inflammatory factors including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and IL-6 were determined using real time-polymerase chain reaction. Western blot analysis was used to detect fibronectin levels in the pancreas.

RESULTS

Compared with the control group, mice with 20% L-arginine-induced CP had obvious macrophage infiltration and a higher level of fibrosis. IL-6 serum concentrations were significantly increased. Double immunofluorescence staining showed that IL-6 and F4/80 were co-expressed in the pancreas. With the administration of DCHD, the infiltration of macrophages and degree of fibrosis in the pancreas were significantly attenuated; IL-6, MCP-1 and MIP-1α mRNA, and fibronectin levels were reduced.

CONCLUSION

The dominant role of macrophages in the development of CP was mainly related to IL-6 production. DCHD was effective in ameliorating pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion in the pancreas.

Key words: Dachaihu decoction; Pancreatic fibrosis; Macrophages; Interleukin-6

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Core tip: Macrophages, important inflammatory cells, can also promote fibrogenesis by interfering with the synthesis and degradation of the extracellular matrix, as confirmed in both liver fibrosis and renal fibrosis models. Our study suggested that macrophages also play an important role in the development of pancreatic fibrosis. We found that macrophages are an important source of interleukin-6, which is involved in the progression of chronic pancreatitis (CP). Dachaihu decoction (DCHD), a traditional Chinese medicinal formula, effectively improves the clinical symptoms of CP patients, but the underlying mechanism remains unclear. We found that DCHD ameliorates pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion.

Duan LF, Xu XF, Zhu LJ, Liu F, Zhang XQ, Wu N, Fan JW, Xin JQ, Zhang H. Dachaihu decoction ameliorates pancreatic fibrosis by inhibiting macrophage infiltration in chronic pancreatitis. *World J Gastroenterol* 2017; 23(40): 7242-7252 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7242.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7242

INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease that is characterized by irreversible injury of the pancreas leading to endocrine and exocrine dysfunction[1]. It has been reported that pancreatic fibrosis is a common pathological feature and characterizes the end-stage of CP of different etiologies[2]. The progressive fibrotic cascade can eventually lead to loss of pancreatic function as well as systemic complications, including malabsorption, diabetes mellitus, and others. However, the underlying molecular mechanisms of CP remain unclear, and the available therapeutic strategies are very few in number[3].

Based on the necrosis-fibrosis sequence playing a key role in the underlying pathogenesis of CP, pancreatic fibrosis commonly arises from inflammation or tissue injury[4]. Recent studies have highlighted the role of macrophages in promoting wound healing and fibrosis[5,6]. Large numbers of macrophages have been observed around areas of pancreatic injury in rat models of CP[7,8]. Detlefsen and colleagues[9] demonstrated that many infiltration macrophages are also found in the pancreas of alcoholic CP patients, which facilitate fibrosis formation by producing transforming growth factor (TGF)-β and platelet derived growth factor (PDGF)-β. Thus, we propose that macrophages may play a critical role in the progression of pancreatic fibrosis, but its definite mechanism remains unclear.

Interleukin-6 (IL-6) is a key pro-inflammatory cytokine that is involved in inflammation and the immune response. It is mainly released by macrophages, fibroblasts, endothelial cells, and myeloid cells[10,11]. In a previous study, Zhang *et al*[12] found that acinar injury results in the infiltration of myeloid cells (*i.e.*, macrophages and neutrophils) into the pancreas during severe acute pancreatitis, and infiltrating macrophages further release IL-6, which can lead to pancreatitis-associated acute lung injury. A study by Lesina *et al*[13] demonstrated that tumor-associated macrophages appear to be the major source of IL-6 in murine and human pancreatic ductal adenocarcinoma. Several reports have confirmed that serum level of IL-6 in patients with CP was significantly increased compared with that in control subjects[12,14]. However, the contribution of IL-6 and the relationship between IL-6 and macrophages in pancreatic fibrosis have not been analyzed in detail.

Dachaihu decoction (DCHD; major Radix bupleuri decoction) is a traditional Chinese medicine formula that was first described by Zhongjing Zhang in "Treatise on Febrile Disease Caused by Cold (Shanghan Lun)". DCHD has been widely used in the clinical treatment of acute pancreatitis (AP)[15]. Recently, it was used to treat patients with CP, and it was found that the effects included improving the clinical symptoms of CP

patients. But whether it can inhibit pancreatic fibrosis and its underlying mechanism remain uncertain. The aim of the present study was to ascertain the role of macrophages in pancreatic fibrosis induced by 20% L-arginine, as well as to determine the effects and mechanism of DCHD on inhibiting pancreatic fibrosis.

MATERIALS AND METHODS

Animals and materials

KunMing mice (20-28 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Certificate No. 0011744) and housed under specific-pathogen-free conditions. The mice received humane care in accordance with the Shaanxi University of Chinese Medicine Animal Care Committee guidelines. Antibodies against fibronectin (FN), F4/80, and IL-6 were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States). All secondary antibodies and immunofluorescence and immunohistochemistry reagents were purchased from Boster Biotechnology Inc. (Wuhan, China). L-arginine was obtained from Sigma-Aldrich Co. (St. Louis, MO, United States). Crude DCHD, including Radix bupleuri, Radix scutellariae, Fructus aurantii immaturus, Paeonia lactiflora, Rhizomapinelliae, Rheum palmatum, Rhizoma zingiberis recens and Fructus jujubae (Table 1), was purchased from the Department of Pharmaceutical Preparation of Chinese Medicine of Shaanxi University. The components of DCHD were extracted twice by boiling in distilled water for 1 h, and the drug was subsequently filtered and reduced by boiling to a volume of 70 mL (1 g crude drug/mL) and stored at 4 ℃ for later use. Masson staining and amylase enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Nanjing Jiancheng Biotechnology Company (Nanjing, China). Reverse transcriptase polymerase chain reaction (PCR) kits and primers were provided by Transgen Biotech Co. (Beijing, China).

L-arginine-induced chronic pancreatitis

The KunMing mice were randomly divided into three groups (n = 36): the control group, the CP group, and the DCHD group. Mice in the CP and DCHC groups were injected intraperitoneally with 20% L-arginine (two 3 g/kg injections separated by 1 h, weekly for 6 wk), while the mice in the control group were injected with saline. Mice in the DCHD group were treated with DCHD (14 g/kg per day) intragastrically 1 wk after the 20% L-arginine injections.

Serum and tissue sample collection

Mice were anesthetized and sacrificed at 2 wk, 4 wk and 6 wk after 20% L-arginine or saline injection (n = 9 at each time point). Blood samples were obtained from the inferior vena cava, centrifuged at 1500 rpm for 10 min, and serum was collected and stored at -80 ℃ for IL-6 analysis. The pancreas was removed, weighed, and mixed well. One part of the pancreas was fixed in 4% formaldehyde solution for hematoxylin and eosin (HE) and immunofluorescence staining, while the other part was frozen in liquid nitrogen and stored at -80 ℃ for RNA and protein extraction.

ELISA test

Plasma was collected at 2 wk, 4 wk and 6 wk after modeling and serum was obtained by centrifuging samples at 1500 rpm for 10 min. IL-6 levels were evaluated using an ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histology and HE and Masson staining

To observe morphological and fibrosis changes in the pancreas, pancreatic sections were subjected to HE or Masson staining. Samples were dehydrated and embedded in paraffin, sectioned (3 μm) after fixation in 4% formaldehyde solution for 12 h. Images were collected using an optical microscope.

Immunohistochemistry and immunofluorescence staining

Immunohistochemical staining and immunofluorescence staining were performed. For immunohistochemistry, antibodies were applied at the following concentrations: F4/80, 1:150 dilution, overnight at 4 ℃; and biotinylated secondary antibody, 1:400 dilution, for 1 h. Bound peroxidase was visualized after a 3,3-diaminobenzidine reaction for 2-5 min and after light counterstaining with hematoxylin, dehydration, and mounting. The slides were examined using a Zeiss Axio1 imager (Carl-Zeiss-Promenade, Jena, Germany). For immunofluorescence, antibodies were applied at the following concentrations: F4/80 and IL-6 antibodies, 1:150 dilution, overnight at 4 ℃ in the dark; FITC-labeled and PE-labeled secondary antibody, 1:400 dilution, for 1 h. DAPI (1:1000) was used to counterstain the nuclei for 5-15 s, and the slides were mounted on coverslips with anti-fade mounting medium. Images were collected using a fluorescent microscope (Olympus IX51, Shinjuku-ku, Tokyo, Japan).

RNA isolation and real-time PCR

Total RNA of pancreatic tissue was extracted using the Trizol reagent (RNeasy Minicolumns, Transgen, Beijing, China) and reverse- transcribed into cDNA using reverse transcriptase kits. RT-PCR was performed using SYBR Green reaction mix and the ABI-7500 Sequence Detection System (Thermo Fisher Scientific, Shanghai, China). The primers, number of cycles, and annealing temperatures are shown in Table 2.

Protein extraction and western blotting

Protein was extracted from pancreas tissue using standard methods. The protein concentrations were measured using a BCA protein assay kit (Boster

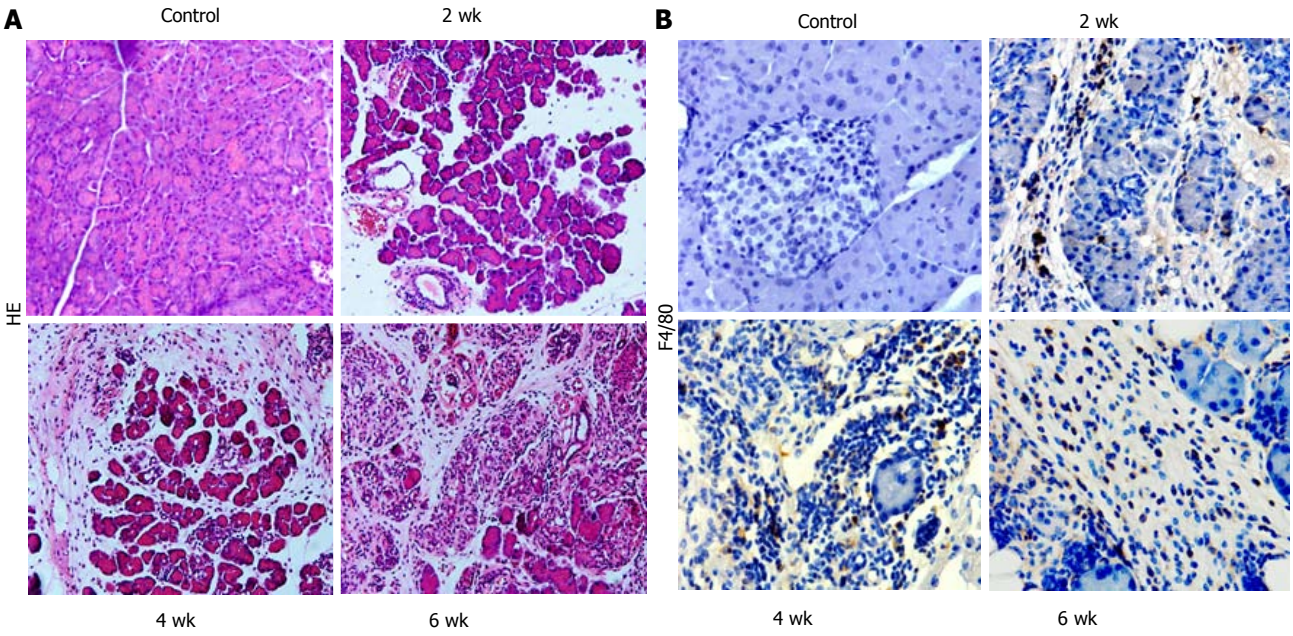


Figure 1 Morphological changes and macrophage infiltration in pancreatic sections obtained from mice with chronic pancreatitis induced by L-arginine. A: Pancreatic morphology at different time points of L-arginine induction using hematoxylin and eosin staining (original magnification, × 100); B: Macrophage infiltration in pancreas was detected using immunohistochemistry. Macrophages were stained using an anti-mouse F4/80 antibody (original magnification, × 200).

Table 1 Contents of Dachaihu decoction

Chinese name	Botanical name	Common name	Genus	Family	Weight, g	Part used
Chai hu	*Bupleurumabchasicum Manden*	Radix bupleuri	Bupleurum	umbelliferae	15	Root
Huang qin	*Scutellaria baicalensis Georgi baicalensis*	Radix scutellariae	Scutellaria	Labiatae	9	Root
Zhishi	*Citrus × aurantium L.*	Fructus aurantii immaturus	Citrus	Rutaceae	9	Fruit
Shaoyao	*Paeonia × suffruticosa Andrews*	Paeonia lactiflora	Paeonia	Paeoniaceae	9	Root
Ban xia	*Pinelliaternate (Thunb.) Makino*	Rhizomapinelliae	pinellia	Araceae	9	Root and rhizoma
Da huang	*Rheum palmatum L.*	Rheum palmatum	Rheum	polygonaceae	6	Root and rhizome
Sheng jiang	*Zingiber officinale Roscoe*	Rhizoma zingiberis recens	Zingiber Adans .	zingiberaceae	15	Root and rhizome
Da zao	*Ziziphus jujube MIll*	Fructus jujuba	Ziziphus	Rhamnales	20	fruit

Biotechnology Inc.) and adjusted to 4 μg/μL. Before loading, the samples were boiled at 95 ℃ for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, United States), and transferred onto PVDF membranes (EMD Millipore, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk followed by incubation with anti-actin (Boster Biotechnology Inc.) and anti-FN antibodies (sc-101759; Santa Cruz Biotechnology Inc.). The sizes of the detected bands were determined by reference to a 10-180 kDa protein marker ladder.

Statistical analysis

Values are presented as the mean ± SD and were analyzed using the unpaired Student's *t*-test and one-way analysis of variance (commonly known as ANOVA). A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Macrophage infiltration is increased in CP

We induced CP in mice using repeated 20% L-arginine injections over 6 wk. Two weeks after modeling, morphological signs of CP were found, including leukocyte infiltration, acinar cell necrosis, and a small amount of connective tissue deposition. At 4 wk and 6 wk, loss of acinar cells, collagen deposition, and aggravated fibrosis in the pancreas were observed (Figure 1A).

Immunohistochemistry using the macrophage marker F4/80 was performed. Two weeks after L-arginine injection, F4/80-positive cells were predominant in mice with CP compared with the normal group (Figure 1B). The positive macrophage ratio was highest at week 4, and levels were sustained until week 6. Thus, macrophages were abundant during CP and

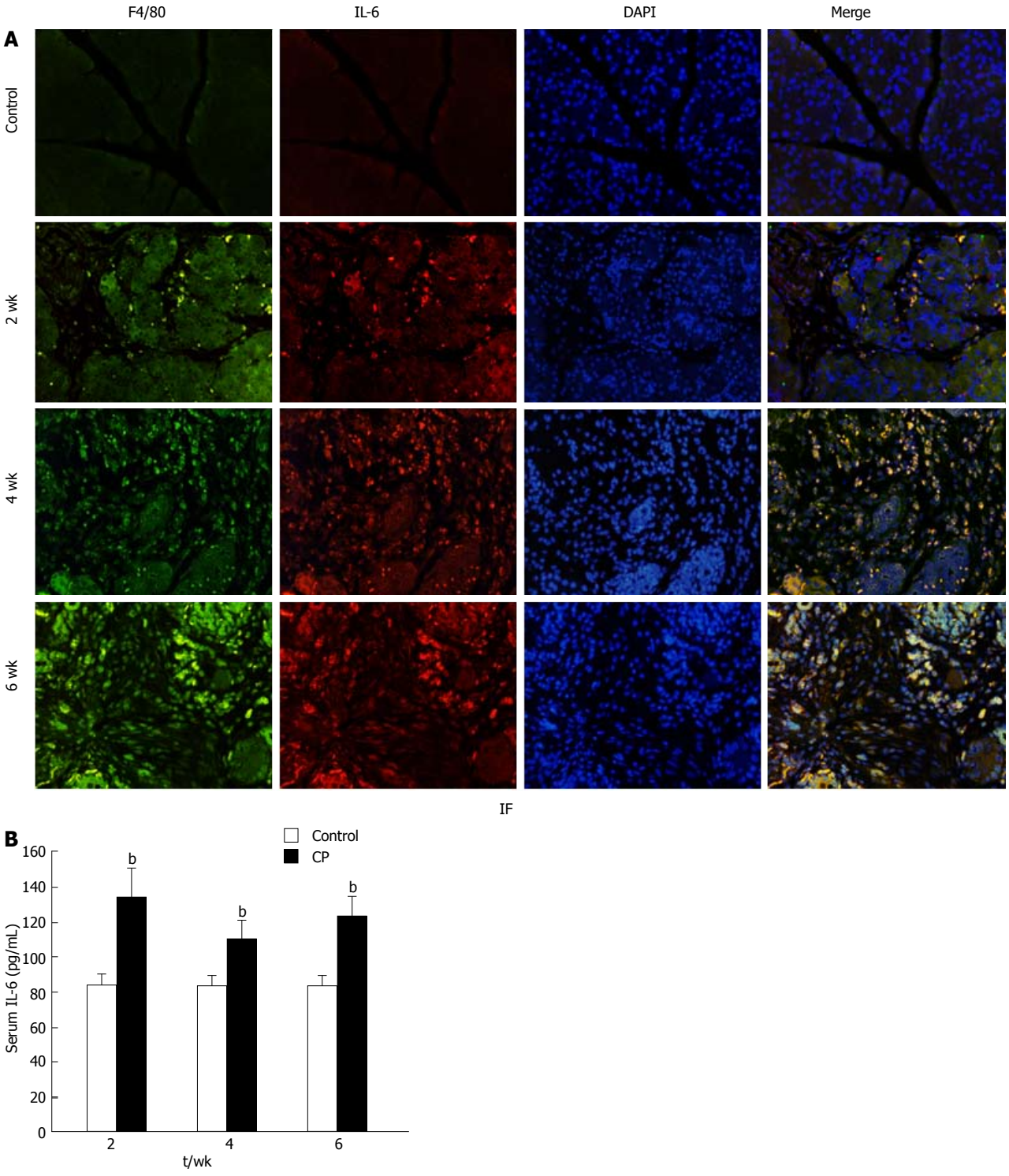


Figure 2 Interleukin-6 production is related to macrophage infiltration. A: Double immunofluorescent signals from F4/80 and interleukin-6 in L-arginine-induced chronic pancreatitis tissue at different time points (original magnification, × 200); B: Interleukin-6 levels in the sera of mice with chronic pancreatitis. Values are shown as mean ± SD (n = 36). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

were involved in the development of fibrosis (Figure 1B).

Macrophage infiltration in the pancreas results in IL-6 production

The serum concentration of IL-6 was significantly increased 2 wk after 20% L-arginine injection. At 4 wk, the IL-6 level was somewhat decreased but remained elevated to week 6 (Figure 2A). These findings demonstrate that high IL-6 levels contribute to the development of CP.

While macrophage infiltration and IL-6 levels were increased in CP, their relationship remains unclear. To determine whether IL-6 is mainly secreted by

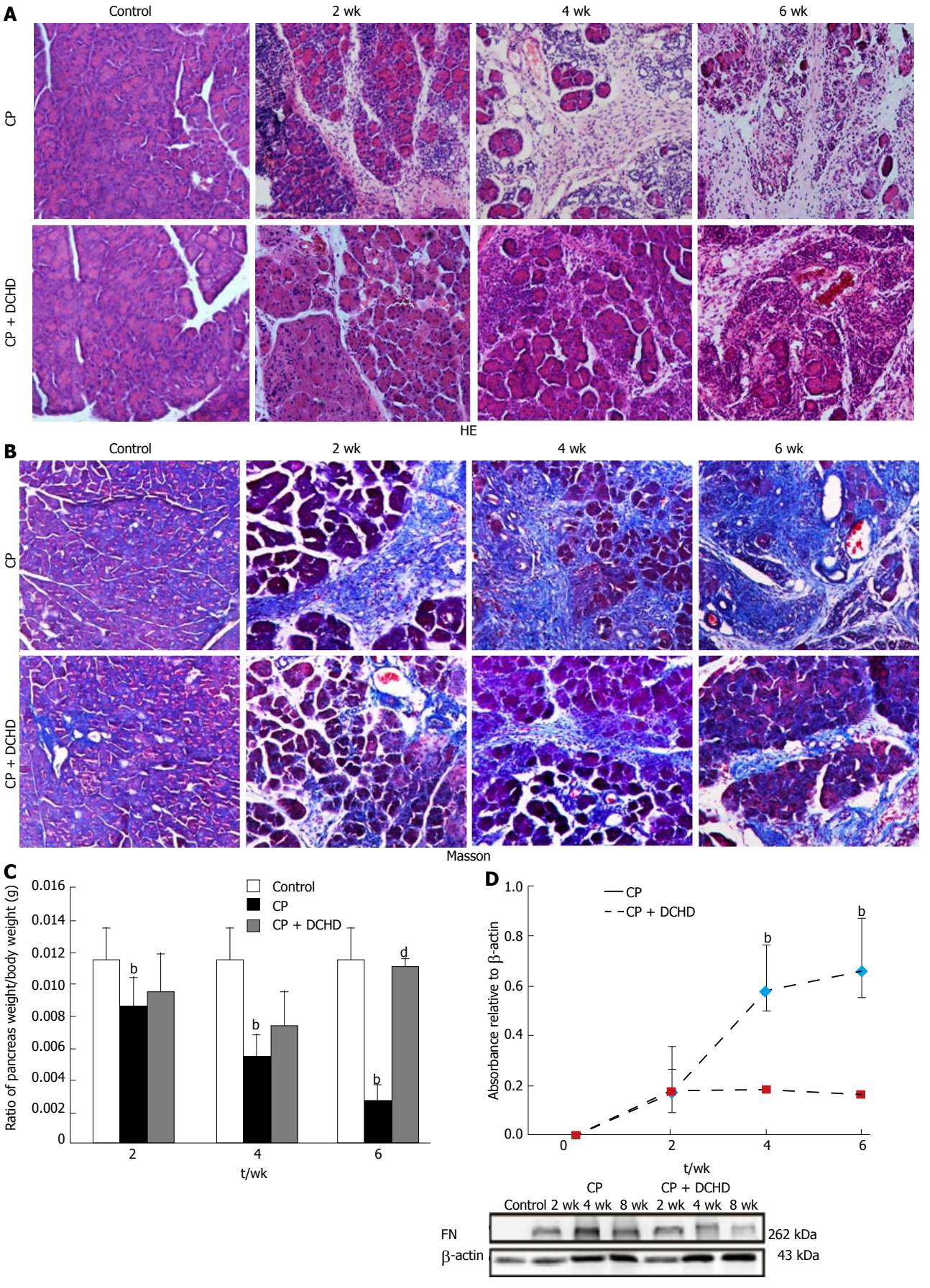


Figure 3 Dachaihu decoction protects against pancreatic injury and pancreatic fibrosis in mice with chronic pancreatitis. A: Changes in pancreatic morphology at different time points were evaluated by hematoxylin and eosin staining (original magnification, × 100); B: Collagen deposition was detected by Masson staining (original magnification, × 100); C: Ratio of pancreas weight/body weight changes in the different groups; D: The expression of fibronectin in the pancreas following the development of chronic pancreatitis. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

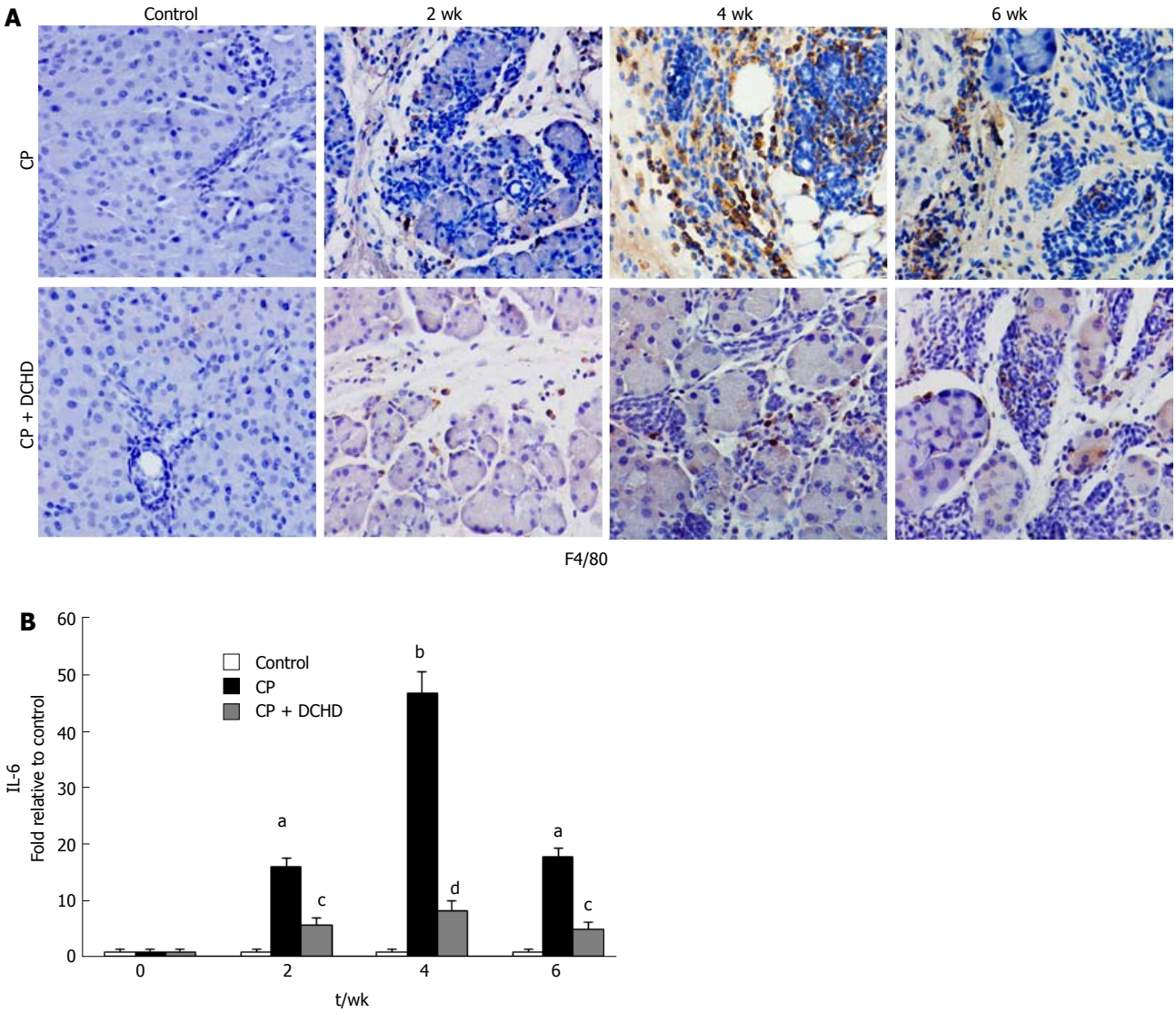


Figure 4 Dachaihu decoction attenuated macrophage infiltration and interleukin-6 expression in the pancreas. A: Immunohistochemistry of macrophages associated with chronic pancreatitis (original magnification, × 200); B: Interleukin-6 mRNA levels in the pancreas determined by RT-PCR. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

Table 2 Primer sequences used for real time-polymerase chain reaction

Gene	Forward primer	Reverse primer	Cycles	Temperature, ℃
IL-6	5′-GAAGTAGGGAAGGCCGTGG-3′	5′-CTCTGCAAGAGACTTCCATCCAGT-3′	40	60
MCP-1	5′-GTTGGCCTCAGCCAGATGCA-3′	5′-AGCGTACTCATTGGGATCATCTTG-3′	40	60
MIP-1α	5′-TGCCCTTGCTHTTCTTCTCTGCACCATGGC-3′	5′-GGCAAATTCCACGAAAATTCATTGCTGACT-3′	40	60
β-actin	5′-CATCCTGCGTCTGGACCT-3′	5′-TCAGGAGGAGCAATGATCTTG-3′	40	60

macrophages, double staining of F4/80 and IL-6 labeled with different fluorescence markers was performed on pancreas paraffin sections. As shown in Figure 2B, no positive F4/80 and IL-6 cells were found in the pancreas of the control group. However, L-arginine administration resulted in a significant increase in the expression of F4/80 and IL-6 in the pancreas. While there was only weak expression in the samples at 2 wk, co-expression of F4/80 and IL-6 increased markedly at 4 wk and 6 wk. These results demonstrate that IL-6 is mainly secreted by macrophages in CP.

DCHD alleviates pancreatic injury and pancreatic fibrosis in mice with CP

We next sought to investigate whether pancreatic injury and subsequent fibrosis were alleviated by DCHD treatment. DCHD administration resulted in

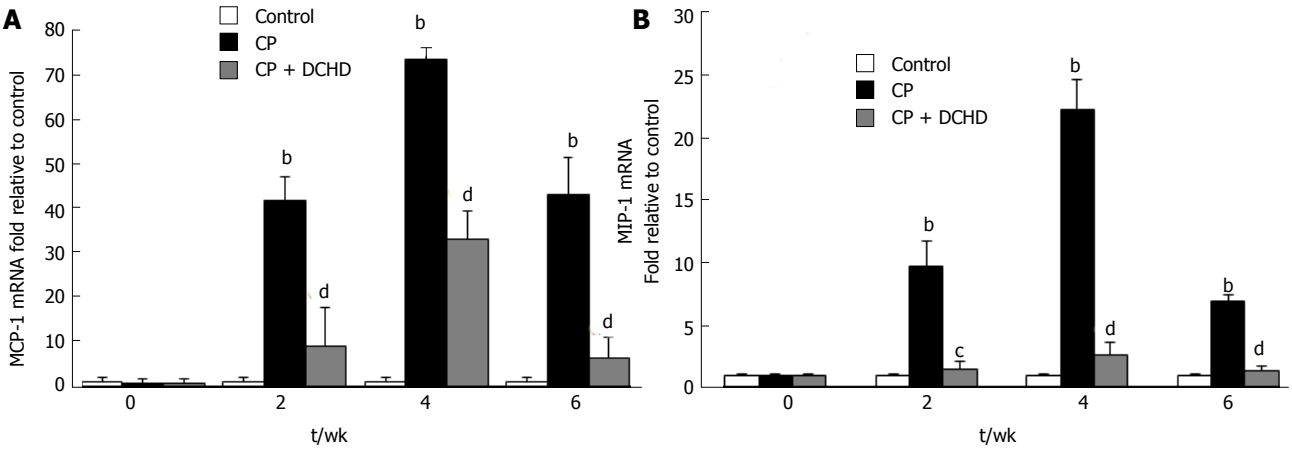


Figure 5 Effects of Dachaihu decoction on MCP-1 and MIP-1α mRNA levels in the pancreas of mice with chronic pancreatitis. A: MCP-1 mRNA levels in the pancreas as determined by real time-polymerase chain reaction; B: MIP-1α levels in the pancreas as determined by real time-polymerase chain reaction. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

significantly improved tissue damage, pancreatic fibrosis, and ratio of pancreas weight/body weight (Figure 3A-C). Moreover, Masson staining revealed that the area of pancreas fibrosis markedly decreased in the DCHD group (Figure 3B).

FN is a major indicator of extracellular matrix deposition and the degree of fibrosis. Western blotting revealed no FN expression in the pancreas of control mice; however, 20% L-arginine treatment resulted in increased FN expression in the pancreas at 2 wk, 4 wk, and (maximally) 6 wk. Interestingly, DCHD treatment attenuated FN levels compared with those in the CP group at the same time point, illustrating that DCHD can suppress the deposition of FN in pancreatic fibrosis (Figure 3D).

DCHD attenuated macrophage infiltration and IL-6 mRNA expression in the pancreas

To understand the mechanism underlying the effects of DCHD treatment in CP, we used immunohistochemistry to detect macrophage infiltration. Compared with the CP group, DCHD treatment significantly decreased pancreatic macrophage infiltration at 2 wk. Accompanied with evident and an improvement in pancreatic fibrosis at 4 wk and 6 wk, macrophage infiltration was also significantly reduced (Figure 4A). Thus, limiting macrophage accumulation in the pancreas may be an underlying mechanism of DCHD in inhibiting the development of pancreatic fibrosis.

Considering the association between IL-6 and macrophages, the expression of IL-6 mRNA in the pancreas was assessed using RT-PCR. IL-6 expression levels were increased in CP and were highest at week 4. Compared with those in the CP group, IL-6 levels in the DCHD group were significantly decreased (Figure 4B). This finding demonstrates that IL-6 is produced by macrophages and thus may be a potential treatment target for DCHD.

DCHD decreased chemokine mRNA levels were associated with macrophage infiltration

To further explore the mechanism underlying macrophage accumulation, pancreatic mRNA levels of the macrophage-associated chemokines MCP-1 and MIP-1α were detected using RT-PCR. MCP-1 and MIP-1α were up-regulated in the CP group compared with the control group at 2 wk, 4 wk and 6 wk (Figure 5A-B), while they were significantly decreased in the DCHD group at the same time points.

DISCUSSION

Generally, pancreatic fibrosis is a common pathological feature of CP. Recurrent acute pancreatic insults trigger necro-inflammation, and inflammatory cells, once in their active states, release cytokines that in turn promote fibrogenesis, which is involved in the progression of pancreatic fibrosis[16]. Recent studies have confirmed the dominant role played by activated macrophages in liver fibrosis by interfering with the synthesis and degradation of the extracellular matrix[17]. In a rat model of liver cirrhosis, activated macrophages were shown to accelerate fibrogenesis by activating hepatic myofibroblasts, while the suppression of macrophage infiltration inhibited liver fibrogenesis[17]. Treiber *et al*[18] found large numbers of macrophages among inflammatory cells infiltrating the pancreas of a mouse model of CP. In our study, the development of pancreatic fibrosis was accompanied by a significant increase in F4/80-positive cells in pancreatic tissue, suggesting that activated macrophages may play a role in the progression of pancreatic fibrosis.

A study by Saeki *et al*[19] revealed that infiltration of macrophages in AP originates mainly from peripheral blood mononuclear cells (PBMCs) due to chemokines. Our CP model was based on repetitively inducing AP, and we speculated that infiltration of macrophages

in CP was attributable to PBMCs' recruitment. MCP-1 and MIP-1α are potent C-C type chemokines that are mainly secreted by activated macrophages and fibroblasts. They recognize the C-C chemokine receptor type (CCR)-2 or CCR-3 on PBMCs and recruit macrophages to injured tissues[20,21]. Therefore, we measured MCP-1 and MIP-1α mRNA levels and found that they were up-regulated in mice with CP, which may contribute to macrophage accumulation in CP.

To obtain a deeper understanding of the role of macrophages in pancreatic fibrosis, other researchers previously stimulated PBMCs with lipopolysaccharide, and found that TGF-β1 and IL-6 were significantly elevated in culture supernatant. Moreover, further co-culture of macrophages with pancreatic stellate cells resulted in increased FN and collagen type I levels[22]. TGF-β1 is considered one of the strongest fibrosis factors[9], but the role of IL-6 in CP has not been recognized. Numerous studies have shown that IL-6 was related to fibrosis progress in the liver and lung[23,24]. Recent experiments also demonstrated that the serum level of IL-6 in CP patients was significantly increased[14].

In our present study, IL-6 levels in serum and IL-6 mRNA levels in the pancreas were also significantly increased in the progression of CP mice. These results indicated that IL-6 participates in the development of pancreatic fibrosis. To determine the source of IL-6 production, we preformed immunofluorescent double-staining of F4/80 and IL-6 to evaluate F4/80 and IL-6 expression in the pancreas and found large numbers of doubly positive cells in the pancreas of the CP group. We can thus infer that macrophage infiltration is the main source of IL-6 secretion, and an excess of IL-6 might aggravate inflammation and further accelerate pancreatic fibrogenesis.

The traditional Chinese herbal compound DCHD is composed mainly of Radix bupleuri, Radix scutellariae, Fructus aurantii immaturus, Paeonia lactiflora, Rhizomapinelliae, Rheum palmatum, Rhizoma zingiberis recens, and Fructus jujubae[25]. In the clinic, DCHD has been effective in treating the characteristics of the "shao-yang signs", such as fever, chills and rib-side fullness, and "yang-ming" signs, such as abdominal pain and fullness[15]. Therefore, DCHD is used mainly to treat acute and chronic digestive diseases, such as AP, inflammatory bowel disease, hepatitis and disorders of the gallbladder[15].

Clearly, CP, a common disease of the digestive system, is associated with the shao-yang and yang-ming signs of abdominal pain and rib-side distention. According to the traditional Chinese medical theory, many doctors consider that the mechanism underlying CP involves "liver-spleen disharmony, qi and blood stasis, and dampness-heat obstruction"[26], while the main actions of DCHD are removal of heat and toxic materials and elimination of blood stasis[27]. DCHD may be effective in treating CP. Modern medical studies have shown that petunidin and quercetin from *Radix bupleuri* exert anti-inflammatory effects and act as antioxidants[15,26,28]. Therefore, DCHD has also been used to treat patients of CP, and some related clinical symptoms such as abdominal pain, abdominal distension and loss of appetite, with significant improvement[15], but the underlying molecular mechanisms remain unclear.

In this study, the effects of DCHD on the development of pancreatic fibrosis in CP were evaluated systematically using an L-arginine-induced CP mouse model. Following DCHD administration, morphological changes indicative of CP, including pancreatic acinar cell injury, inflammatory cells infiltration, and pancreatic fibrosis were improved. This suggests that DCHD can attenuate inflammatory lesions and intervene in the progression of pancreatic fibrosis, and led us to perform further study on its underlying mechanism of action.

We next found that DCHD treatment significantly attenuated macrophage infiltration and IL-6 expression in the pancreas. Combined with our previous results, DCHD may suppress pancreatic fibrosis *via* the inhibition of macrophage infiltration, which in turn reduces the release of IL-6. In addition, MCP-1 and MIP-1α expression was also significantly decreased, suggesting that the DCHD-induced decrease in macrophage infiltration was due to a reduction in the release of macrophage-associated chemokines.

The present study suggests that macrophages play a critical role in CP and pancreatic fibrosis, and their dominant role in CP may be related to the release of cytokines, specifically IL-6. We demonstrated that macrophages are the main source of IL-6 production in CP. In addition, DCHD can ameliorate pancreatic fibrosis by inhibiting the macrophage infiltration of CP, which provides a new experimental basis for the clinical promotion of DCHD.

However, our work had certain limitations. We confirmed that the effect of macrophages on CP is related to IL-6, but the detailed mechanism of IL-6 on CP progression was not further explored. In addition, DCHD is effective in CP treatment, but its in-depth mechanism is not further studied, and these are our next steps.

ARTICLE HIGHLIGHTS

Research background

Pancreatic fibrosis is a common pathological feature characteristic of chronic pancreatitis (CP). As the necrosis-fibrosis sequence plays an important role in the underlying pathogenesis of CP, pancreatic fibrosis commonly develops after repeated inflammation. Macrophages are important inflammatory cells that can also promote fibrogenesis by interfering with the synthesis and degradation of the extracellular matrix. This has been confirmed in models of liver fibrosis and renal fibrosis, but any role of macrophages in pancreatic fibrosis has remained unclear. A traditional Chinese medicinal formula, Dachaihu decoction (DCHD), has been widely used in the clinical treatment of AP, and recently, DCHD was

used to treat CP, which significantly improved the patient's clinical symptoms, but, again, the underlying mechanism has been unclear.

Research motivation

CP is a progressive inflammatory disease characterized by irreversible injury to the pancreas leading to endocrine and exocrine dysfunctions that pose serious threats to human health, because the pathogenesis is unclear, and few treatments are available. Recent studies suggested that DCHD can significantly improve the clinical symptoms of CP patients, such as abdominal pain, abdominal distension, loss of appetite and others. However, DCHD cannot be widely used clinically because the therapeutic mechanism of action remains unclear.

Research objectives

The aim was to explore the role played by macrophages in the pathogenesis of CP to further elucidate the mechanisms of CP. In addition, they assessed the efficacy of DCHD in treating CP in animal experiments to determine the mechanism involved, providing an experimental basis for promoting the use of DCHD in clinical practice.

Research methods

KunMing mice were randomly divided into a control group, CP group, and DCHD group. In the CP and DCHD groups, mice were intraperitoneally injected with 20% L-arginine (3 g/kg twice a day, 1 d a week). Mice in the DCHD group were given DCHD (14 g/kg per day) intragastrically for 1 wk after CP induction. The animals were anesthetized and sacrificed at 2 wk, 4 wk and 6 wk after study commencement. Pancreatic morphology and the extent of fibrosis were assessed using hematoxylin and eosin and Masson staining. Serum interleukin-6 (IL-6) levels were assessed by enzyme-linked immunosorbent assay. Double immunofluorescence staining was performed to assess the co-expression of F4/80 and IL-6 in the pancreas. The mRNA levels of inflammatory factors including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and IL-6 were determined by real time-polymerase chain reaction. Western blotting was used to measure fibronectin (FN) levels in the pancreas.

Research results

We found that macrophage infiltration was significantly increased in the CP group, and the IL-6 levels in serum and IL-6 mRNA levels in the pancreas were also increased in the CP group. Further, immunofluorescent double-staining of F4/80 and IL-6 revealed that macrophages were the main source of the IL-6. In addition, DCHD ameliorated pancreatic fibrosis, inhibited the pancreatic infiltration of macrophages in the CP group, and reduced the release of cytokines IL-6, MCP-1 and MIP-1α mRNA, and FN levels. However, our work had certain limitations. We confirmed that the effect of macrophages on CP is related to IL-6, but the detailed mechanism of IL-6 on CP progression was not further explored. In addition, DCHD is effective in CP treatment, but its in-depth mechanism was not studied further.

Research conclusions

The authors confirmed that macrophages are involved in the development of pancreatic fibrosis and may constitute a new therapeutic target. In addition, DCHD can ameliorate pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion in the pancreas.

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ORIGINAL ARTICLE

Basic Study

Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression

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Abstract

AIM

To investigate the protective effect of prostaglandin E1 (PGE1) against endoplasmic reticulum (ER) stress-induced hepatocyte apoptosis, and to explore its underlying mechanisms.

METHODS

Thapsigargin (TG) was used to induce ER stress in the human hepatic cell line L02 and hepatocarcinoma-derived cell line HepG2. To evaluate the effects of PGE1 on TG-induced apoptosis, PGE1 was used an hour prior to TG treatment. Activation of unfolded protein response signaling pathways were detected by western blotting and quantitative real-time RT-PCR. Apoptotic index and cell viability of L02 cells and HepG2 cells were determined with flow cytometry and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium] assay.

RESULTS

Pretreatment with 1 μmol/L PGE1 protected against TG-induced apoptosis in both L02 cells and HepG2 cells. PGE1 enhanced the TG-induced expression of C/EBP homologous protein (CHOP), glucose-regulated protein (GRP) 78 and spliced X box-binding protein 1 at 6 h. However, it attenuated their expressions after 24 h. PGE1 alone induced protein and mRNA expressions of GRP78; PGE1 also induced protein expression of DNA damage-inducible gene 34 and inhibited the expressions of phospho-PKR-like ER kinase, phospho-eukaryotic initiation factor 2α and CHOP. Treatment with protein kinase A (PKA)-inhibitor H89 or KT5720 blocked PGE1-induced up-regulation of GRP78. Further, the cytoprotective effect of PGE1 on hepatocytes was not observed after blockade of GRP78 expression by H89 or small interfering RNA specifically targeted against human GRP78.

CONCLUSION

Our study demonstrates that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Key words: Hepatocytes; Endoplasmic reticulum stress; Thapsigargin; Glucose-regulated protein 78; Protein kinase A; Apoptosis

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Core tip: The mechanism underlying the hepatoprotective effect of prostaglandin E1 (PGE1) remains unclear. In this study, we found that pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP)78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression with either H89 or siRNA. Our study demonstrates for the first time that PGE1 protects against endoplasmic reticulum stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Yang FW, Fu Y, Li Y, He YH, Mu MY, Liu QC, Long J, Lin SD. Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression. *World J Gastroenterol* 2017; 23(40): 7253-7264 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7253.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7253

INTRODUCTION

Hepatocyte apoptosis can be triggered by intra- or extracellular signals[1]. The intracellular signals for hepatocyte apoptosis are induced by DNA damage, oxidative stress, growth factor deprivation, mitochondrial dysfunction, ATP depletion and endoplasmic reticulum (ER) stress[2,3]. A complex interaction occurs among these intracellular apoptotic signaling pathways. ER stress is known to induce hepatocyte apoptosis under various pathological conditions[4]. ER stress is implicated in the pathogenesis of various liver diseases, such as obesity-associated fatty liver disease[5,6], viral hepatitis[7], alcohol-induced liver injury[8], drug-induced liver injury[9] and ischemia/reperfusion injury of the liver[10,11]. Devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

ER is a multifunctional intracellular organelle responsible for the synthesis, processing and trafficking of proteins that are essential for cell growth and survival. ER also serves as a storage organelle for calcium[12]. When the homeostasis of ER is disturbed under various pathophysiological conditions, ER stress is induced and the unfolded protein response (UPR) is activated. The UPR activates three ER transmembrane transducers: inositol-requiring enzyme (IRE) 1a, PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6α[12-14]. Activation of these three UPR pathways enhances the ER's protein folding *via* up-regulation of the synthesis of glucose-regulated protein (GRP) 78. UPR signals also accelerate the degradation of misfolded proteins and reduce the synthesis of new proteins. Therefore, UPR during ER stress facilitates restoration of homeostasis. However, sustained or unresolved ER stress can activate a cascade of apoptotic signals that eventually result in cell death[15,16].

In several experimental models of liver injury, prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis[17-19]. PGE1 is also effective in the treatment of patients with fulminant hepatitis and those with primary graft non-function after liver transplantation[20,21]. Thus, PGE1 appears to protect hepatocytes against apoptosis through various mechanisms[22-24]. However, the underlying mechanism of its hepatoprotective effect is not well understood.

In two recent studies, PGE1 was shown to induce expressions of heat-shock protein (HSP) and GRP78 in animal models of liver injury caused by ischemia reperfusion and hepatectomy[25,26]. These findings suggest that modulation of UPR may mediate the hepatoprotective effects of PGE1. However, the role of PGE1 in ER stress-induced apoptosis of hepatocytes is largely unknown.

In this study, we evaluated the protective effect of PGE1 against ER stress-induced hepatocyte apoptosis in both the normal human hepatocyte cell line L02 and

the hepatocarcinoma-derived cell line HepG2[27].

MATERIALS AND METHODS

Chemical reagents

RPMI 1640 was obtained from Thermo-Fisher Biochemical Products Co. Ltd (Beijing, China). Fetal bovine serum (FBS), thapsigargin (TG), protein kinase A (PKA) inhibitor H89 and KT5720, 4-phenylbutyric acid (PBA) and PGE1 were purchased from Sigma (St. Louis, MO, United States). Antibodies against GRP78, PERK, eukaryotic translation initiation factor-2α (eIF-2α), phospho-PERK (p-PERK) and phospho-eIF2α (p-eIF2α), C/EBP homologous protein (CHOP), spliced X box-binding protein 1 (sXBP1), growth arrest and DNA damage-inducible gene 34 (GADD34) and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Small interfering (si)RNA scramble control and validated human GRP78-siRNA were purchased from Santa Cruz Biotechnology. All other chemicals and reagents were obtained from Sigma, unless stated otherwise.

Cell culture

The human hepatocyte cell line L02 and hepatocarcinoma-derived cell line HepG2 were obtained from the cell bank of the Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China). The L02 and HepG2 cells were propagated at 37 ℃ in 5% CO_2 in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin, and were passaged every 5-7 d. The L02 and HepG2 cells were cultured until they acquired 80%-100% confluence. Thereafter, the cells were rinsed three times with 10 mL of phosphate-buffered saline (PBS) and cultured in a medium lacking FBS for 24-36 h. To evaluate the effects of PGE1 or PBA on TG-induced apoptosis, PGE1 or PBA was used an hour prior to TG treatment and, thereafter, the medium was not changed. TG and H89 were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 40 μmol/L and 20 μmol/L, respectively, as stock solution. PGE1 was dissolved in ethanol at a concentration of 2.82 mmol/L as stock solution. PBA was dissolved in RPMI 1640 medium. All control conditions included corresponding vehicles at the appropriate concentrations (ethanol for PGE1 and DMSO for TG and H89).

Cell apoptosis analysis

Apoptosis was determined using the annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's instructions. Briefly, 2×10^6 cells were harvested using 0.05% trypsin with 0.5% mmol/L EDTA. To analyze the whole apoptotic cell population, non-adherent cells present in the culture medium were added to the harvested cells. The cells were then washed twice with pre-chilled PBS and resuspended in 500 μL annexin binding buffer. Then, 5 μL of annexin V-FITC and 5 μL of PI were added to each sample and incubated in dark at room temperature for 10 min. Flow cytometry (Gallios; Beckman Coulter, Brea, CA, United States) was performed according to the manufacturer's specifications. The apoptotic index was calculated as the percentage of annexin V^+ cells divided by the total number of cells in the gated region.

Cell viability assay

Cell viability was assessed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, United States) according to the manufacturer's instructions. In brief, early passage of L02 or HepG2 cells were plated in triplicate on 96-well plates (10000 cells/well) and cultured in modified RPMI 1640 for 24 h. Thereafter, the cells were rinsed three times in 200 μL of PBS and cultured in a medium lacking fetal calf serum. Cell viability was determined by replacing the medium with 20 μL of MTS. After incubation of the cells at 37 ℃ for 3 h, the absorbance was measured at 490 nm using a microplate reader (Bio-Rad model 680; Bio-Rad, Hercules, CA, United States). Cell viability was normalized as a percentage of control. This experiment was performed five times.

Western blotting

Cell lysates containing 40 μg of protein were resolved by SDS-PAGE using 4%-20% polyacrylamide gradient gel, and the fractioned proteins were subsequently transferred to nitrocellulose membranes. After blocking with Tris-based saline buffer containing 5% dry milk and 0.1% Tween 20 for 1 h, the membranes were blotted with the corresponding antibodies. The primary and secondary antibodies used were: rabbit anti-human GRP78 (1:500 dilution), PERK (1:1000 dilution), phospho (p)-PERK (1:500 dilution), eIF-2α (1:500 dilution), p-eIF-2α (1:250 dilution), sXBP1 (1:500 dilution), CHOP (1:1000 dilution), GADD34 (1:1000 dilution), mouse anti-human β-actin, goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP), and goat anti-mouse IgG conjugated with HRP. The membranes were developed using a chemiluminescence detection system and thereafter exposed to BioMax Light Film (Kodak, Rochester, NY, United States). The band intensity for each protein was measured using ImagePro Plus analysis software (MediaCybernetics, Silver Spring, MD, United States) and the expression normalized to that of β-actin.

Quantitative real-time RT-PCR

Total RNA was isolated from L02 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNase-free DNase I (TaKaRa, Dalian, China) was applied before cDNA synthesis to remove any genomic contamination. A total of 1 μg RNA from each sample was used for cDNA synthesis with a reverse transcription kit (TaKaRa). Reverse transcription (final volume of 20 μL) was performed at 42 ℃ for 10 min, followed by 75 ℃ for 5 min. The real-time PCR reaction (25 μL) containing 12.5 μL of 2 × SYBR Premix Ex*Taq* II (Tli RNaseH Plus; TaKaRa), 1 μL of each 10 μmol/L primers, 2 μL of cDNA template, and 8.5 μL RNAse/DNAse-free water was performed on a CFX96 PCR system (Bio-Rad). The reaction process was as follows: denaturation at 95 ℃ for 3 min, followed by 40 cycles of amplification (95 ℃ for 10 s and 60 ℃ for 30 s), ending with a melt curve ranging from 60 ℃ to 95 ℃ with a heating rate of 0.3 ℃/15 s. All samples were run in triplicate. Relative expression of GRP78 was calculated using the delta-delta-Ct method with β-actin as the reference control.

Primers used in the PCR were: GRP78 forward, 5′-AAATAAGCCTCAGCGGTTTCTT-3′ and reverse, 5′-TCAAGTTCTTGCCGTTCAAGG-3′; β-actin forward, 5′- CGGGAAATCGTGCGTGAC-3′ and reverse, 5′-CAGGAAGGAAGGCTGGAAG-3′ (TaKaRa). Quantitative real-time PCR was performed according to the MIQE guidelines[28].

GRP78-siRNA transfection

Briefly, cell culture plates containing 6-wells were seeded with 2 × 10^5 cells/well and cultured in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin. siRNA scramble control and validated human GRP78-siRNA were used. GRP78 inhibition was performed using a commercially available siRNA kit (Santa Cruz Biotechnology). Knock-down of the target molecule, GRP78, was monitored by western blotting and real-time PCR.

Statistical analysis

Results of cell apoptosis and cell viability are expressed as mean ± SD. One-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed to compare multiple groups. The Student's *t*-test was used to assess between-group differences. The level of significance was set at $P < 0.05$.

RESULTS

TG-induced ER stress and apoptosis in L02 cells and PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis

We confirmed TG-induced ER stress in L02 cells (Figure 1). At 48 h, TG (1 μmol/L) caused significant

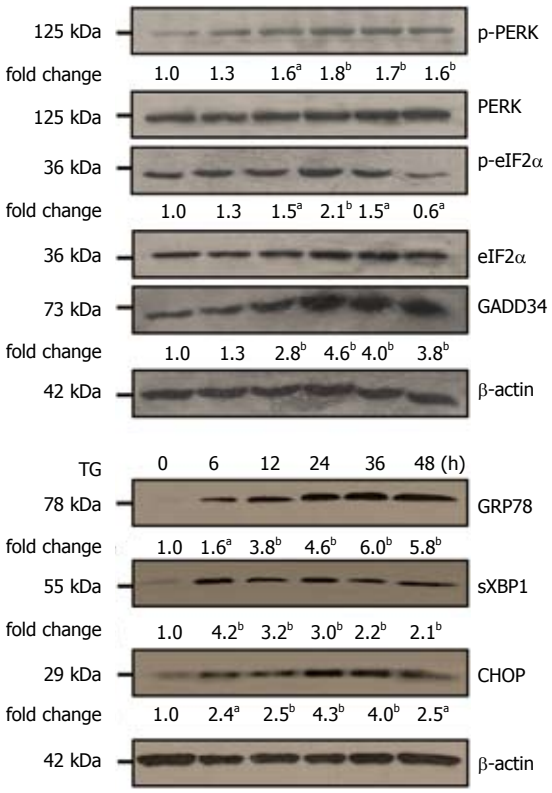


Figure 1 TG induced endoplasmic reticulum stress in L02 cells. L02 cells were treated with 1 μmol/L TG for 6, 12, 24, 36 and 48 h. Expressions of p-PERK, p-eIF2α, GADD34, GRP78, sXBP1 and CHOP were assessed by western blotting. Representative blots from three independent experiments are presented. The results of densitometric analysis are presented as a fold-change compared to that at 0 h ($^aP < 0.05$; $^bP < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene34; GRP78: Glucose-regulated protein 78; p-eIF2α: Phospho-eukaryotic translation initiation factor-2α; p-PERK: Phospho-PKR-like ER kinase; sXBP1: Spliced X box-binding protein1; TG: Thapsigargin.

enhancement in phosphorylation of PERK and GADD34 proteins, as compared to that observed at 6 h. Further, phosphorylation of eIF2α at 24 h was also up-regulated, as compared to that at 6 h. TG also induced a significant increase in the expressions of GRP78, CHOP and sXBP1 proteins.

We also confirmed TG-induced apoptosis in both L02 and HepG2 cells by means of flow cytometry and MTS assay. As shown in Figure 2A, the apoptotic index of L02 cells after TG treatment was significantly higher than that of control from 6 h to 48 h ($P < 0.01$). At 48 h, the apoptotic index reached 32.66%. TG also showed a dose-dependent increase in apoptotic index with increase in the concentration of TG (1 μmol/L, 2 μmol/L and 3 μmol/L; data not shown). Cell viability of both L02 and HepG2 cells significantly decreased from 24 h to 48 h after TG treatment (Figure 2B and C).

We assessed the effect of PGE1 on ER stress-induced apoptosis in L02 cells (Figure 2A). From 6 h to 48 h, 1 μmol/L PGE1 pretreatment significantly decreased TG-induced apoptotic index. At 48 h, 0.5 μmol/L and 1 μmol/L PGE1 showed dose-dependent

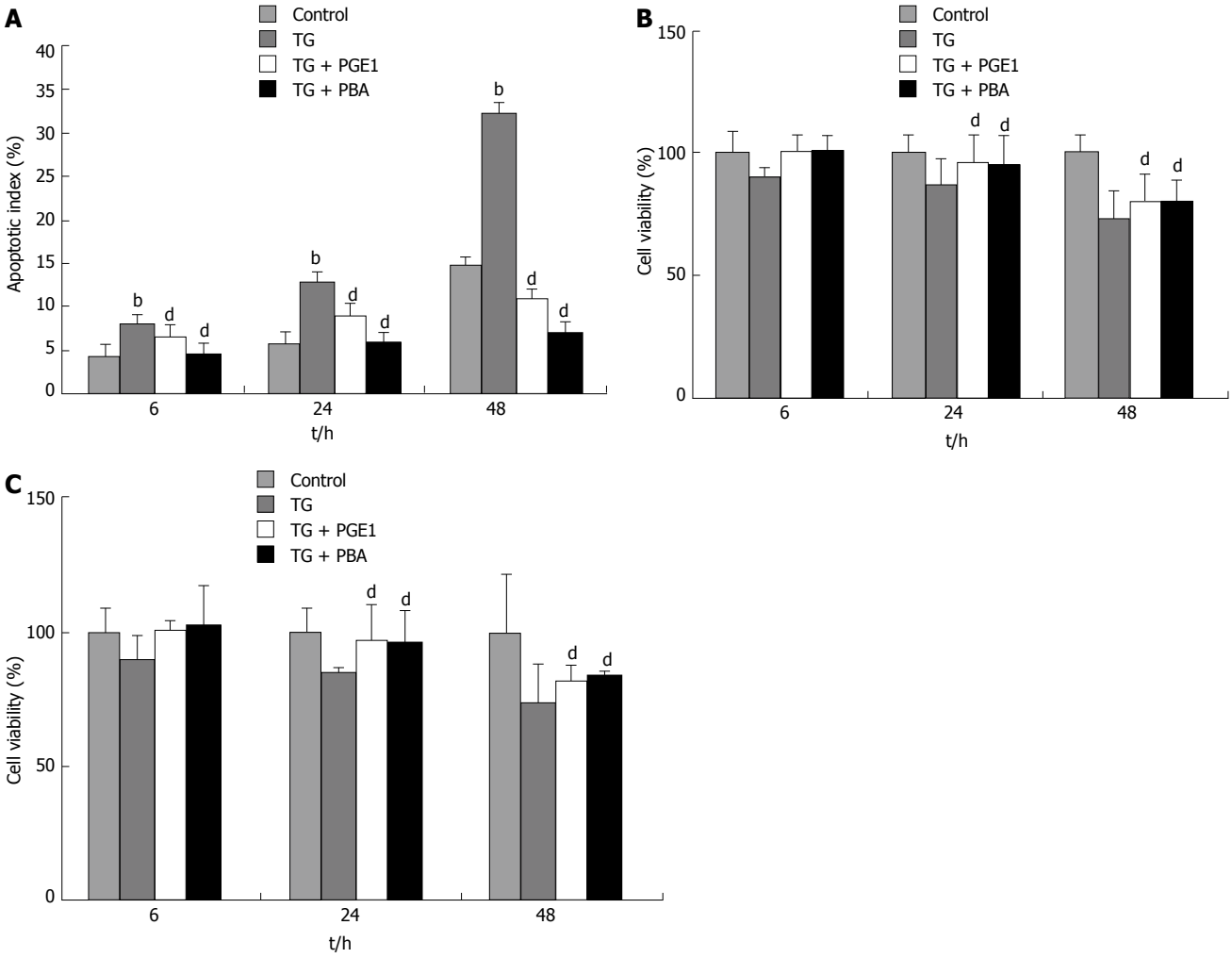


Figure 2 Prostaglandin E1 protected against thapsigargin-induced apoptosis in both L02 cells and HepG2 cells. L02 cells and HepG2 cells were pretreated with PGE1 or PBA for 1 h and treated with a final concentration of 1 μmol/L TG for 6, 24 and 48 h. A: Apoptotic index of L02 cells was determined by flow cytometry. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. $^{b}P < 0.01$ *vs* control at the same time point; $^{d}P < 0.01$ *vs* TG at the same time point; B and C: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. The absorbance was measured at 490 nm and cell viability was normalized as a percentage of control. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. $^{d}P < 0.01$ *vs* TG at the same time point. MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PBA: 4-phenylbutyric acid; PGE1: Prostaglandin E1; TG: Thapsigargin.

protection against TG-induced apoptosis (data not shown). PBA is a low molecular weight chemical chaperone and an ER stress inhibitor. PBA at 10 nmol/L significantly inhibited TG induced apoptosis (Figure 2A). MTS assay revealed that PGE1 significantly increased the viability of TG-treated L02 cells and HepG2 cells (Figure 2B and C). These results demonstrate the protective effect of PGE1 on ER stress-induced apoptosis in L02 cells and in HepG2 cells.

PGE1 promoted rapid recovery of ER stress

After demonstration of the cytoprotective effect of PGE1, we investigated the effects of PGE1 on UPR signals induced by TG. We selected three indicators from UPR, GRP78, CHOP and sXBP1, as GRP78 is a common downstream target of the three UPR signal pathways. CHOP is the major protein involved in apoptotic signals induced by ER stress, and sXBP1 is activated through the oligomerization of IRE1a[29]. As shown in Figure 3, 1 μmol/L PGE1 appeared to increase TG-induced GRP78, CHOP and sXBP1 expressions till 6 h post-treatment. However, from 24 h to 48 h, PGE1 suppressed TG-induced GRP78 and CHOP expressions. These results indicate that although PGE1 increased the early expression of UPR signals, it promoted the rapid recovery of ER stress.

PGE1 induced GRP78 expression via the PKA pathway

The observation that PGE1 attenuated the ER stress-induced apoptosis and promoted the rapid recovery from ER stress prompted us to investigate the effect of PGE1 on GRP78, CHOP and GADD34 expressions. GRP78 is a critical chaperone which determines the outcome of ER stress. GADD34 is involved in both recovery and resumption of protein synthesis as well as in the ER stress-induced apoptosis[14,30]. We also investigated the effects of PGE1 on phosphorylation of eIF2α and PERK, since the activation of PERK and

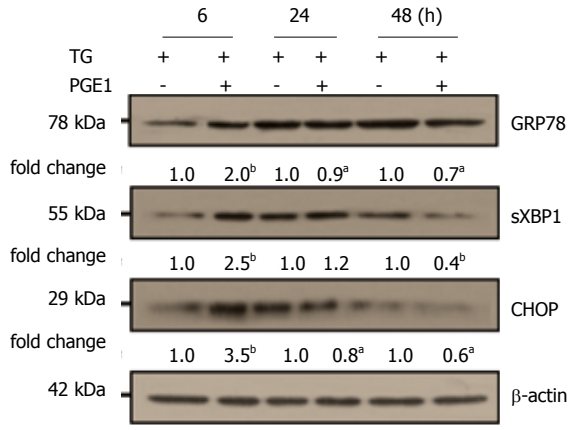


Figure 3 Prostaglandin E1 promoted rapid recovery of endoplasmic reticulum stress. L02 cells were pretreated with 1 μmol/L PGE1 for 1 h and at a final concentration of 1 μmol/L TG for 6, 24 and 48 h. Expressions of GRP78, CHOP and sXBP1) were assessed on western blotting. One representative blot each of three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of TG at the same time point ($^{a}P < 0.05$, $^{b}P < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; sXBP1: Spliced X box-binding protein 1; TG: Thapsigargin.

eIF2α is known to induce CHOP and GADD34. From 3 h to 24 h after treatment with 1 μmol/L PGE1, a significant increase in GRP78 expression was observed in both L02 cells and HepG2 cells (Figure 4A and B). PGE1 also decreased CHOP expression in L02 cells and induced the expression of GADD34; however, it suppressed the expression of p-PERK and p-eIF2α proteins (Figure 4A). Results of quantitative real-time RT-PCR also demonstrated that PGE1 induced the mRNA expression of GRP78 from 3 h to 24 h (Figure 4C).

To explore the signal pathways that may mediate the effect of PGE1 on GRP78 expression, we inhibited the PKA pathway by H89 or KT5720. Treatment with 10 μmol/L of H89 or 1 μmol/L KT5720 appeared to counteract the effect of PGE1 on GRP78 expression (Figure 5A and B). These results indicate that PGE1 increased GRP78 expression *via* a PKA-dependent pathway.

PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis via PKA-dependent induction of GRP78 expression

H89 at 10 μmol/L appeared to inhibit the protective effect of PGE1 on hepatocyte apoptosis, which suggests that the cytoprotective effect of PGE1 was mediated *via* the PKA signaling pathway (Figure 5C). To further test whether the protective effect of PGE1 was dependent on the induction of GRP78 expression, we inhibited GRP78 expression by transfection with siRNA specifically targeted against human GRP78. On blocking the PGE1-induced up-regulation of GRP78 expression by siRNA (Figure 6A-C), PGE1 appeared to lose its cytoprotective effect in both L02 cells and HepG2 cells (Figure 6D-G). These findings imply that PGE1 protected against hepatocyte apoptosis *via* PKA-dependent induction of GRP78 expression.

DISCUSSION

The key findings of this study are that PGE1 protected hepatocytes from ER stress-induced apoptosis, that PGE1 enhanced the expression of GRP78 *via* the PKA pathway, and that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* PKA-dependent GRP78 induction.

TG is known to induce ER stress by blocking ER Ca^{2+} uptake, which leads to depletion of ER Ca^{2+} stores[31]. PGE1 can also increase intracellular Ca^{2+} level by promoting the influx of Ca^{2+} from the external medium as well as by mobilization of Ca^{2+} from intracellular stores[32]. As intracellular Ca^{2+} level is an important factor in hepatocyte apoptosis and necrosis, cotreatment with TG and PGE1 may have modulated apoptotic signals of L02 cells, which eventually led to cell death by necrosis. Therefore, we assessed cell apoptosis and viability on flow cytometry and by MTS assay. Our results strongly suggest that PGE1 protects hepatocytes from ER stress-induced apoptosis.

PGE1 is known to have a direct vasodilator as well as an anti-platelet effect[22,33]. Several *in vivo* studies have indicated that PGE1 protects against hepatocyte apoptosis and promotes hepatocyte proliferation *via* inducing down-regulation of proinflammatory cytokine levels[24,26], suppression of tumor necrosis factor-α receptor and adhesion molecule expression[23,24], and by up-regulating cyclin C and cyclin D1 expressions[34]. PGE1 also inhibits oxidative stress and nitrosative stress-induced hepatocyte death by inhibiting production of superoxide anion, by enhancing nitric oxide synthase expression and by inhibiting nuclear factor-κB activation *in vitro*[19,35,36]. In this study, we demonstrated that PGE1 protected hepatocytes from ER stress-induced apoptosis. Our findings suggest that modulation of UPR may mediate the cytoprotective effect of PGE1 in various pathological conditions.

The mechanisms involved in ER stress-induced apoptosis are yet to be elucidated. The adaptive capacity of cells to ER stress is an important determinant of the outcomes of ER stress. Apoptosis results from sustained or strong ER stress when UPR fails to restore ER homeostasis[37,38]. In the present study, PGE1 treatment enhanced TG-induced CHOP, GRP78 and sXBP1 expressions at 6 h, and significantly attenuated TG-induced CHOP, GRP78 and sXBP1 expressions, as assessed at 48 h. These results suggest that PGE1 boosted the initial expression of UPR and promoted restoration of ER homeostasis during ER stress.

During ER stress, UPR may have been activated to restore ER homeostasis. UPR is precisely regulated by three ER stress transducers and their downstream signals. GRP78 is considered as a master regulator of response to ER stress[39,40]. GRP78 binds with all three major UPR sensors (PERK, IRE1a and ATF6) and

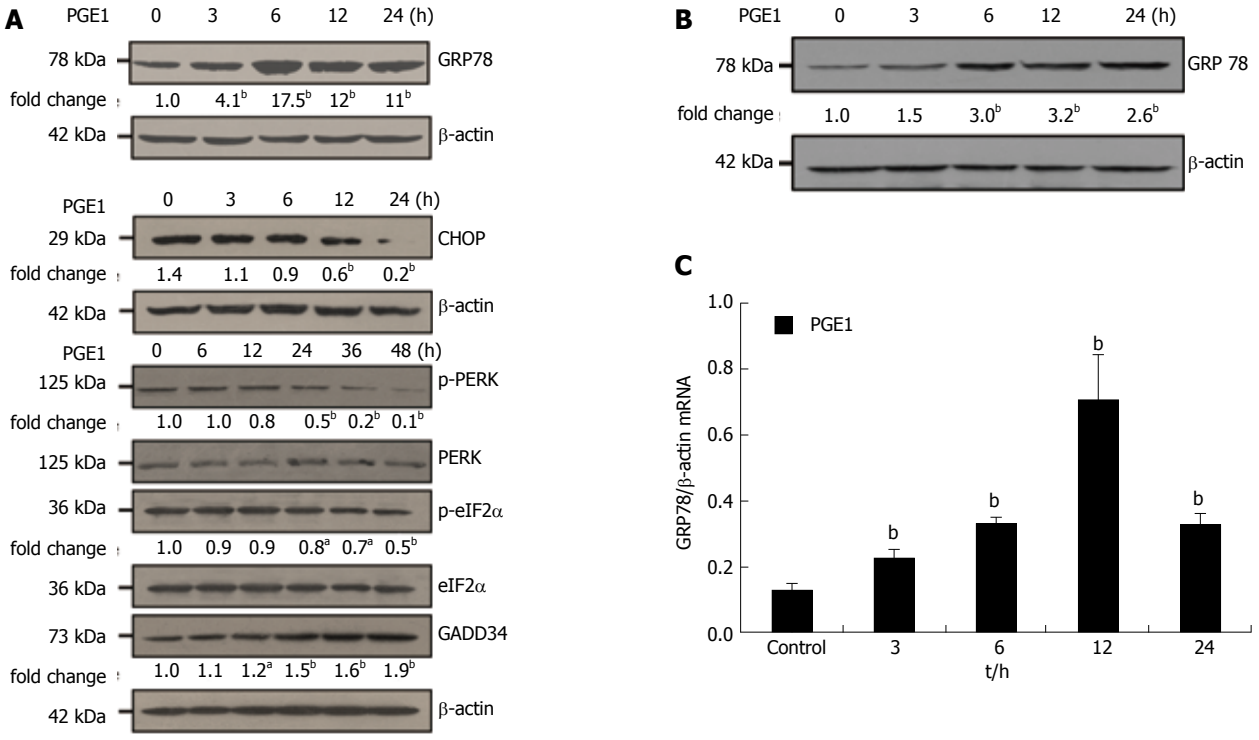


Figure 4 Prostaglandin E1 induced glucose-regulated protein 78 protein and mRNA expressions. To determine GRP78 and CHOP protein or mRNA expressions, L02 cells and HepG2 cells were treated with 1 μmol/L PGE1 for 3, 6, 12 and 24 h; for detecting GADD34 and the p-PERK and p-eIF2α, L02 cells were treated with 1 μmol/L PGE1 for 6, 12, 24, 36 and 48 h. A: Expressions of GRP78, CHOP, GADD34, p-PERK and p-eIF2α in L02 cells were assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^aP < 0.05$, $^bP < 0.01$); B: Expression of GRP78 in HepG2 cells was assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^bP < 0.01$); C: mRNA expression of GRP78 in L02 cells was assessed by quantitative real-time PCR (E). Histograms represent mean ± SD of three experiments ($^bP < 0.01$ *vs* those at 0 h). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene 34; GRP78: Glucose-regulated protein 78; p-eIF2α: Phospho-eukaryotic translation initiation factor-2α; PGE1: Prostaglandin E1; p-PERK: Phospho-PKR-like ER kinase; TG: Thapsigargin.

keeps them inactivated while the homeostasis of ER is maintained. During ER stress, GRP78 dissociates from PERK, ATF6 and IRE1a and binds to unfolded or misfolded proteins, promotes their proper folding or directs them to degradation. The dissociation of GRP78 from PERK, ATF6 and IRE1a, triggers the UPR response and further enhances the expression of GRP78. Increased GRP78 augments the folding capacity of the ER, inactivates the three ER sensors and promotes restoration of ER homeostasis[29].

The protective role of GRP78 against apoptosis during ER stress has been demonstrated both *in vivo* and *in vitro*[41-44]. The insulin signaling pathway has been found to promote cell proliferation and improve cell survival *via* up-regulation of GRP78 expression[45]. In this study, PGE1 significantly inhibited TG-induced apoptosis. PGE1 induced protein and mRNA expressions of GRP78. Further, inhibition of GRP78 expression *via* either H89 or siRNA hindered the protective role of PGE1 on TG-induced apoptosis. These results demonstrate that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* induction of GRP78 during ER stress. The protein expression of GRP78 induced by PGE1 peaked at 6 h; however, mRNA expression of GRP78 induced by PGE1 peaked at 12 h. It is difficult to explain the difference in the time frame for attainment of peak levels of mRNA and protein expressions of GRP78. One explanation is that PGE1 regulated the GRP78 expression not only at the transcriptional level but also at the translational or posttranslational level. Whether PGE1 regulates the expression of GRP78 at the translational or posttranslational level warrants further studies.

The other important signal pathways in ER stress-induced apoptosis are mediated *via* induction of CHOP and GADD34. During UPR, phosphorylation of eIF2α *via* the PERK pathway results in inhibition of mRNA translation and general protein synthesis. However, mRNA for activating transcription factor (ATF) 4 is selectively up-regulated. Activation of ATF4 results in induction of CHOP and GADD34 expressions. GADD34 and protein phosphatase 1 were shown to promote dephosphorylation of eIF2α and allow protein synthesis[46]. However, recent studies have shown that induction of GADD34 exacerbates the disturbance of ER homeostasis and leads to cell apoptosis by increasing oxidative stress[47]. CHOP has been identified as one of the most important mediators of ER stress-induced apoptosis; it induces apoptosis through various signal pathways[48].

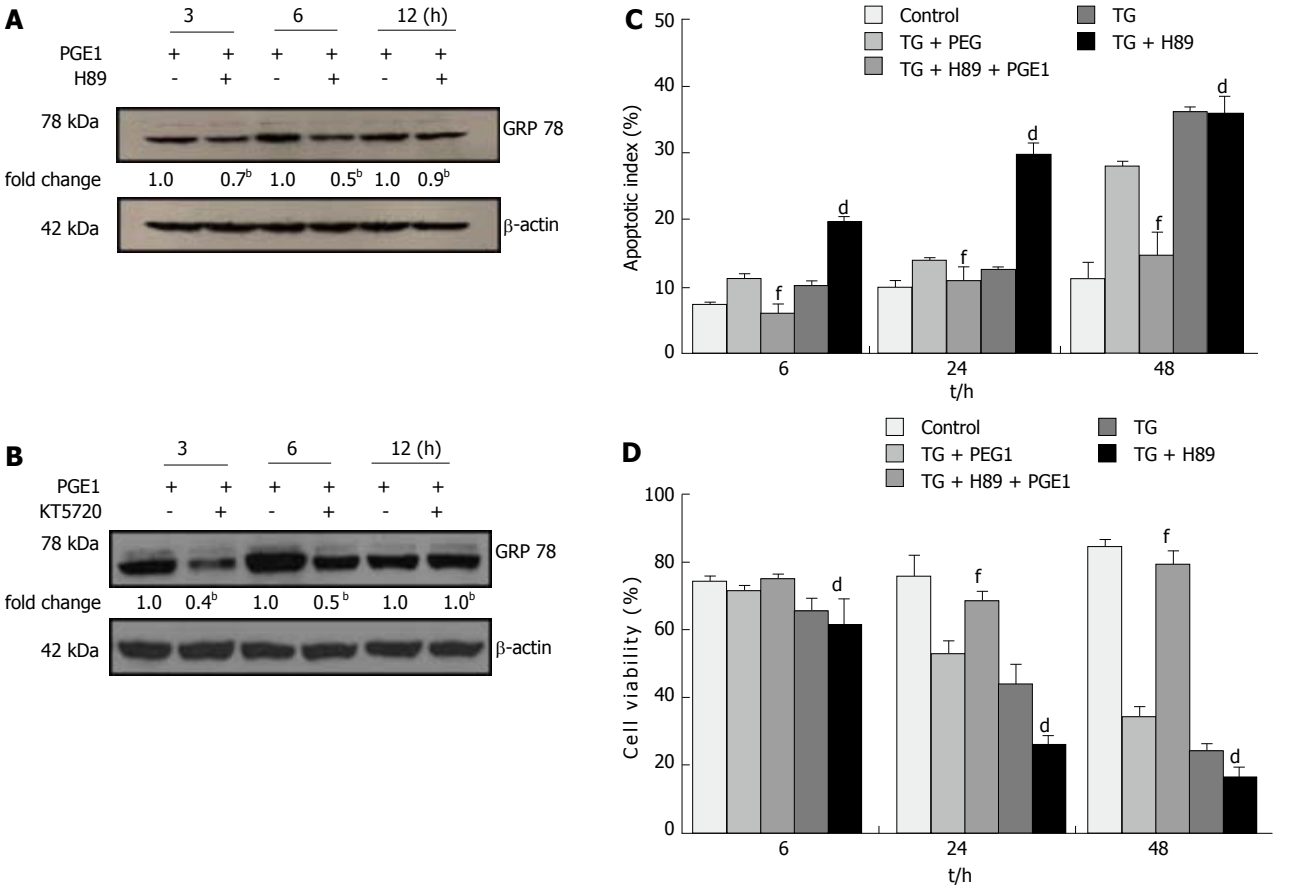


Figure 5 Prostaglandin E1 induced glucose-regulated protein 78 expression and protected L02 cells against endoplasmic reticulum stress-induced apoptosis *via* a protein kinase a-dependent pathway. A and B: L02 cells were pretreated with or without PKA inhibitor H89 (10 μmol/L) or KT5720 (1 μmol/L), and then 1 μmol/L PGE1 for 3, 6 and 12 h. The expressions of GRP78 were detected by western blotting. One representative blot each from three independent experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of PGE1 at the same time points ($^bP < 0.01$); C: L02 cells were pretreated with or without 10 μmol/L H89, and then 1 μmol/L PGE1 and a final concentration of 1 μmol/L TG for 6, 24 and 48 h. The apoptotic index was determined by flow cytometry. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point); D: L02 cells were pretreated with or without 10 μmol/L H89, and then 1 μmol/L PGE1 and a final concentration of 1 μmol/L TG for 6, 24 and 48 h. Cell viability of L02 cells was determined by MTS assay. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point). GRP78: Glucose-regulated protein 78; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PGE1: Prostaglandin E1; PKA: Protein kinase A; TG: Thapsigargin.

In the current study, we had anticipated inhibition of GADD34 by PGE1. However, we observed an enhanced expression of GADD34 and significant inhibition of p-PERK, CHOP and p-eIF2α expressions in L02 cells. Our results indicate that the cytoprotective effect of PGE1 against hepatocyte apoptosis does not depend on the inhibition of GADD34. Since PGE1 lost its protective effects after inhibition of GRP78 expression in this study, it is possible that the increased expression of GADD34 in our study was the result of induction of GRP78 by PGE1 and that this represented restoration of ER homeostasis. Further study is needed to clarify the role of GADD34 in ER stress-induced apoptosis.

The biological effects of PGE result from its binding to its receptors, EP1 to EP4, and previous studies have shown that L02 cells express EP1 receptors[49]. Binding of PGE1 with its receptors stimulates production of the second messenger cyclic 3, 5 adenosine monophosphate (cAMP). cAMP may act *via* distinct intracellular signaling effectors, such as PKA and the exchange proteins activated by cAMP[50]. A previous study has shown that cAMP has PKA-independent interaction with Ca^{2+} stored in lymphocytes[51]. To test whether the hepatoprotective effect of PGE1 is mediated *via* PKA-independent interaction with Ca^{2+} stores in L02 cells, we used H89 to block the PKA pathway. The results showed that the protective effects of PGE1 were largely dependent on the PKA pathway. However, H89 inhibited GRP78 expression most effectively at 6 h, and then at 12 h the inhibitory effect was alleviated; the increased apoptotic index lasted from 6 h to 48 h. It is difficult to explain this result. One explanation is that H89 may also have inhibited other kinases in addition to PKA. H89 has been shown to inhibit at least 8 kinases beside PKA[52]. The role of PKA and other kinases in ER stress-induced apoptosis remains to be studied in the future.

It is known that GRP78 expression is regulated at the transcriptional level by ER stress. Previous

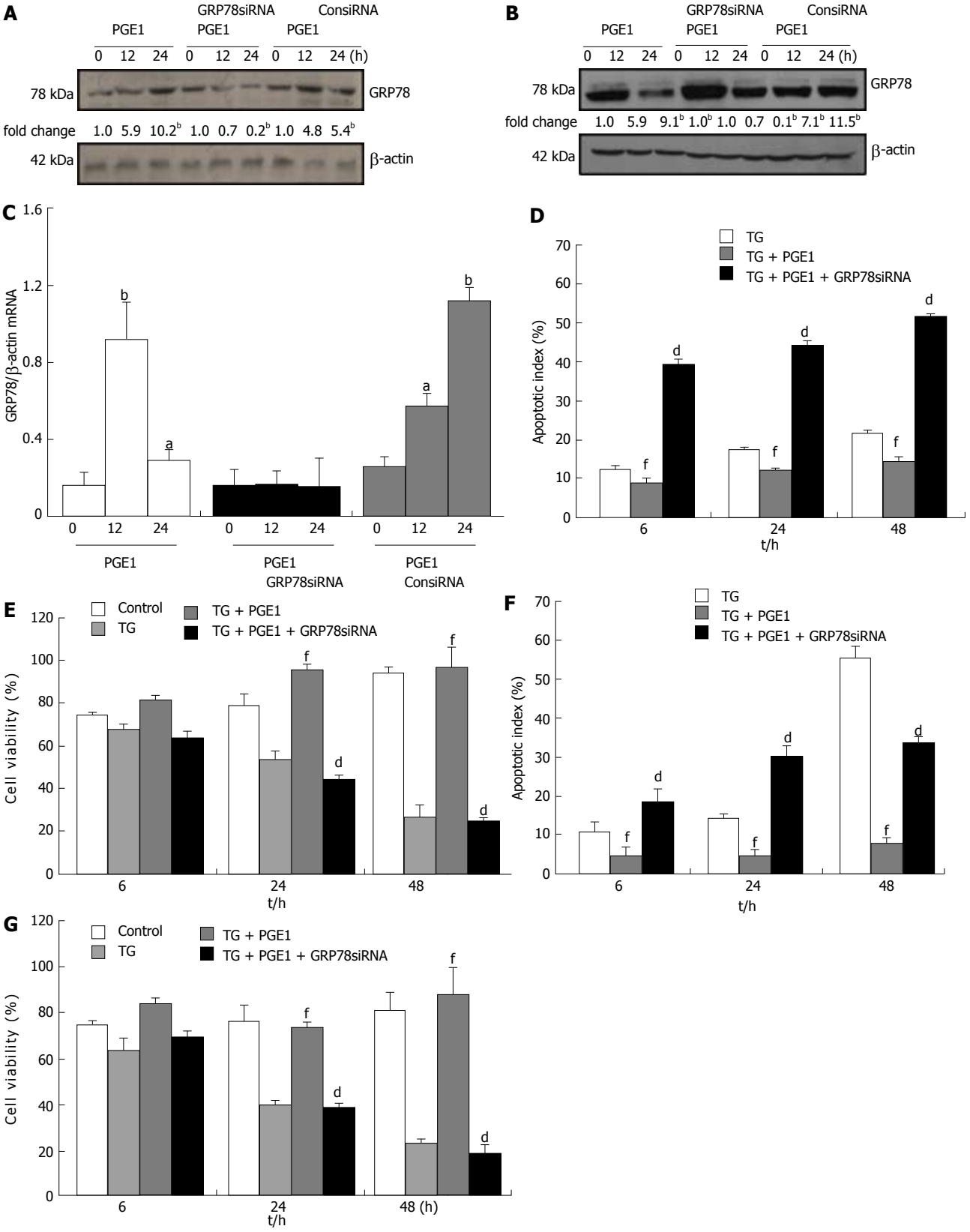


Figure 6 Prostaglandin E1 protected against endoplasmic reticulum stress-induced apoptosis *via* induction of glucose-regulated protein 78 expression in both L02 and HepG2 cells. L02 and HepG2 cells were transfected with either siRNA scramble control (ConsiRNA) or siRNA against human GRP78 (GRP78 siRNA) for 48 h. The cells were treated with 1 μmol/L PGE1 for 12 h and 24 h. A and B: Expressions of GRP78 in L02 cells and in HepG2 cells were detected by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^bP < 0.01$); C: mRNA expression of GRP78 in L02 cells was detected by real-time PCR. Histograms represent mean ± SD of three experiments ($^aP < 0.05$; $^bP < 0.01$ *vs* those at 0 h). D and F: Apoptotic indices of L02 cells and HepG2 cells were determined by flow cytometry. Histograms represent mean ± SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point); E and G: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. Histograms represent mean ± SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 and $^fP < 0.01$ *vs* those of TG at the same time point). GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; siRNA: Small interfering RNA; TG: Thapsigargin.

studies have shown that preconditioning to ER stress protects against cell death *via* induction of GRP78 or autophagy[53]. In this study, although PGE1 pretreatment significantly induced GRP78 expression and enhanced TG-induced early CHOP and sXBP1 expressions, PGE1 alone inhibited the expressions of p-PERK and p-eIF2α and CHOP. Therefore, whether PGE1 induced GRP78 expression *via* induction of ER stress is not known. In previous studies, leptin was shown to induce GRP78 expression in neuronal cells through the PI3K-mTOR pathway[54]; further, oncostatin M was also shown to induce GRP78 expression without triggering ER stress[55]. The mechanisms involved in the induction of GRP78 expression by PGE1 warrant further investigation.

In conclusion, this is the first study to demonstrate that the cytoprotective effect of PGE1 against ER stress-induced apoptosis is mediated *via* PKA-dependent induction of GRP78 expression in hepatocytes. Further studies are required for devising treatment strategies to protect hepatocytes against ER stress-induced apoptosis, which will be of much clinical relevance in the context of liver diseases.

COMMENTS

Background

Prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis; however, the role of Prostaglandin E1 (PGE1) in endoplasmic reticulum (ER) stress-induced apoptosis of hepatocytes is largely unknown.

Research frontiers

ER stress has been implicated in the pathogenesis of various liver diseases. Understanding the mechanisms underlying ER stress-induced apoptosis and devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

Innovations and breakthroughs

Pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP) 78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression by either H89 or siRNA. Our study demonstrates, for the first time, that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Applications

PKA and GRP78 may be new targets for pharmacological treatment in patients with liver diseases.

Peer-review

The strengths of this study lie in the experimental design, methodology and that the authors describe the caveats and potential pitfalls in great detail. Overall, the study was well-designed and the paper is interesting and sound.

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ORIGINAL ARTICLE

Retrospective Study

Genetic associations with adverse events from anti-tumor necrosis factor therapy in inflammatory bowel disease patients

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Abstract

AIM

To study the type and frequency of adverse events associated with anti-tumor necrosis factor (TNF)

therapy and evaluate for any serologic and genetic associations.

METHODS

This study was a retrospective review of patients attending the inflammatory bowel disease (IBD) centers at Cedars-Sinai IBD Center from 2005-2016. Adverse events were identified *via* chart review. IBD serologies were measured by ELISA. DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol. SNPs underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling. Standard and rigorous QC criteria were applied to the genetic data, which was generated using immunochip. Genetic association was assessed by logistic regression after correcting for population structure.

RESULTS

Altogether we identified 1258 IBD subjects exposed to anti-TNF agents in whom Immunochip data were available. 269/1258 patients (21%) were found to have adverse events to an anti-TNF-α agent that required the therapy to be discontinued. 25% of women compared to 17% of men experienced an adverse event. All adverse events resolved after discontinuing the anti-TNF agent. In total: *n* = 66 (5%) infusion reactions; n = 49 (4%) allergic/serum sickness reactions; *n* = 19 (1.5%) lupus-like reactions, *n* = 52 (4%) rash, *n* = 18 (1.4%) infections. In Crohn's disease, IgA ASCA (*P* = 0.04) and IgG-ASCA (*P* = 0.02) levels were also lower in patients with any adverse events, and anti-I2 level in ulcerative colitis was significantly associated with infusion reactions (*P* = 0.008). The logistic regression/human annotation and network analyses performed on the Immunochip data implicated the following five signaling pathways: JAK-STAT (Janus Kinase-signal transducer and activator of transcription), measles, IBD, cytokine-cytokine receptor interaction, and toxoplasmosis for any adverse event.

CONCLUSION

Our study shows 1 in 5 IBD patients experience an adverse event to anti-TNF therapy with novel serologic, genetic , and pathways associations.

Key words: Genetic associations; Inflammatory bowel disease; Anti-tumor necrosis factor; Adverse events

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Core tip: Tumor necrosis factor-α (TNF-α) plays a key role in the development and progression of inflammatory bowel disease (IBD). Anti-TNF therapy is highly efficacious in treating IBD patients, but many experience adverse events. Few studies have evaluated factors associated with adverse events to anti-TNF therapy. In this study, we found some genetic associations and pathways that are enriched for genes associated with the development of adverse events. Future studies will need to confirm these findings as the ability to identify subjects at high risk may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Lew D, Yoon SM, Yan X, Robbins L, Haritunians T, Liu Z, Li D, McGovern DPB. Genetic associations with adverse events from anti-tumor necrosis factor therapy in inflammatory bowel disease patients. *World J Gastroenterol* 2017; 23(40): 7265-7273 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7265.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7265

INTRODUCTION

Tumor necrosis factor-α (TNF-α) plays a key role in the development and progression of inflammatory bowel disease (IBD)[1-3]. While anti-TNF therapy is an effective therapeutic option for IBD patients[1,4,5], response to these agents is highly heterogeneous and a high proportion of patients either fail initial induction therapy or lose response during maintenance therapy[6-8]. Predicting response to these agents has been studied extensively by evaluating a multitude of factors including potential genetic components[7], but an important addition would be the ability to predict the development of adverse events associated with these agents including: infusion reactions; infections; rash; allergic reactions; serum sickness like reactions; and lupus-like reactions[9-11]. Minimizing the risks is important to increase patient compliance and improve response to therapy, and will also become more important as physicians struggle with decisions around where to position biologic therapies as therapeutics targeting novel mechanisms become available[12,13].

Currently, there are few studies designed to determine factors associated with adverse events to anti-TNF-α therapy. The objectives of this study were to describe the type and frequency of adverse events associated with anti-TNF-α therapy in a large cohort and evaluate for any serologic and genetic associations.

MATERIALS AND METHODS

Study population

This study was a retrospective review of patients attending the IBD centers at Cedars-Sinai IBD Center from 2005-2016. Patients included in the study were

those that had given consent, had available genotype data, carried a diagnosis of IBD (Crohn's disease, ulcerative colitis, or IBDU), and who had been treated with anti-TNF-α agents (infliximab, adalimumab, certolizumab pegol). All research-related activities were approved by the Cedars-Sinai Medical Center Institutional Review Board (IRB #3358).

Data gathering

Detailed clinical information for each patient was obtained *via* chart review. Clinical information included age at disease diagnosis, type of IBD (CD, UC, or IBDU), gender, and type of anti-TNF-α agent used. All patients were seen by gastroenterologists, experienced in managing patients with IBD treated with anti-TNF agents, at the IBD centers at Cedars-Sinai Medical Center, Los Angeles.

Adverse events

Adverse events were identified *via* chart review by evaluating the "Allergies" section and the progress notes written by the gastroenterologist. Potential adverse events include infusion reactions, serum sickness-like reactions, drug-induced lupus, rash, infections, and non-specific symptoms (arthralgias, shortness of breath, rash, *etc*). An infusion reaction was defined as any significant adverse experience that occurred during or within two hours of infusion[14]. An allergic or a serum sickness-like reaction was defined clinically as the occurrence of myalgias, arthralgias, fever, or rash within 1-14 d after reinfusion of infliximab[15].

The likelihood of a causal relationship for each adverse event was determined based on the assessment of the gastroenterologist as documented in the progress note and as evidenced by the following: time elapsed between a dose and adverse event, resolution of the adverse event when the therapy was discontinued, and return of the adverse event if the therapy was resumed[16].

Serological analysis

IBD serologies (ANCA, anti-nuclear cytoplasmic antibodies; anti-CBir1, anti-flagellin; anti-I2, anti-*Pseudomonas fluorescens*-associated sequence I2; anti-OmpC, anti-outer membrane porin C; ASCA, anti-*Saccharomyces cerevisiae* antibodies) were measured by enzyme-linked immunosorbent assay (ELISA) as previously described[17]. Results were expressed as ELISA units (EU/mL) relative to Cedars-Sinai Medical Center laboratory or a Prometheus laboratory standard derived from a pool of patient sera with well-characterized disease found to have reactivity to these antigens. All assays were performed in a blinded fashion.

Genotype data

DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol (Illumina, San Diego, CA, United States). Average genotyping call rate for samples that passed quality control was 99.8%; average replicate concordance and average heritability rates were > 99.99% and 99.94%, respectively. Single-nucleotide polymorphisms (SNPs) underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling[18].

Statistical analysis

χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events. For continuous variables with skewed distribution (*e.g.*, serology levels), Wilcox test signed rank test was performed. SNPs association with adverse events was evaluated using PLINK. Principal components (PCs) from population stratification analysis were included in the PLINK analysis to control for potential confounding[19]. Two-sided *P*-value of 0.05 was considered statistically significant.

Genetic pathway, and network analyses

For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analysis was performed with statistically significant SNPs ($P < 0.001$). These SNPs were first annotated into corresponding genes, and the genes were further analyzed with multiple biological functional databases including human protein reference databases (http://www.hprd.org), Reactome, NCI/Nature pathway interaction database and others. The final networks were then constructed form the known interactions from any of these databases. Pathways and gene set enrichment analysis was performed with STRING (http://string-db.org/) and cytoscape (http://www.cytoscape.org).

RESULTS

Patient demographics and characteristics

1258 IBD (954 CD patients, 260 UC, 44 IBDU) patients qualified for this study. The average age of onset was 25.7 years and, and the overwhelming majority were of European ancestry.

Adverse events

A total of 269/1258 patients (21%) were found to have experienced an adverse event. The different

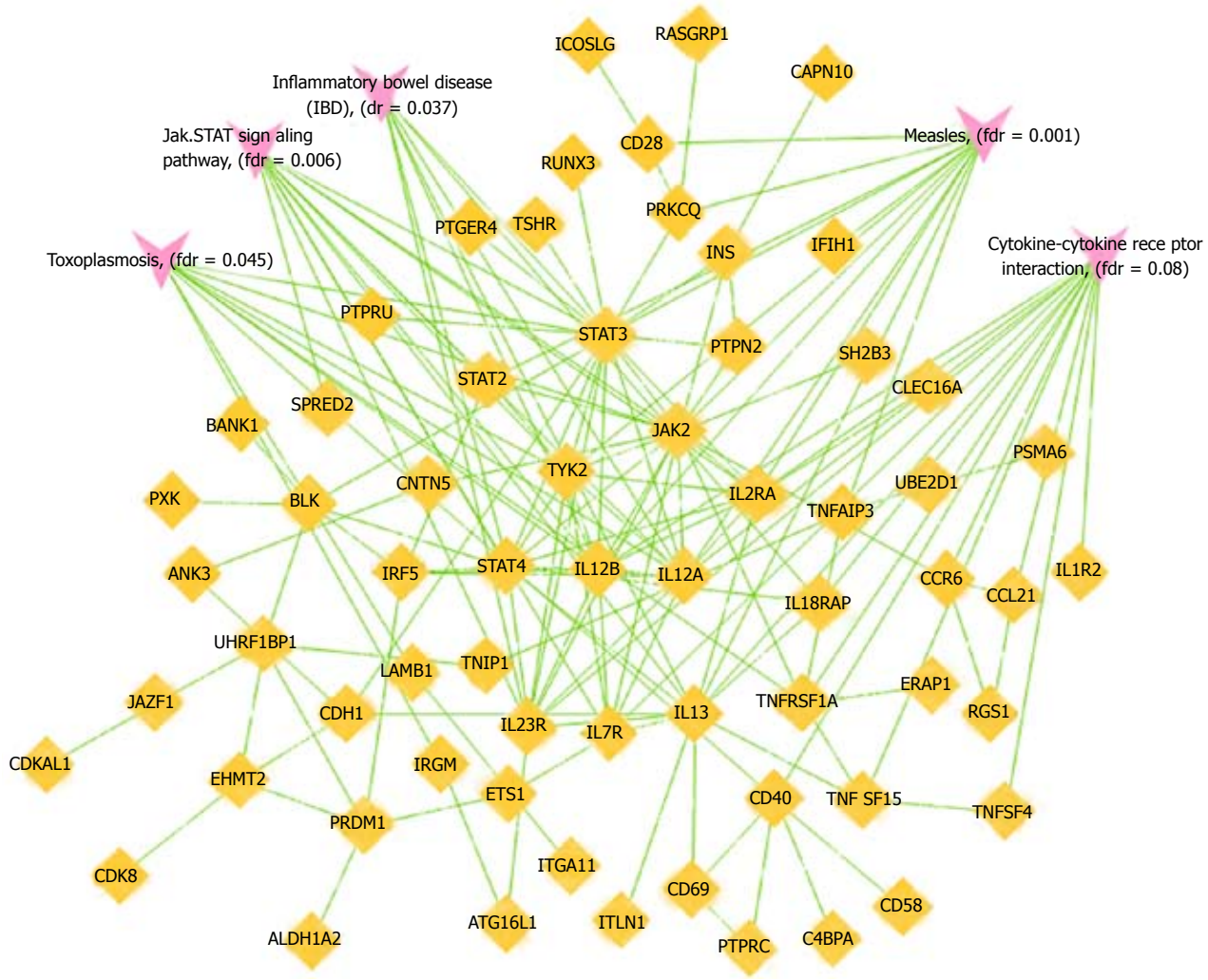


Figure 1 Network analyses of allergic reactions to anti-tumor necrosis factor agents.

types of adverse events were similar among women and men, except for lupus-like reactions and rashes, which were both more commonly seen in women (Table 1).

Serology

In CD patients we observed that IgA ASCA+/- was associated with a lower risk of developing any adverse event (OR = 0.68, *P* = 0.047), while IgG ASCA+/- and total ASCA+/- show a similar trend and borderline association respectively (OR = 0.72 and 0.70, *P* = 0.09 and 0.05, respectively). IgA ASCA (*P* = 0.04) and IgG ASCA (*P* = 0.02) levels were also lower in patients with any adverse events (Table 2). Anti-I2 level in UC was significantly associated with infusion reactions (*P* = 0.008) (Table 2). No other associations were seen with IBD-associated serologies (data not shown).

Genetic analysis

IBD-associated SNPs that achieved a nominal level of significance with adverse events are shown in Table 3. For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analyses performed on the Immunochip data implicated the following five signaling pathways: JAK-STAT (Janus Kinase-signal transducer and activator of transcription), Measles, IBD, Cytokine-cytokine receptor interaction, and Toxoplasmosis (Table 4 and Figure 1). After False Discovery Rate correction, all associations remained statistically significant except for the Cytokine-cytokine receptor interaction for infusion and allergic reactions.

DISCUSSION

In our cohort approximately 1 in 5 IBD patients experienced adverse events to anti-TNFs that eventually led to the discontinuation of the therapy in keeping with the current literature, although most studies only report the presence or not of adverse events and do not comment on whether patients had to

Table 1 Adverse events based on type of inflammatory bowel disease and gender *n* (%)

Clinical traits	All adverse events	Infusion reactions	Allergic reactions	Lupus-like reactions	Rash	Other
Type of IBD						
Crohn's disease (*n* = 954)	220 (23)	52 (5)	45 (5)	14 (1)	40 (4)	69 (7)
Ulcerative colitis (*n* = 260)	42 (16)	14 (5)	4 (2)	4 (2)	10 (4)	10 (4)
IBDU (*n* = 44)	7 (16)	0 (0)	0 (0)	1 (2)	2 (5)	4 (9)
Total	269 (21)	66 (5)	49 (4)	19 (1.5)	52 (4)	83 (7)
Gender						
Male (*n* = 624)	108 (17)	28 (4)	24 (4)	3 (0.5)	17 (3)	36 (6)
Female (*n* = 634)	161 (25)	38 (6)	25 (4)	16 (3)	35 (6)	47 (7)

All values expressed as *n* (%). IBD: Inflammatory bowel disease.

Table 2 Serological associations with anti-tumor necrosis factor adverse reactions in patients with ulcerative colitis and Crohn's disease (anti-I2, anti-*Pseudomonas fluorescens*-associated sequence I2; ASCA, anti-Saccharomyces cerevisiae antibodies)

IBD type	Adverse event	Serological marker	Serology levels in positive (U/mL)	Serology levels in negative (U/mL)	*P* value
CD	Any	IgA ASCA	7	10	0.040
CD	Any	IgG ASCA	18	26.5	0.020
UC	Infusion	Anti-I2	0	7	0.008

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Inflammatory bowel disease-associated single nucleotide polymorphisms associated with different type of adverse events

Adverse event	Risk allele	SNP	Gene (s) of interest	*P* value	OR (95%CI)
Infusion	C	rs6740462	*SPRED2*	0.003	0.4 (0.2-0.7)
Infusion	G	rs1182188	*GNA12*	0.007	1.8 (1.2-2.6)
Infusion	G	rs10061469	*TMEM174, FOXD1*	0.010	0.5 (0.3-0.9)
Infusion	A	rs477515	*HLA-DRB1*	0.010	1.7 (1.1-2.5)
Allergic	A	rs4692386	*SMIM20, RBPJ*	0.010	0.5 (0.3-0.9)
Allergic	A	rs10761659	*ZNF365*	0.010	0.6 (0.3-0.9)
Lupus-like	G	rs13407913	*ADCY3*	0.003	3.5 (1.6-7.9)
Lupus-like	C	rs10051722	*CHSY2, HINT1*	0.010	2.7 (1.2-5.7)
Rash	A	rs1363907	*ERAP2*	0.003	3.0 (1.5-6.2)
Rash	G	rs11010067	*PARD3*	0.003	0.2 (0.1-0.6)
Rash	C	rs7746082	*PREP*	0.005	2.7 (1.3-5.3)
Any	C	rs6740462	*SPRED2*	0.0007	0.6 (0.5-0.8)
Any	C	rs10774482	*ERC1*	0.003	1.4 (1.1-1.8)

SNP: Single nucleotide polymorphisms.

discontinue[20]. The incidence of serious lupus-like reactions requiring the discontinuation of anti-TNFs was found to be 1.1%[21], which was comparable to that seen in our population of 1.5%. To our knowledge, this is the largest study examining adverse events with anti-TNF agents.

We identified a number of genetic associations with known IBD loci including two (*HLA-DRB1* and *ERAP2* [endoplasmic reticulum aminopeptidase 2]) that are associated with a number of immune-mediated diseases as well as IBD and also, in the case of *HLA-DRB1*, with the development of extra-intestinal manifestations in IBD[22-24]. Furthermore, both are involved in peptide presentation by HLA molecules[25,26]. We also observed associations at other IBD genes including *ZNF365,* a transcription factor in maintaining genomic stability during DNA replication in the brain, heart, lung, pancreas, small intestine and colon[27,28]. Our genetic findings also implicated genes that maintain colonic wall permeability including *SPRED* (Sprouty-related EVH1 domain-containing protein) and *PARD3* (Partitioning defective 3 homolog)[29,30]. In addition, *GNA12* (Guanine nucleotide-binding protein alpha-12), a modulator of different transmembrane signaling systems, has also been implicated in the loss of barrier integrity[31]. Our pathway analyses strongly implicated

Table 4 Pathway analyses from genetic associations with adverse events from immunochip analyses

Type of adverse event	Pathway	Number of genes	*P* value	*P* value (Bonferroni)
Any	JAK-STAT signaling pathway	13	3.98×10^{-6}	7.96×10^{-4}
Any	Measles	12	8.87×10^{-6}	0.0018
Any	IBD	8	3.05×10^{-5}	0.0061
Any	Cytokine-cytokine receptor interaction	16	5.03×10^{-5}	0.0100
Any	Toxoplasmosis	10	5.49×10^{-5}	0.0110
Infusion	JAK-STAT signaling pathway	12	3.68×10^{-5}	0.0074
Infusion	Measles	12	8.24×10^{-6}	0.0016
Infusion	IBD	7	2.11×10^{-4}	0.0420
Infusion	Cytokine-cytokine receptor interaction	14	4.98×10^{-4}	0.0990
Infusion	Toxoplasmosis	11	9.02×10^{-6}	0.0018
Allergic	JAK-STAT signaling pathway	12	2.96×10^{-5}	0.0060
Allergic	Measles	12	6.6×10^{-6}	0.0010
Allergic	IBD	7	1.84×10^{-4}	0.0370
Allergic	Cytokine-cytokine receptor interaction	14	4.02×10^{-4}	0.0800
Allergic	Toxoplasmosis	9	2.25×10^{-4}	0.0450

IBD: Inflammatory bowel disease.

five signaling pathways including JAK-STAT signaling pathway, Cytokine-cytokine receptor interaction pathway, Measles signaling pathway, Toxoplasmosis signaling pathway, and the IBD signaling pathway. The network analyses for allergic reactions (Figure 1) show a number of key nodes including *TYK2, BLK* and *IL13*, which have previously been shown to be associated with allergic susceptibility[32-34].

IBD serologies (ANCA, anti-CBir1, anti-I2, anti-OmpC, and ASCA) can distinguish CD from UC, risk stratify IBD patients, and also predict postoperative complications and occur as a result of an aberrant or exaggerated response to commensal flora[35]. The association with ASCA and I2 are interesting. Perhaps these markers identify patients with a predilection towards small bowel involvement. Patients with colonic disease tend to respond less to anti-TNFs or require higher doses[8,36] and, perhaps therefore, these patients are more likely to develop antibodies or reactions to anti-TNFs. Further studies will be needed to confirm these borderline associations.

There are several potential limitations of this study including, the relatively small sample size and the retrospective nature of the study (despite it being the largest of its kind to date). Additionally, we did not have information on anti-drug antibody formation as the majority of these patients developed adverse events prior to the widespread use of these parameters in clinical practice. It is also important to note that our study population was predominantly of European ancestry. While IBD is rising in non-Europeans, the highest prevalence is still seen in European ancestry populations. For this reason, and the location of Cedars-Sinai Medical Center in west Los Angeles, the majority of our patients are "European". Previous work have shown ethnic differences in genetic associations with adverse events[37], and a study similar to this one should be performed for other ethnic groups.

In conclusion, our study revealed that approximately 1 in 5 IBD patients experienced an adverse event to anti-TNF therapies that required cessation of therapy. The majority of these were infusion/allergic reactions but approximately 1 in 30 women will develop a lupus-like reaction and we also observed other serious adverse events including pancreatitis and vascultitis but these were rare. We have demonstrated some genetic associations and pathways that are enriched for genes associated with development adverse events. Future studies will need to confirm these findings as the ability to identify subjects at high risk may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

ATRICLE HIGHLIGHTS

Research Background

Tumor necrosis factor (TNF) inhibitors are highly efficacious in treating inflammatory bowel disease (IBD). Response to these agents is highly heterogenous, and there have been a multitude of studies aimed at predicting the response to these agents. An important addition is the ability to predict the development of adverse events associated with these agents such as infusion reactions, infections, or rash. Minimizing the risk is important to increase patient compliance and improve response to therapy.

Research motivation

Recognizing the type and frequency of adverse events to anti-TNF therapy, and the potential genetic and serologic associations can help identify subjects at high risk, and may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Research objectives

The objectives of this study were to describe the type and frequency of adverse

events associated with anti-TNF-α therapy in a large cohort and evaluate for any serologic and genetic associations. The significance of realizing these objectives is that it can identify subjects at high risk for developing adverse events and can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Research methods

This study was a retrospective review, and detailed clinical information was collected *via* manual chart review. χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events.

The serological data was measured by ELISA assay at Cedars-Sinai, which was performed in a blinded fashion, and analyzed with Wilcox test signed rank test.

DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol (Illumina, San Diego, CA, United States). Average genotyping call rate for samples that passed quality control was 99.8%; average replicate concordance and average heritability rates were > 99.99% and 99.94%, respectively. Single-nucleotide polymorphisms (SNPs) underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling. SNPs association with adverse events was evaluated using PLINK, with two-sided p-value of 0.05 was considered statistically significant.

For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analysis was performed with statistically significant SNPs ($P < 0.001$). These SNPs were first annotated into corresponding genes, and the genes were further analyzed with multiple biological functional databases. The final networks were then constructed form the known interactions from any of these databases.

The research methods described above are standard for a retrospective review analyzing genetic and serologic data.

Research results

About 1 in 5 patients were found to have adverse events to an anti-TNF-α agent that required the therapy to be discontinued. All adverse events resolved after discontinuing the anti-TNF agent. The majority of patients developed infusion reactions. In CD patients we observed that IgA ASCA +/- was associated with a lower risk of developing any adverse event. IgA ASCA and IgG ASCA levels were also lower in patients with any adverse events. Anti-I2 level in UC was significantly associated with infusion reactions. The authors identified a number of genetic associations with known IBD loci including *HLA-DRB1, ERAP2, ZNF365*. Their pathway analyses strongly implicated JAK-STAT signaling pathway, Cytokine-cytokine receptor interaction pathway, Measles signaling pathway, Toxoplasmosis signaling pathway, and the IBD signaling pathway. The network analyses for allergic reactions showed a number of key nodes including *TYK2, BLK* and *IL13*, which have previously been shown to be associated with allergic susceptibility.

They have demonstrated some novel genetic associations and pathways that are enriched for genes associated with development adverse events. Future studies should be performed to confirm our results, and incorporate other ethnic groups besides European ancestry, and include data on anti-drug antibody formation.

Research conclusions

The genetic and serologic associations found in concordance with adverse events to anti-TNF therapy. This is the first study to evaluate and describe these associations. There are potentially genetic and serologic associations with adverse events to anti-TNF therapy that can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Current studies describe in great detail the efficacy of anti-TNF therapy and the ability to predict response to therapy. However, current studies are lacking in evaluating the ability to predict the development of adverse events. The results from this study reveal that there indeed are genetic and serologic associations with anti-TNF therapy that can potentially be targeted to prevent or avoid these adverse events in the future. The new hypothesis proposed by this study is that there serologic and genetic associations with anti-TNF therapy.

The methods used in this study were similar to other retrospective studies analyzing genetic and serologic data. Manual chart review was performed to generate detailed clinical information; ELISA assay was performed to gather serologic data; and genetic data was generated using Illumina Infinium Immunochipv1 array. χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events; serological data were analyzed with Wilcox test signed rank test; and SNPs association with adverse events were evaluated using PLINK, with two-sided *P*-value of 0.05 considered statistically significant.

The genetic and serologic associations found in concordance with adverse events to anti-TNF therapy are novel and have not been described elsewhere. This study confirmed that there are genetic and serologic associations with adverse events from anti-TNF therapy. Identifying the potential factors associated with adverse events from anti-TNF therapy can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

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ORIGINAL ARTICLE

Retrospective Study

Clinical features of alcoholic hepatitis in latinos and caucasians: A single center experience

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Abstract

AIM

To study differences of presentation, management, and prognosis of alcoholic hepatitis in Latinos compared to Caucasians.

METHODS

We retrospectively screened 876 charts of Caucasian and Latino patients who were evaluated at University of California Davis Medical Center between 1/1/2002-12/31/2014 with the diagnosis of alcoholic liver disease. We identified and collected data on 137 Caucasians and 64 Latinos who met criteria for alcoholic hepatitis, including chronic history of heavy alcohol use, at least one episode of jaundice with bilirubin ⩾ 3.0 or

coagulopathy, new onset of liver decompensation or acute liver decompensation in known cirrhosis within 12 wk of last drink.

RESULTS

The mean age at presentation of alcoholic hepatitis was not significantly different between Latinos and Caucasians. There was significant lower rate of overall substance abuse in Caucasians compared to Latinos and Latinos had a higher rate of methamphetamine abuse (12.5% *vs* 0.7%) compared to Caucasians. Latinos had a higher mean number of hospitalizations (5.3 ± 5.6 *vs* 2.7 ± 2.7, $P = 0.001$) and mean Emergency Department visits (9.5 ± 10.8 *vs* 4.5 ± 4.1, $P = 0.017$) for alcohol related issues and complications compared to Caucasians. There was significantly higher rate of complications of portal hypertension including gastrointestinal bleeding (79.7% *vs* 45.3%, $P < 0.001$), spontaneous bacterial peritonitis (26.6% *vs* 9.5%, $P = 0.003$), and encephalopathy (81.2% *vs* 55.5%, $P = 0.001$) in Latinos compared to Caucasians.

CONCLUSION

Latinos have significant higher rates of utilization of acute care services for manifestations alcoholic hepatitis and complications suggesting poor access to outpatient care.

Key words: Alcoholic hepatitis; Latino; Hispanic; Caucasian; Alcoholic liver disease

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Core tip: We conducted a retrospective chart review on Caucasian and Latino patients with alcoholic hepatitis. We showed that Latinos had significantly higher rates of gastrointestinal bleeding, encephalopathy, spontaneous bacterial peritonitis, recurrence of alcoholic hepatitis, and utilization of acute care services for alcohol related issues compared to Caucasians. However, the survival rates were not significantly different between Latinos and Caucasians. Our findings suggest that Latino patients have poor access to outpatient care and management of complications of portal hypertension.

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INTRODUCTION

Alcoholism is estimated to cause 2.5 million deaths annually worldwide, and it represents 4% of all deaths[1]. Forty percent of all mortality from cirrhosis is attributed to alcoholic liver disease (ALD). ALD includes different clinical entities with different severity and prognosis, ranging from alcoholic fatty liver disease characterized by hepatic steatosis, to severe alcoholic hepatitis (AH), and ultimately alcoholic cirrhosis[1] with portal hypertension and its complications. Disparities exist in the incidence and severity of ALD among various racial and ethnic groups in the United States.

Ethnicity data from the National Center for Health Statistics for 1991-1997 showed greatest liver cirrhosis mortality among Latino men compared to Black and Caucasian men followed by Latina females[2]. A comprehensive discharge study of all patients with ALD in non-federal hospitals in Los Angeles County in 1999 showed that ALD accounted for 1.2% of all deaths and was most prevalent in middle aged Latino men as compared to other ethnicities[3]. A more recent study showed that the Latino population in the United States has a higher incidence and more aggressive pattern of chronic liver diseases than the Caucasian population[4]. Longevity data from a multicenter Veteran Affairs cooperative study showed that the 5-year survival from active ALD was only 28% among Latino men compared to 40% in Caucasian men[5]. Among heavy alcohol drinkers, Latinos were 9.1 times more likely to have a 2-fold elevation in AST and GGT levels when compared to Caucasians[6], and Mexican American men are more likely to be heavy binge drinkers than Caucasians and African American men or Mexican American women[7]. A recent review highlights the linkage of excessive alcohol intake and dependence among Mexicans to the presence of functional SNPs in genes that regulate both alcohol craving mechanisms in the brain and alcohol metabolism in the liver[8]. In our own experience[9], Latino patients treated at UC Davis for ALD were significantly younger than Caucasians at the time of presentation and were more likely than Caucasian patients to present with AH or cirrhosis. Despite these well documented differences, there are no ethnic-specific data on the various manifestations of ALD. AH is the most severe manifestation of ALD is characterized by an inflammatory process of the liver with neutrophilic infiltration leading to ballooning degeneration of hepatocytes, hepatocyte necrosis, steatosis and presence of Mallory bodies. AH clinic presentation is associated with jaundice, fever, hepatomegaly, liver tenderness, encephalopathy, and gastrointestinal bleeding (GIB)[1]. The goal of the present study was to determine the clinical features of AH in Latino patients compared to Caucasian patients.

MATERIALS AND METHODS

We performed a single-center retrospective chart review of 137 Caucasian patients and 64 Latino patients older than 18 years old diagnosed with AH and who were seen at the University of California Davis

Hepatology Clinic or hospitalized at the University of California Davis Medical Center in Sacramento, California between 1/1/2002-12/31/2014. Of note, it is estimated that 83% of Latinos living in the Sacramento area are Mexican Americans (http://quickfacts.census.gov/qfd/states/06/06067.html). We performed a chart review of 876 Caucasian and Latino patients with ICD9 codes for AH, alcoholic cirrhosis, ALD, and unspecified liver damage identify subjects with AH of each group. When possible, we applied the NIAAA Alcoholic Hepatitis Consortia criteria[10] with few modifications given the retrospective nature of the study: (1) chronic history of heavy alcohol use; (2) at least one episode of jaundice with bilirubin ⩾ 3 or coagulopathy with INR ⩾ 1.2 (modification); (3) new onset of liver decompensation; (4) diagnosis acute AH per ICD-9 code on known cirrhosis; and (5) within 12 wk (modification) of last drink and where no other causes of liver decompensation were identified. Otherwise, ICD9 codes for AH were applied to ensure the highest number of subjects was included in the study. We excluded patients with known active hepatitis B (positive HBsAg and Hepatitis B viral load > 20,000 IU/mL) and active hepatitis C (positive HCV Ab and HCV viral load > 615 IU/mL).

We used the demographics section of the electronic medical record and/or from history and physical examination notes to identify our research population, ethnicity, gender, and age of onset of AH. We defined the drinking patterns as follow: moderate drinking was defined as no more than four drinks per day, or 14 drinks per week for men, and no more than three drinks per day, or 7 drinks per week for women, and heavy drinking defined as 15 or more drinks per week, or four drinks in a day, for men or, more than seven drinks per week, or three drinks a day for women. We defined binge drinking as five or more drinks for male or 4 or more drinks in female in about 2 h on multiple occasions. We also gathered drinking duration occurred in our patient population. We included data pertaining to body mass index (BMI) and whether subjects had a diagnosis of metabolic syndrome and diabetes mellitus (DM). We recorded the development of complications associated with AH and/or portal hypertension including GIB, encephalopathy, spontaneous bacterial peritonitis (SBP), and presence of other infections, pancreatitis, development of acute kidney injury (AKI) and/or hepatorenal syndrome, disseminated intravascular coagulopathy (DIC), and hepatocellular carcinoma. We recorded intensive care unit (ICU) admission, length of ICU stay, development of respiratory failure, and length of hospital stay, and we determined the number of emergency room visits and hospitalizations for alcohol related morbidity.

We made the distinction of AH without known history of cirrhosis and AH with known cirrhosis and recorded the number of times a patient met our criteria for AH. To assess severity of AH, we calculated the Discriminant Function (DF) [4.6 × (Pt's PT - Control PT) + TBili]. If ⩾ 32, it is considered severe AH[11] with related increased risk of mortality[12]. We determined Glasgow score for AH, Model for End-Stage Liver Disease (MELD) score, and Child-Turcotte-Pugh class. We collected data on modality of medical treatment of severe AH (supportive care, steroids, pentoxifylline including their duration and doses). Finally, we collected survival data that included total survival days after episode of AH (total number of days since date of AH to last day to be alive or seen at the UC Davis Medical Center or clinic).

Statistical analyses

Categorical variables were compared between ethnicities using χ^2 tests, followed by examination of adjusted residuals in the case of a significant test. Continuous variables were compared between ethnicities using *t*-tests, with duration data and lab data log-transformed prior to testing. Survival was compared between ethnicities using log rank tests.

The effects of subject disease characteristics and treatment on overall survival were analyzed using Cox proportional hazards models. Analyses were conducted for all subjects and separately for each ethnicity. All analyses were adjusted for age, sex, BMI, and, for analyses in all subjects, ethnicity. Analyses were conducted using R, version 3.3.1 (R Core Team, 2016).

RESULTS

The mean age at presentation for all patients was 48.6 ± 10.4 years and was not significantly different between Latinos and Caucasians. Among men, there was a marginally significant higher proportion of male Latino subjects with AH compared to Caucasians subjects (71.9% *vs* 56.2%, $P = 0.049$). There were no significant differences between Latinos and Caucasians in BMI, presence of DM, or metabolic syndrome (Table 1). The mean duration of drinking was longer in Latinos compared to Caucasians (24.3 ± 11.0 years *vs* 20.5 ± 12.7 years, $P = 0.027$). There were no significant differences between Latinos and Caucasians regarding pattern of drinking or daily mean average number of alcoholic drinks (Table 1). Latinos and Caucasians presented different patterns of non-alcoholic substance abuse. Caucasians had significant higher rates of having no history of substance abuse (73.0% *vs* 57.8%), whereas more Latinos had a history of methamphetamine abuse (12.5% *vs* 0.7%) and a remote history of substance abuse (9.4% *vs* 4.4%) than would be expected under homogeneity of variance ($P = 0.003$) (Table 1).

Latinos had about twice the number of emergency department (ED) and hospitalizations visits due to alcohol intoxication and alcohol related issues compared to Caucasians (Figures 1 and 2). There was no significant difference in the mean length of hospital stay between Latinos (52 subjects admitted out of 64)

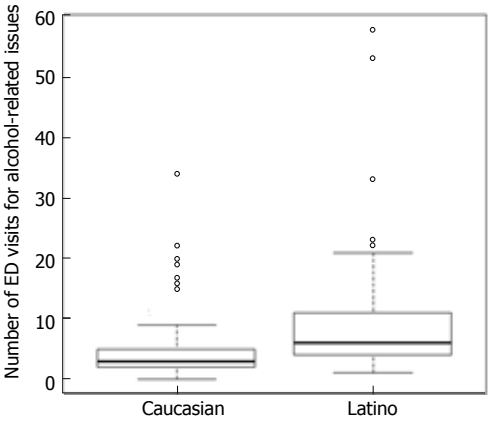


Figure 1 Emergency department visits for alcohol-related issues. There was a significant difference in the mean number of ED visits for alcohol related issues between ethnicities. Latinos had a significant higher mean number of ED visits for alcohol related issues compared to Caucasians (9.5 ± 10.8 *vs* 4.1 ± 4.1 ED visits, $P \leqslant 0.001$). ED: Emergency department.

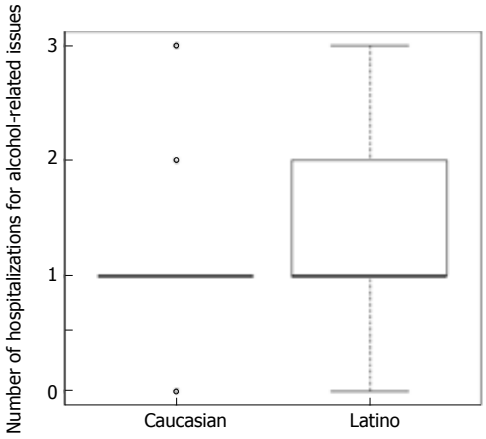


Figure 2 Hospitalizations for alcohol-related issues. There was a significant difference in the mean number of hospital admissions for alcohol related issues between ethnicities. Latinos had a higher mean number of hospital admissions for alcohol related issues compared to Caucasians (5.3 ± 5.6 *vs* 2.7 ± 2.7 admissions, $P = 0.001$).

Table 1 Patient Characteristics *n* (%)

	Caucasian (*n* = 137)	Latino (*n* = 64)	All Patients (*n* = 201)	*P* value
Age of presentation with AH (yr)				0.082
mean ± SD	49.5 (10.4)	46.8 (10.1)	48.6 (10.4)	
Sex				0.049
Male	77 (56.2)	46 (71.9)	123 (61.2)	
Female	60 (43.8)	18 (28.1)	78 (38.8)	
BMI				0.167
mean ± SD	28.1 (5.9)	29.4 (6.2)	28.5 (6)	
DM				0.061
No	105 (76.6)	41 (64.1)	146 (72.6)	
Yes	25 (18.2)	20 (31.2)	45 (22.4)	
Unknown	7 (5.1)	3 (4.7)	10 (5)	
Metabolic syndrome				0.405
No	37 (27)	13 (20.3)	50 (24.9)	
Yes	53 (38.7)	28 (43.8)	81 (40.3)	
Unknown	47 (34.3)	23 (35.9)	70 (34.8)	
Duration of drinking (yr)				0.027
mean ± SD	20 (12.7)	24.3 (11)	21.6 (12.3)	
Pattern of drinking				0.283
Heavy drinking	5 (3.6)	0	5 (2.5)	
Binge drinking	130 (94.9)	64 (100.0)	194 (96.5)	
Unknown	2 (1.5)	0	2 (1.0)	
Number of drinks/d				0.093
mean ± SD	13.9 (10.9)	16.1 (7.2)	14.6 (9.9)	
None	100 (73.0)	37 (57.8)	137 (68.2)	
Cocaine	2 (1.5)	0	2 (1.0)	
Heroin	1 (0.7)	0	1 (0.5)	
Methamphetamine	1 (0.7)	8 (12.5)	9 (4.5)	
Marijuana	9 (6.6)	3 (4.7)	12 (6.0)	
Other	2 (1.5)	0	2 (1.0)	
Polysubstance abuse	10 (7.3)	9 (14.1)	19 (9.5)	
Remote history	6 (4.4)	6 (9.4)	12 (6.0)	
Unknown	6 (4.4)	1 (1.6)	7 (3.5)	

AH: Alcoholic hepatitis; BMI: Body mass index; DM: Diabetes mellitus.

and Caucasians (111 admitted out of 137) admitted to the hospital with AH and there was no significant difference between Latinos and Caucasians requiring ICU admission and length of ICU stay in days (Table 2).

There were no significant differences in the proportions of Latinos and Caucasians who presented with AH without history of cirrhosis (76.6% *vs* 68.6%) and with history of cirrhosis (23.4% *vs* 31.4%) ($P = 0.321$). The mean DF score of the whole group was 46.7 ± 33.9 and there was no significant difference in prevalence of severe AH, as determined by DF > 32, between Latinos and Caucasians, mean MELD score, and

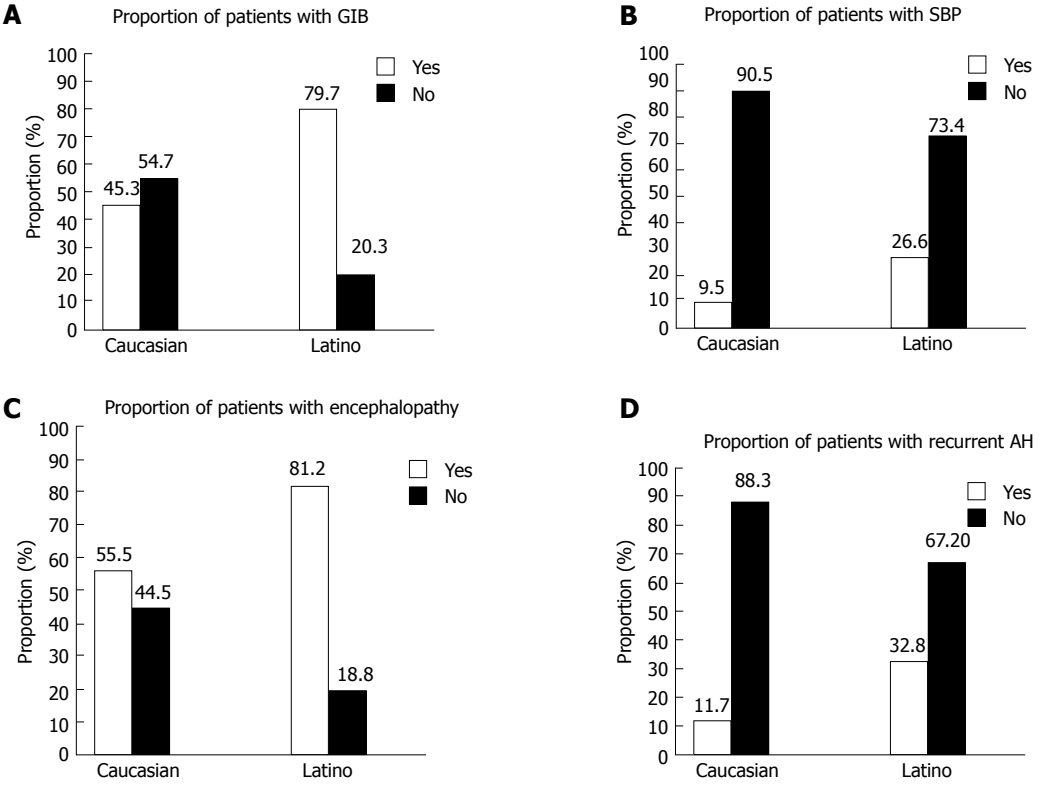


Figure 3 Clinical manifestations of alcoholic hepatitis. There was a significant difference in the proportion of patients who experienced GIB, SBP, encephalopathy, and recurrence of AH between ethnicities. A: A significant higher proportion of Latinos experienced GIB compared to Caucasians (79.7% *vs* 45.3%, $P < 0.001$); B: A significantly higher proportion of Latinos experienced SBP compared to Caucasians (26.6% *vs* 9.5%, $P = 0.003$); C: A significant higher proportion of Latinos experienced encephalopathy compared to Caucasians (81.2% *vs* 55.5%, $P = 0.001$); D: A higher proportion of Latinos had recurrent AH compared to Caucasians (32.8% *vs* 11.7%, $P = 0.005$). AH: Alcoholic hepatitis; GIB: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis.

Table 2 Level of care *n* (%)

	Caucasian	Latino	All patients	*P* value
Hospitalization duration (in days)	111	52	163	0.239
mean ± SD	14.4 (12.8)	13.8 (15.9)	14.2 (13.8)	
ICU admission	111	52	163	0.756
Yes	39 (35.1)	17 (32.7)	56 (34.4)	
No	72 (64.9)	35 (67.3)	107 (65.6)	
ICU stay (in days)	39	15	54	0.587
mean ± SD	7.7 (9.9)	12.2 (16.6)	8.9 (12.1)	

111 out of the 137 Caucasians and 52 out of 64 Latino Subjects were admitted to the hospital for management of alcoholic hepatitis. The other Caucasians and Latino patients were seen in the ED or outpatient setting. ICU: Intensive care unit.

initial mean Glasgow score for AH (Table 3). Among patients who received medical treatment for severe AH (Latinos $n = 15$ and Caucasians $n = 37$), patients from both ethnicities were treated similarly with similar proportions of patients treated with steroids, pentoxyfilline or combination of both (Table 3). There was no significant difference in the Lille score after 7 d of treatment between Latinos ($n = 14$) and Caucasians ($n = 35$) (Table 3).

Latinos and Caucasians admitted with AH presented a similar rate of complications including respiratory failure, acute pancreatitis, AKI, hepatorenal syndrome, and DIC (Table 4). However Latinos had higher rates of portal hypertension complications including overall GIB (Figure 3A), SBP (Figure 3B), encephalopathy (Figure 3C), and recurrence of AH (Figure 3D) compared to Caucasians. Despite these higher rates of complications, Latinos presented a longer median survival compared to Caucasians but the difference was not significant (12.1 *vs* 4.6 years, $P = 0.055$) (Figure 4).

Table 5 shows results of Cox proportional hazards analysis of overall survival. In the analysis of all subjects, after adjusting for age, BMI, sex, and ethnicity, a higher MELD score, a Child-Turcotte-Pugh score of C, an initial Maddrey score > 32, abnormal renal function, and presence of hepatorenal syndrome were all associated with significantly higher hazards of death.

Table 3 Type of alcoholic hepatitis, parameters of liver injury and treatment *n* (%)

	Caucasian	Latino	All patients	*P* value
Type of AH	137	64	201	0.321
Acute AH with no history of cirrhosis	94 (68.6)	49 (76.6)	143 (71.1)	
AH on chronic cirrhosis	43 (31.4)	15 (23.4)	58 (28.9)	
DF score at day 0	137	64	201	0.794
mean ± SD	47.7 (37.2)	46.6 (22.1)	47.3 (33.1)	
DF < 32	44 (32.1)	12 (18.8)	56 (27.9)	
DF > 32	93 (67.9)	52 (81.2)	145 (72.1)	
Initial MELD score	137	64	201	0.661
mean ± SD	21.1 (7)	21.6 (6.7)	21.3 (6.9)	
< 9	2 (1.5)	0	2 (1)	
10-19	61 (44.5)	30 (46.9)	91 (45.3)	
20-29	53 (38.7)	25 (39.1)	78 (38.8)	
30-39	19 (13.9)	9 (14.1)	28 (13.9)	
> 40	2 (1.5)	0	2 (1.0)	
Glasgow AH score at day 0				0.991
mean ± SD	7.5 (1.6)	7.4 (1.5)	7.5 (1.6)	
< 9	109 (71.7)	53 (69.7)	162 (71.1)	
> 9	43 (28.3)	23 (30.3)	66 (28.9)	
Type of treatment	37	15	52	0.862
Steroids	16 (11.7)	8 (12.5)	24 (11.9)	
Pentoxifylline	13 (9.5)	4 (6.2)	17 (8.5)	
Both	8 (5.8)	3 (4.7)	11 (5.5)	
Lille score after 7 d				0.829
< 0.45	18 (13.1)	8 (12.5)	26 12.9)	
> 0.45	17 (12.4)	6 (9.4)	23 (11.4)	

AH: Alcoholic hepatitis; MELD: Model for end stage liver disease; DF: Maddrey discriminant function is defined as DF = 4.6 x [prothrombin time (s) - control prothrombin time (s)] + (serum bilirubin in mg/dL)[11].

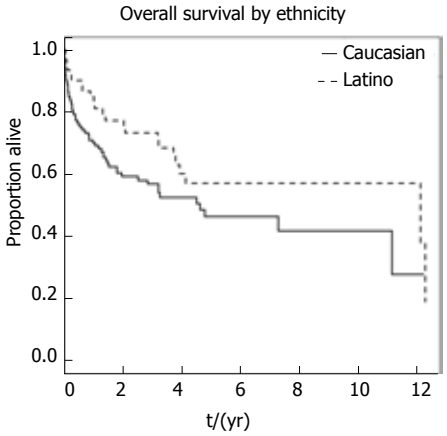


Figure 4 Survival by ethnicity. There was no significant difference in the median survival between ethnicities as shown on Kaplan-Meier Plot. The median survival in years between Latinos and Caucasians was not significantly different (12.1 yr *vs* 4.6 yr, *P* = 0.055).

In Latinos, after adjusting for age, BMI, and sex, a higher MELD score, a Child-Turcotte-Pugh score of C, an initial Glasgow score above 9, and the presence of hepatorenal syndrome were associated with significantly higher hazards of death. In Caucasian subjects, after adjusting for age, BMI, and sex, a higher MELD score, a Child-Turcotte-Pugh score of C, abnormal renal function, and presence of hepatorenal syndrome were associated with significantly higher hazards of death.

DISCUSSION

AH is an acute hepatic inflammation that usually occurs in heavy drinkers and is associated with significant morbidity and mortality in severe cases of up to 30%-50% at 6 mo[13]. Several previous studies indicated that Latino patients with ALD had earlier onset of disease and poorer prognosis compared to Caucasians[9] but specific data on ALD subtypes are lacking. The goal of the present study was to focus on a sample of Latino patients with AH followed at UC Davis Medical Center in Sacramento, California and describe any similarities and differences of AH in comparison to Caucasian patients, with the ultimate goal to identify specific needs and potential gaps in the care of this population. First, Latinos and Caucasians appeared to differ primarily in the duration of alcohol drinking, which was longer for the Latinos, and methamphetamine use, which was also more prevalent in Latinos. These findings are corroborated by previous large epidemiological studies showing that Mexican migrants in California presented a high prevalence of methamphetamine use associated with alcohol use and other psychiatric issues[14]. The two groups received similar medical treatment once admitted for severe AH. However, Latinos appear to present more frequently with manifestations of severe portal hypertension in association with more frequent utilization of the acute services but not ICU. Overall, these disparities did not seem to affect the survival of our studied Latino population. Several clinical studies

Table 4 Complications associated with alcoholic hepatitis *n* (%)

	Caucasian	Latino	All patients	*P* value
Development of respiratory failure	137	64	201	0.687
Yes	13 (9.5)	8 (12.5)	21 (10.4)	
No	124 (90.5)	56 (87.5)	180 (89.6)	
Development of acute pancreatitis				0.413
No	110 (80.3)	52 (81.2)	162 (80.6)	
Yes	13 (9.5)	3 (4.7)	16 (8.0)	
Unknown	14 (10.2)	9 (14.1)	23 (11.4)	
Development of AKI				0.520
Yes	33 (24.1)	19 (29.7)	52 (25.9)	
No	104 (75.9)	45 (70.3)	149 (74.1)	
Hepatorenal syndrome				0.328
No	119 (86.9)	60 (93.8)	179 (89.1)	
Yes, type 1	15 (10.9)	3 (4.7)	18 (9.0)	
Yes, type 2	3 (2.2)	1 (1.6)	4 (2.0)	
Development of DIC				0.497
Yes	3 (2.2)	0	3 (1.5)	
No	89 (65)	50 (78.1)	139 (69.2)	
Unknown	45 (32.8)	14 (21.9)	59 (29.4)	

AKI: Acute kidney injury; DIC: Disseminated intravascular coagulation.

Table 5 Cox proportional hazards analyses of overall survival by subject characteristics[1]

	All subjects		Caucasian subjects		Latino subjects	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
History of GI bleeding: yes *vs* no	0.79 (0.49, 1.28)	0.339	0.91 (0.53, 1.54)	0.718	0.39 (0.09, 1.72)	0.213
History of SBP: yes *vs* no	0.95 (0.50, 1.80)	0.874	0.86 (0.37, 2.01)	0.727	0.98 (0.37, 2.62)	0.97
ICU Admission: yes *vs* No	1.32 (0.80, 2.19)	0.278	1.42 (0.79, 2.56)	0.237	1.31 (0.45, 3.81)	0.618
MELD score[2]	1.88 (1.39, 2.55)	< 0.001	1.86 (1.29, 2.69)	0.001	2.15 (1.19, 3.87)	0.011
Child-turcotte-pugh score: C *vs* A or B	2.85 (1.62, 5.00)	< 0.001	2.62 (1.36, 5.07)	0.004	4.3 (1.24, 14.9)	0.021
Initial glasgow score: > 9 *vs* < 9	1.86 (1.18, 2.93)	0.008	1.6 (0.93, 2.74)	0.091	2.91 (1.21, 6.97)	0.017
Initial maddrey score: > 32 *vs* < 32	2.41 (1.29, 4.49)	0.006	1.82 (0.95, 3.50)	0.072	[3]	[3]
Treatment with steroids: yes *vs* no	1.63 (0.89, 2.98)	0.113	1.68 (0.81, 3.51)	0.164	2.05 (0.61, 6.88)	0.247
Duration of treatment with steroids: ≥ 28 d *vs* < 28 d	2.23 (0.66, 7.58)	0.198	5.28 (0.69, 40.5)	0.110	1.03 (0.01, 83.3)	0.988
Renal function: CKD 1 or higher *vs* CKD 0	7.43 (2.74, 20.1)	< 0.001	7.74 (2.8, 21.4)	< 0.001	[3]	[3]
Hepatorenal syndrome: yes *vs* no	7.07 (3.95, 12.7)	< 0.001	7.77 (3.92, 15.4)	< 0.001	5.65 (1.66, 19.2)	0.006

[1]A separate analysis was conducted for each risk factor shown. All analyses were adjusted for age, sex, BMI, and, for analyses in all subjects, ethnicity; [2]Change in hazard of death for 10 point increase in actual MELD score; [3]Not enough data to analyze. GI bleeding: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis; ICU: Intensive care unit; MELD: Model for end stage liver disease; CKD: Chronic kidney disease.

indicate that Latinos develop more severe liver diseases than other ethnicities and this has been confirmed in common conditions including ALD[9,15], non-alcoholic steatohepatitis[16], and chronic hepatitis C[17,18] or more rare diseases including primary biliary cholangitis[19]. In addition, Latinos might have an increased risk of hepatocellular carcinoma[20]. Ethnic matching between patients with alcohol-related injury or alcohol problems improved the effectiveness of the brief alcohol intervention on drinking outcomes of Latino patients, especially the foreign-born and less acculturated[21]. Our data indicate that, at least in our center, Latinos with AH are receiving similar inpatient medical management compared to Caucasians. It should be noted that only a small number of patients received treatment for severe AH during the studied period. This could be

related to the presence of contraindications to the use of steroids (for example active infections or GIB) or to the lack of proper diagnosis of severe AH by providers. The main issue regards the long term management of the complications of portal hypertension. Previous published data have shown that Latinos are more likely to visit the ED and are more often admitted through the ED for alcohol related issues[22]. Similarly, our study showed that Latinos present more frequently at the ED and are hospitalized more often compared to Caucasians for alcohol related issues, but also present more frequently severe complications of portal hypertension. This suggests a poor outpatient management of chronic complications of liver disease and inadequate access or use of outpatient clinics to receive preventive measures, including GIB prophylaxis with upper endoscopies and beta blockers, encephalopathy prophylaxis with lactulose, or prophylaxis with SBP when indicated. We did not observe any difference in survival between the two subpopulations and this may be in contradiction with the data showing worse outcome for Latinos with ALD. It is also interesting to note that the overall Latino population has better survival rate compared to Caucasians[23]. Our data reflect a small population in a highly diverse urban area but this initial data should help in identifying some of the major challenges in the care of underserved populations. Given the retrospective nature of the data collection and given the fact that is based on single center experience, we could not identify the actual background of the Latino population, which could be very heterogeneous.

This single retrospective study did not confirm previous findings that Latinos developed AH at earlier age compared to Caucasians[9]. In fact, there was no significant difference between the ages of presentation of AH between Latinos and Caucasians. Our study also suggested that there is no difference in the severity of AH at presentation and management between the ethnicities. On the other hand, this study showed Latinos had higher rates of complications associated with AH which include GIB, encephalopathy, and SBP. Latinos also had higher rates of AH recurrence. Interestingly, there was no significant difference in the overall mortality between ethnicities. In fact, Latinos may have better survival than Caucasians even though this is was not statistically significant in our study. Future research is needed to elucidate the effects of alcohol on Latinos and the predisposing factor that lead to the development of AH as well as the study of measures that can improve access to care and adequate outpatient follow-up for this underserved population.

ATRICLE HIGHLIGHTS

Research background

There are well known differences in the presentation of alcoholic liver disease (ALD) between Caucasian and Mexican-American patients. However, there are no ethnic-specific data on alcoholic hepatitis.

Research motivation

Alcoholic hepatitis is the most severe manifestation of ALD and is characterized by an inflammatory process of the liver with neutrophilic infiltration leading to ballooning degeneration of hepatocytes, hepatocyte necrosis, steatosis and presence of Mallory bodies. Alcoholic hepatitis clinic presentation is associated with jaundice, fever, hepatomegaly, liver tenderness, encephalopathy, and gastrointestinal bleeding. Ultimately, alcoholic hepatitis is characterized by a high risk of mortality. Latino and Caucasian patients with ALD and alcoholic hepatitis may have different progression and prognosis compared to other ethnicities.

Research objectives

The goal of the present study was to determine the clinical features of alcoholic hepatitis in Latino patients compared to Caucasian patients. The overarching goal is to optimize the care of patients of different ethnicities and identify their challenges when accessing medical care in the United States.

Research methods

The authors conducted a single-center retrospective chart review of 137 Caucasian patients and 64 Latino patients older than 18 years old diagnosed with alcoholic hepatitis. We collected extensive clinical data on their diagnosis, treatment, and outcome.

Research results

The major finding was a significantly higher rate of complications of portal hypertension including gastrointestinal bleeding, spontaneous bacterial peritonitis, and encephalopathy in Latino compared to Caucasian patients with alcoholic hepatitis. Despite these differences, the two ethnic groups did not present differences in survival. Future studies should prospectively confirm these findings in larger populations.

Research conclusions

According to our results, Latinos with alcoholic hepatitis presents with more severe complications of alcoholic hepatitis and appear to have limitations in access to medical treatment of long term complications of portal hypertension. Efforts should be made to ensure improved access to care and compliance.

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ORIGINAL ARTICLE

Retrospective Study

Predictive factors associated with carcinoid syndrome in patients with gastrointestinal neuroendocrine tumors

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Author contributions: All authors were equally involved in the design of the study; Chang conducted the statistical analyses; and all authors contributed equally in the interpretation of results and writing of the manuscript.

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Institutional review board statement: We conducted a retrospective case-control study using the Truven Health Analytics MarketScan Database and the IMS Health PharMetrics Database, both commercial health insurance claims database for employer-insured beneficiaries in the United States. The databases are fully compliant with the Health Insurance Portability and Accountability Act and meet the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Informed consent statement: This study involved analyses of Health Insurance Portability and Accountability Act-compliant secondary databases, MarketScan and PharMetrics, thus no informed consent was feasible or necessary.

Conflict-of-interest statement: Cai is an employee of Novartis Pharmaceuticals Corporation. Broder, Chang, and Yan are employees of the Partnership for Health Analytic Research, LLC, which received funding from Novartis to conduct the researchdescribed in this manuscript. Metz is an employee of Northwestern University and was paid by Novartis to consult as a subject matter expert. Metz is Chair of the North American Neuroendocrine Tumor Society (NANETS) and also a consultant for Ipsen. Metz has received commercial research grants from Lexicon and Advanced Accelerator Applications (AAA), and is a consultant/on the advisory board for AAA.

Data sharing statement: The study statistician, Eunice Chang, conducted all statistical analysis for this study using Health Insurance Portability and Accountability Act-compliant commercial-insurance secondary databases MarketScan and PharMetrics.

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Abstract

AIM

To discover unknown factors associated with carcinoid syndrome (CS) with the goal of earlier diagnosis of CS.

METHODS

In this retrospective case-control study using United States administrative claims, patients (⩾ 18 years) newly-diagnosed with gastrointestinal neuroendocrine tumors (GI NETs) without CS (controls) were exactly matched to patients with CS (cases) based on NET diagnosis date at a 3-to-1 ratio. Study index date was first CS diagnosis (controls: same distance from NET diagnosis as cases). The most observed conditions, excluding CS-associated symptoms/diagnoses, during the year before index date were assessed. Forward-stepwise logistic regression models were used to derive predictors, and were validation within another claims database.

RESULTS

In the development database, 1004 patients with GI NETs were identified; 251 (25%) had CS and 753 (75%) were controls. In the validation database, 724 patients with GI NETs were identified; 181 (25%) had CS and 543 (75%) were controls. A total of 33 common diagnoses (excluding conditions already known to be associated with CS) in the development database were entered in forward step-wise logistic regression models. In the final, validated logistic regression model, three factors prior to CS diagnosis were found consistently associated with higher risks for CS, including liver disorder [odds ratio (95%CI): 3.38 (2.07-5.51)], enlargement of lymph nodes [2.13 (1.10-4.11)], and abdominal mass [3.79 (1.87-7.69)].

CONCLUSION

GI NET patients with CS were 2-4 times as likely to have preexisting diagnoses (*i.e.*, liver disorder, enlarged lymph nodes, abdominal mass) than non-CS patients.

Key words: Carcinoid syndrome; Gastrointestinal neuroendocrine tumors; Predictive factors; Data mining

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Core tip: By assessing patients with gastrointestinal neuroendocrine tumors from two independent United States claim databases, this study found that patients with carcinoid syndrome (CS) were 2-4 times as likely to have a preexisting diagnosis of a liver disorder, enlargement of lymph nodes, or abdominal mass than patients without CS.

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INTRODUCTION

Neuroendocrine tumors (NETs), the most of common of which are carcinoid tumors[1], are characterized by a relatively indolent rate of growth and an ability to secrete a variety of peptide hormones. These tumors can be functional or non-functional and benign or malignant[1]. Even though NETs can develop anywhere in the body, the majority of them occur in the gastrointestinal (GI) tract (67.5%)[2]. Within the GI tract, sites of origin include the stomach, small intestine, appendix, and colorectum[2]. A 6-fold increase in the age-adjusted incidence of diagnosed NETs has been observed from 1.09 new cases per 100000 individuals in 1973 to 6.98 new cases per 100000 individuals in 2012[3,4].

One of the more common functional NET syndromes is carcinoid syndrome (CS), occurring in 8% to 35% of NET patients[5]. CS occurs when functional carcinoid tumors metastasize to the liver or outside the GI tract, and the vasoactive hormones secreted by metastases, such as serotonin, histamine, or tachykinins, are no longer metabolized and inactivated by the liver and reach the general circulation[6,7]. 5-hydroxyindoleacetic acid (5-HIAA), a product of serotonin metabolism of urinary excretion, is used as a first-line test for biochemical detection of suspected CS, in which 5-HIAA excretion is usually elevated[8]. CS includes an array of signs and symptoms. The classical manifestations of episodic flushing and diarrhea affect more than 80% of patients with CS[6]. Other signs include pellagra, wheezing, abdominal pain, telangiectasia and heart disease (usually right sided valvular regurgitation). The clinical presentation with diarrhea and/or abdominal pain dominating often leads to misdiagnosis of CS as irritable bowel disease or small bowel obstruction[9,10] especially in middle-aged perimenopausal females. Delays in the correct diagnosis are common; reports on median time from onset of symptoms to diagnosis range from 2 to 20 years[9].

Other than the typical signs associated with CS, there is limited knowledge on the existence of any other predictors that may be associated with the risk of developing CS. We conducted the current study in patients with GI NETs, the most common type of NETs, to describe factors associated with CS with the intention of assisting physicians with making an earlier diagnosis of CS.

MATERIALS AND METHODS

Data source

We conducted a matched case-control study using data from two large United States healthcare claims databases - IMS PharMetrics Plus and Truven Health Analytics MarketScan. We used IMS PharMetrics Plus as the development database to derive risk factors for CS and MarketScan databases to validate the factors for CS. We used data from 1/1/2009 to 12/31/2014 from both databases.

The PharMetrics Plus database comprises adjudicated

medical and pharmacy claims for approximately 150 million patients enrolled in United States health insurance plans, with an annual capture of 40 million. This database is representative of the United States commercially insured population for individuals under age 65 years. The MarketScan Research Database combines 2 separate databases-the Commercial Claims and Encounters database, and the Medicare Supplemental and Coordination of Benefits database. The commercial database contains the inpatient, outpatient, and outpatient prescription drug experience of about 40 million employees and their dependents, who are covered under a variety of fee-for-service and managed care health plans. The Medicare database contains the healthcare experience of about 3 million retirees with Medicare supplemental insurance paid for by employers. Both databases contain detailed cost, use, and outcomes data for inpatient and outpatient healthcare services. The medical claims are linked to outpatient prescription drug claims and person-level enrollment data through the use of unique enrollee identifiers. Both PharMetrics and MarketScan are Health Insurance Portability and Accountability Act compliant[11,12]. The study was exempt from review by an institutional review board

Study population

The study population consisted of patients who were new diagnosed with GI NETs during the identification (ID) period (1/1/2010-12/31/2014). We identified patients with at least one inpatient or two outpatient claims with International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes for any NET (209.xx.) Among these, patients who had ICD-9-CM codes for GI NET (209.0, 209.1, 209.2, 209.4, 209.5, and 209.6) as the most frequent NET codes were classified as GI NET. To ensure that patients were newly diagnosed, we excluded patients with GI NETs during the 1-year prior to the first diagnosis date. We excluded patients with pancreatic NETs and Merkel cell carcinoma at any time during the study period.

We matched patients with GI NETs without CS (controls) to patients with CS (cases) based on the diagnosis date (month and year) of the first GI NET diagnosis at a 3-to-1 ratio. For cases, the index date was the first CS diagnosis date. For controls, the index date was assigned to have the same distance from the date of first NET diagnosis as the matched case patients. All patients were required to have at least 1-year continuous enrollment prior to the index date (study baseline).

The algorithm used to identify cases was developed based on a series of analyses performed before undertaking the main study. First, we examined overall prevalence of CS and the distribution of all NET locations (*e.g.*, GI, lung, pancreas) in patients with a CS by identifying cases using a diagnosis (ICD-9-CM code 259.2) and found it did not match the expected distribution. Specifically, we found that 6% of patients with pancreatic neuroendocrine tumor (PNET) had at least two claims with a code for the CS, as did 23% of patients with GI NET. We suspected that the diagnosis code was being applied at an early point in the diagnostic process (*e.g.*, as a "rule out"). We tested the application of more stringent criteria, which required two claims with ICD-9-CM code 259.2 and either a urine 24-h 5-HIAA [Current Procedural Terminology (CPT) code: 83497] or a serum serotonin (CPT code: 84260). In addition, the tests must have been ordered in the period 3 mo before or 3 mo after the CS diagnosis. This stricter algorithm resulted in only 1% of patients with PNET and 11% of GI NET being identified as having CS. As a result, we used the 2 CS diagnosis plus testing algorithm to identify cases with CS for this study.

Patient demographic characteristics included age in years on index date, sex, and geographic region (Midwest, Northeast, South, and West). Clinical characteristics included Charlson Comorbidity Index[13,14], and number of chronic condition indicators [15].

The outcome of interest in this study was the presence of CS.

Statistical analysis

We performed descriptive analyses to assess differences between case/control cohorts and patient demographic and clinical characteristics. χ^2 tests were used for categorical variables and two sample *t*-tests were used for continuous variables.

Data mining is a process of selecting, exploring and modeling large amounts of data in order to discover unknown patterns or relationships that provide a clear and useful result[16]. We used data mining to explore unknown factors associated with CS. Specifically, using the development database (PharMetrics Plus), we assessed the top 50 most frequently observed conditions (based on CS patients) other than symptoms/diagnoses known to be associated with CS during the 1 year prior to the index date. We eliminated conditions or symptoms already known to be associated with CS, those related to screening or health maintenance, other GI cancers, and factors occurring in < 10% of both case and control groups. We began the multivariate analysis by forcing demographics and two general comorbidity measures (Charlson Comorbidity Index and number of chronic conditions) into the model. We used forward-stepwise logistic regression to estimate, for each condition in the final model, the extent to which a patient who went on to be diagnosed with CS would have greater odds of having the condition than a patient who went on to be diagnosed with NET, but not CS. In the forward selection, significant factors ($P < 0.05$) from the list of conditions referred to above were retained. Demographic or comorbidity measures were removed from the final model if they were highly

insignificant ($P \geqslant 0.1$). To validate these predictors, we re-ran the model with completely independent data from another administrative claims database (Truven MarketScan).

RESULTS

Descriptive statistics

Of the 23815 NET patients identified from the IMS PharMetrics Plus, the development database, we excluded 23310 patients who did not meet the inclusion criteria, such as enrollment, age, and definition of CS. Of the 505 patients who met the study selection criteria 251 were GI NET patients with CS (cases) (Figure 1). These 251 cases along with the 753 matched controls (GI patients without CS) were included in the final analytic sample in the development database. Case patients were slightly younger than control patients (mean ± SD: 52.8 ± 10.9 years *vs* 54.4 ± 13.3 years; $P = 0.06$). The majority of patients were in the 55-64 age range for both cohorts (37.5% in CS cohort; 34.1% in non-CS; $P = 0.04$). There were no significant differences in sex, region, and number of chronic conditions between the two cohorts (Table 1).

In the validation database 724 patients with GI NETs were identified, among whom 181 (25%) had CS and 1158 (75%) were controls (Table 1). The mean (SD) age in the CS cohort was 51.8 (9.33) years, compared to 51.7 (10.03) years in the non-CS cohort. There were no significant differences in age, sex, and region (Table 1). With this approach, we identified a total of 33 most common conditions in both CS and non-CS cohorts (Table 2) of the development database for further assessment. In both cohorts, abdominal pain (66.1% of CS cohort; 51.5% of non-CS cohort), hypertension (50.6%; 52.2%), and dyslipidemia (49.4%; 46.1%) were the most prevalent diagnoses (Table 2).

Multivariable analysis

Eight of the 33 common diagnoses tested remained significant in the forward step-wise logistic regression models and were further validated using the validation database (Table 3). In the final, validated logistic regression model, three factors were associated with higher risks for CS, including liver disorder [odds ratio (95%CI): 3.38 (2.07-5.51)], enlargement of lymph nodes [2.13 (1.10-4.11)], and abdominal mass [3.79 (1.87-7.69)] (Table 4).

DISCUSSION

By assessing patients from two independent United States claim databases, this study suggested that in patients with proven GI NETs, patients with CS were 2-4 times as likely to have a preexisting diagnosis of a liver disorder, enlargement of lymph nodes, or abdominal mass than patients without CS.

Among patients with NETs, preexisting enlarged lymph nodes, abdominal mass lesions, and a liver disorder seem to jointly indicate increasing tumor burden, which implies that tumor progression may be associated with a higher risk of developing CS. Although there is no obvious clinical explanation for these findings, we propose the following two considerations: first, patients with CS have a larger systemic tumor bulk at diagnosis than patients who do not manifest CS, implying that increasing tumor burden correlates with increasing tumor products, which in turn led to the syndrome; and second, that patients with CS have more aggressive tumors than those without CS, which leads to more rapid growth of tumor and a larger burden of disease when diagnosed. The first consideration appears less likely since patients with small strategically located tumors (*e.g.*, the ovary or lung) commonly present with CS prior to large bulky liver disease developing. On the other hand, it is well known that patients with CS have a poorer quality of life and outcome than those without CS suggesting that the latter explanation is more likely. To the best of our knowledge, this is only the second study assessing distinctions between CS and non-CS patients. The only other study we could identify in the existing literature is a recent study by Halperin and colleagues[17] that used SEER-Medicare data to assess the clinical factors that may be associated with CS specifically. They found that their CS cohort included more patients with regional and distant metastases, while their non-CS cohort included more patients with local disease; for each distinct site of tumor origin, the percentage of CS increased with tumor progression. In the aforementioned study, CS was also associated with shorter survival compared without patients without CS, further supporting the second explanation for our findings[17]. Of note, our findings extend Halperin's data in that we have now identified three specific predictors of the CS that can be used to direct physicians to specifically consider CS over a more general diagnosis of NET, and to monitor those patients more closely by 5-HIAA or serum serotonin testing In our study, we matched the CS and non-CS cohorts on duration of disease as carefully as possible, knowing that one of the limitations of using claims databases for research is that the precise diagnosis date is not the true date of tumorgenesis. Consequently the duration between the first NET diagnosis and first CS diagnosis was the best proxy for disease duration that we could use. Conditional on the reliability of this proxy, our findings suggest that patients who were eventually diagnosed with CS tended to have additional diagnoses indicating tumor advancement, compared with the non-CS patients who had the same duration of disease.

In general, early diagnosis of cancer can improve quality of life and survival[18]. Patients with CS have a significantly worse quality of life than patients with

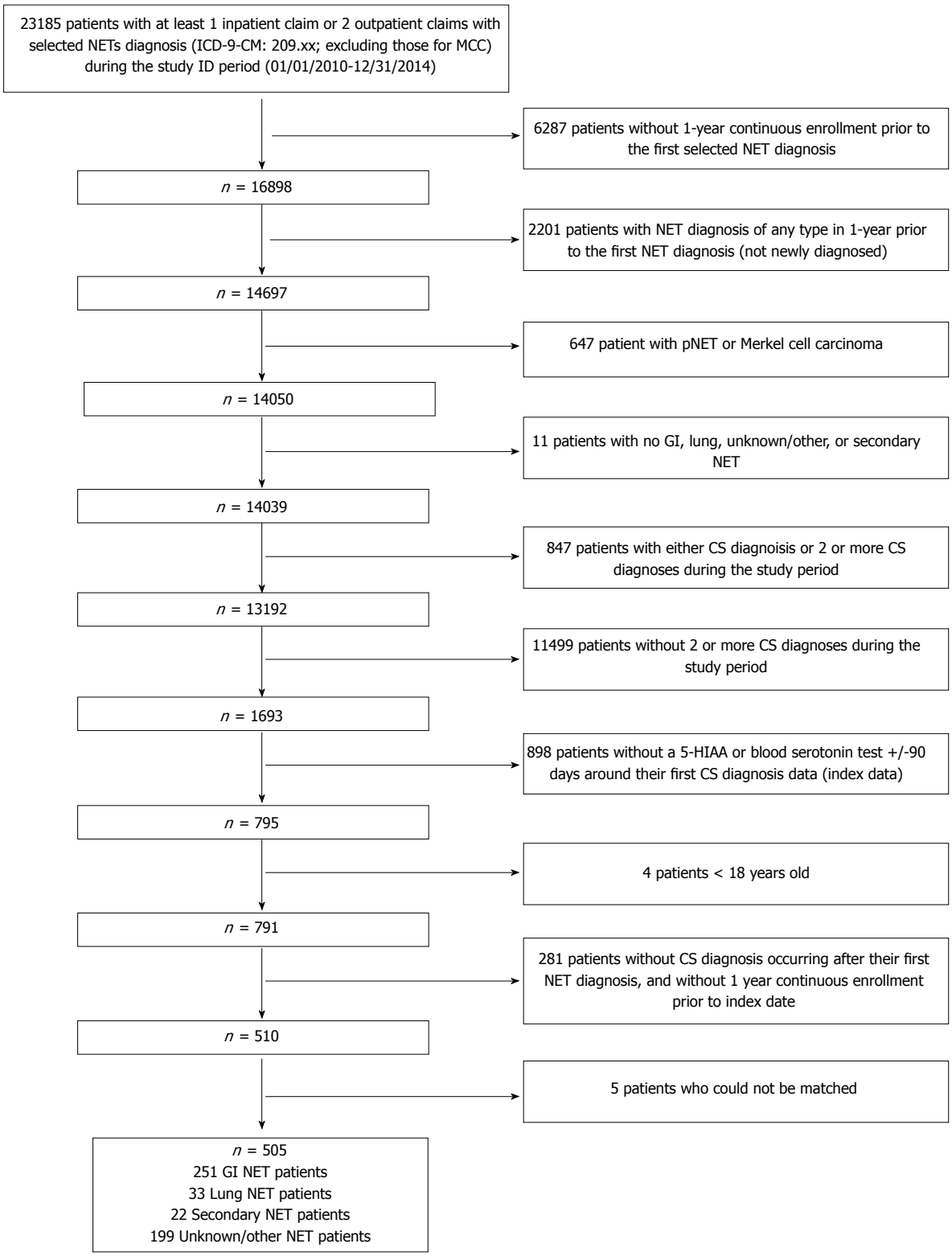


Figure 1 Patient identification. There were 23185 patients with at least one inpatient claim or two outpatient claims with selected NETs diagnosis (ICD-9-CM: 209.xx; excluding those for MCC) during the study ID period (01/01/2010-12/31/2014) in the development database. After excluding patients who were not newly diagnosed and did not have a NET diagnosis of interest; did not have 2 or more CS diagnosis during study period; did not have a 5-HIAA or blood serotonin test +/-90 days around their first CS diagnosis date (index date); were < 18 years old; did not have CS diagnosis occurring after their first NET diagnosis; could not be matched; or were not continuously enrolled in the 1 year pre-index period, there remained 505 NET patients who met the study inclusion criteria. Out of these, 251 were GI NET patients with CS. GI NETs: Gastrointestinal neuroendocrine tumors; CS: Carcinoid syndrome; 5-HIAA: 5-hydroxyindoleacetic acid.

NETs but without CS[19]. Therefore, reducing delays in diagnosis may help improve quality of life in CS. In addition, our findings indicate that the poorer quality of life among patients with CS may also be a composite result of both tumor progression and CS symptoms, as the CS patients also tended to have more advanced

Table 1 Patient demographics *n* (%)

	Development Database				Validation Database			
	CS Cohort	Non-CS Cohort	All	*P* value	CS Cohort	Non-CS Cohort	All	*P* value
n	251	753	1,004		181	543	724	
Age (yr)	52.77 ± 10.88	54.37 ± 13.28	53.97 ± 12.74	0.058	51.76 ± 9.33	51.67 ± 10.03	51.69 ± 9.85	0.913
18-44	52 (20.7)	143 (19.0)	195 (19.4)	0.034	33 (18.2)	100 (18.4)	133 (18.4)	0.637
45-54	80 (31.9)	219 (29.1)	299 (29.8)		64 (35.4)	203 (37.4)	267 (36.9)	
55-64	94 (37.5)	257 (34.1)	351 (35.0)		83 (45.9)	231 (42.5)	314 (43.4)	
65+	25 (10.0)	134 (17.8)	159 (15.8)		1 (0.6)	9 (1.7)	10 (1.4)	
Female	133 (53.0)	407 (54.1)	540 (53.8)	0.770	109 (60.2)	297 (54.7)	406 (56.1)	0.195
Region				0.306				0.667
Midwest	67 (26.7)	219 (29.1)	286 (28.5)		37 (20.4)	118 (21.7)	155 (21.4)	
Northeast	57 (22.7)	200 (26.6)	257 (25.6)		41 (22.7)	100 (18.4)	141 (19.5)	
South	106 (42.2)	269 (35.7)	375 (37.4)		80 (44.2)	254 (46.8)	334 (46.1)	
West	21 (8.4)	65 (8.6)	86 (8.6)		23 (12.7)	71 (13.1)	94 (13.0)	

tumor. Therefore, therapy for CS patients should address both symptom control as well as tumor progression to maintain quality of life.

In addition, we find it rather curious that there were no gender differences in our study. Since flushing is a common symptom of the CS, and since flushing is often confused with the hot flashes of menopause, one might have expected males with CS to be diagnosed earlier than females if the propensity is to misdiagnose CS until the disease is well established (with a known liver disorder, abdominal mass or lymphadenopathy). On the other hand, the delay in identifying CS in NET patients may simply be because flushing is not often sought out by physicians when interviewing patients. Indirectly then, these data suggest that educating physicians about the unusual manifestation of CS may help lead to an earlier diagnosis. The gender equivalence in our study may also be evidence that our algorithm for identifying CS, which required the presence of 5-HIAA or serotonin test around the diagnosis date, was reasonably specific for the condition, so that we minimized the misdiagnosed CS in our CS cohort by requiring this criterion for diagnosis.

Our study had limitations. First, GI NET and CS diagnoses were identified from healthcare claims coded for reimbursement, not research, and misclassification was possible. Errors in coding could bias our analysis. Specifically, patients with CS who have less severe symptoms may never be coded as having the syndrome. Nevertheless, health insurance claims data remain a valuable source of information as they constitute a fairly valid, large sample of patient characteristics and outcomes in a real-world setting. A strength of this study was that it was drawn from two very large underlying databases covering nearly 200 million patients enrolled in United States health insurance plans. The rate of CS among GI-NET patients in our study was higher than in some prior studies[20], and it was lower than at least one other[17]. The criteria we used to identify CS were more restrictive than the study by Halperin *et al*[17]. Current recommendations for diagnosing CS include measuring 5-HIAA[1,21]. We incorporated that recommendation into our identification algorithm, requiring two claims with an ICD-9-CM code for CS and a claim for either a urine 24-hour 5-HIAA or a serum serotonin in the period surrounding that diagnosis, whereas the prior study required two claims for CS, diarrhea, or flushing. Second, because ICD-9-CM codes may be inaccurate, we were not able to identify the specific anatomic location (*e.g.*, portion of large or small bowel) of the GI NET in our study. This could lead to confounding. For instance, small bowl NETs are more commonly associated with CS than large bowel tumors, and previous studies have suggested that hepatic and lymph node metastasis are usually present at time of small bowel NET diagnosis[22]. If our CS cohort systematically includes more small bowel patients than the non-CS cohorts, and the small bowel patients tend to be more advanced at diagnosis, this could explain why we see that CS patients were more likely to have this diagnosis. However, due to the lack of specificity of ICD-9-CM codes, we cannot confirm the anatomic section of the GI tract in which the tumor was identified. Therefore, we could not validate if small bowel NETs may present at a more advanced stage than other NETs based only on the data from a claims database. A study in a data source where the exact location of the tumor could be identified with confidence would be very useful to confirm our findings. Third, our results are reflective of a commercially-insured population, but may not be generalizable to patient populations with other insurance types.

In summary, our study suggests that a liver disorder, enlargement of lymph nodes, or an abdominal mass is associated with higher risk of a future CS diagnosis. There are two aspects with regards how this finding may be applicable to a real world clinical setting. First, CS develops insidiously, and patients who grow accustomed to their syndromic features such as flushing may not think of reporting these symptoms, even when prompted. If physicians are not aware of the presence of CS symptoms in their patients, our findings offer additional signs to prompt them to inquire specifically.

Table 2 Patients with gastrointestinal neuroendocrine tumors: Most frequent diagnoses1 in carcinoid syndrome patients, ordered by prevalence in carcinoid syndrome cohort *n* (%)

Diagnosis description	No. of patients		Difference in Rate (CS-Non-CS)	Relative Risk (CS *vs* Non-CS)
	CS cohort *n* = 251	Non-CS cohort *n* = 753		
Abdominal pain[2]	166 (66.14)	388 (51.53)	14.61%	1.28
Hypertension[2]	127 (50.60)	393 (52.19)	-1.59%	0.97
Dyslipidemia[2]	124 (49.40)	347 (46.08)	3.32%	1.07
Benign neoplasm large bowel	79 (31.47)	241 (32.01)	-0.53%	0.98
Esophageal reflux	74 (29.48)	190 (25.23)	4.25%	1.17
Diverticulosis of colon (without mention of hemorrhage)	68 (27.09)	159 (21.12)	5.98%	1.28
Liver disorder[2]	56 (22.31)	79 (10.49)	11.82%	2.13
Chest pain, unspecified	53 (21.12)	147 (19.52)	1.59%	1.08
Anemia, unspecified	51 (20.32)	141 (18.73)	1.59%	1.09
Pain in limb	41 (16.33)	117 (15.54)	0.80%	1.05
Internal hemorrhoids without mention of complication	41 (16.33)	87 (11.55)	4.78%	1.41
Neoplasm of uncertain behavior of stomach, intestine, and rectum	39 (15.54)	95 (12.62)	2.92%	1.23
Nonspecific (abnormal) findings on radiological and other examination of gastrointestinal tract	38 (15.14)	66 (8.76)	6.37%	1.73
Lumbago	38 (15.14)	79 (10.49)	4.65%	1.44
Enlargement of lymph nodes	36 (14.34)	40 (5.31)	9.03%	2.70
Malignant neoplasm of colon, unspecified site	35 (13.94)	114 (15.14)	-1.20%	0.92
Hypothyroidism, unspecified	34 (13.55)	106 (14.08)	-0.53%	0.96
Abdominal or pelvic swelling, mass, or lump, unspecified site (begin 1994)	34 (13.55)	60 (7.97)	5.58%	1.70
Other and unspecified noninfectious gastroenteritis and colitis	32 (12.75)	73 (9.69)	3.05%	1.32
Type 2 diabetes mellitus[2]	32 (12.75)	158 (20.98)	-8.23%	0.61
Other specified disorders of intestine	29 (11.55)	70 (9.30)	2.26%	1.24
Obesity, unspecified	28 (11.16)	82 (10.89)	0.27%	1.02
Diaphragmatic hernia	28 (11.16)	65 (8.63)	2.52%	1.29
Headache	28 (11.16)	74 (9.83)	1.33%	1.14
Anxiety state, unspecified	27 (10.76)	71 (9.43)	1.33%	1.14
Acute appendicitis, unspecified	26 (10.36)	91 (12.08)	-1.73%	0.86
Reflux esophagitis	26 (10.36)	41 (5.44)	4.91%	1.90
Abdominal or pelvic swelling, mass, or lump, other specified site	26 (10.36)	35 (4.65)	5.71%	2.23
Dyspepsia and other specified disorders of function of stomach	26 (10.36)	26 (3.45)	6.91%	3.00
Urinary tract infection, unspecified	26 (10.36)	96 (12.75)	-2.39%	0.81
Pre-operative cardiovascular examination	25 (9.96)	102 (13.55)	-3.59%	0.74
Tobacco use disorder	24 (9.56)	77 (10.23)	-0.66%	0.94
Constipation, unspecified	24 (9.56)	79 (10.49)	-0.93%	0.91

[1]Claims in 1 year prior to the index date, excluding those for CS, NET, or symptoms/conditions known to be associated with CS; [2]Abdominal Pain (ICD-9-CM 789.0x, 789.6x); Type 2 DM (ICD-9-CM 250.x0, 250.x2); Hypertension (ICD-9-CM 401.xx, 402.xx, 403.xx, 404.xx, 405.xx, 437.2x); Dyslipidemia (ICD-9-CM 272.xx); Liver disorder (ICD-9-CM 573.8x, 573.9x). Variables not shown: Screening (Routine medical examination, Other screening mammogram, Long-term (current) use of other medications, Routine gynecological examination, Vaccination for influenza, Screen for malignant neoplasms of colon, Other specified pre-operative examination; GI cancer (Digestive neoplasm, unspecified); Prevalence < 10% in both CS and non-CS cohort (Screening for malignant neoplasms of prostate, Backache unspecified, Other abnormal blood chemistry, Vitamin D deficiency unspecified, Lumbar or lumbosacral intervertebral disc degeneration, Atrophic gastritis without mention of hemorrhage, Personal history of colonic polyps, Cervicalgia, Iron deficiency anemia unspecified). CS: Carcinoid syndrome; NET: Neuroendocrine tumors.

Table 3 Patients with gastrointestinal neuroendocrine tumors: Selected measures *n* (%)

	Development database				Validation database			
	CS Cohort	Non-CS Cohort	All	*P* value	CS Cohort	Non-CS Cohort	All	*P* value
n	251	753	1004		181	543	724	
Abdominal pain	166 (66.1)	388 (51.5)	554 (55.2)	< 0.001	104 (57.5)	253 (46.6)	357 (49.3)	0.011
Dyslipidemia	124 (49.4)	347 (46.1)	471 (46.9)	0.361	67 (37.0)	209 (38.5)	276 (38.1)	0.724
Diverticulosis of colon	68 (27.1)	159 (21.1)	227 (22.6)	0.050	37 (20.4)	93 (17.1)	130 (18.0)	0.314
Liver disorder	56 (22.3)	79 (10.5)	135 (13.4)	< 0.001	50 (27.6)	44 (8.1)	94 (13.0)	< 0.001
Enlarged lymph nodes	36 (14.3)	40 (5.3)	76 (7.6)	< 0.001	23 (12.7)	25 (4.6)	48 (6.6)	< 0.001
Type 2 diabetes	32 (12.7)	158 (21.0)	190 (18.9)	0.004	30 (16.6)	91 (16.8)	121 (16.7)	0.954
Abdominal mass	26 (10.4)	35 (4.6)	61 (6.1)	0.010	23 (12.7)	16 (2.9)	39 (5.4)	< 0.001
Dyspepsia and other specified disorders of function of stomach	26 (10.4)	26 (3.5)	52 (5.2)	< 0.001	8 (4.4)	35 (6.4)	43 (5.9)	0.318

CS: Carcinoid syndrome

Table 4 Patients with gastrointestinal neuroendocrine tumors: Results of final model

Independent variable[1]	Development database		Validation database	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age group				
18-44 *vs* 65+	2.30 (1.26-4.23)	0.007	4.88 (0.56-42.11)	0.150
45-54 *vs* 65+	1.94 (1.14-3.31)	0.015	3.63 (0.43-30.41)	0.235
55-64 *vs* 65+	2.01 (1.20-3.38)	0.008	3.85 (0.46-31.93)	0.212
Number of chronic conditions	0.94 (0.87-1.01)	0.093	1.13 (1.02-1.24)	0.014
Abdominal pain	1.50 (1.08-2.09)	0.016	1.22 (0.83-1.77)	0.309
Dyslipidemia	1.52 (1.08-2.15)	0.016	0.83 (0.55-1.25)	0.373
Diverticulosis of colon	1.55 (1.08-2.24)	0.019	1.16 (0.72-1.86)	0.537
Liver disorder[2]	2.12 (1.40-3.20)	< 0.001	3.38 (2.07-5.51)	< 0.001
Enlarged lymph nodes	2.33 (1.38-3.92)	0.001	2.13 (1.10-4.11)	0.025
Type 2 diabetes	0.59 (0.38-0.92)	0.021	0.89 (0.54-1.48)	0.653
Abdominal mass	1.89 (1.06-3.37)	0.031	3.79 (1.87-7.69)	< 0.001
Dyspepsia and other specified disorders of function of stomach	2.95 (1.60-5.43)	< 0.001	0.54 (0.23-1.26)	0.154

[1]Variables found consistently significantly associated with risk of CS are bolded; [2]Liver disorder includes other specified disorders of liver (ICD-9-CM 573.8x, 573.9x). CS: Carcinoid syndrome.

Second, patients with a liver disorder, enlarged nodes, or abdominal mass may also not yet have clinically detectable CS symptoms, but may be destined to develop them in the future. In this context, our data suggest that in a patient with NET, even in the absence of typical symptoms, if one of these three findings is noted, consideration should be given to ordering a 5-HIAA or serotonin level. Although validation studies using patients' medical charts are warranted, such a practice could potentially aid physicians in speeding the diagnosis of CS.

ATRICLE HIGHLIGHTS

Research background

Functional neuroendocrine tumors (NETs) have the ability to secrete hormones that may cause carcinoid syndrome (CS), the most common symptoms of which include flushing and diarrhea. Carcinoid syndrome occurs in 8% to 35% of NET patients. Delays in diagnosis of CS are common, ranging from 2 to 20 years. Little is known about predictors that might be related the risk of developing CS.

Research motivation

Patients with CS often face delays in getting the correct diagnosis. Their symptoms, such as diarrhea, are often mistaken for other diseases, such as irritable bowel disease. Finding risk factors that are associated with CS might help physicians with making an earlier diagnosis of CS.

Research objectives

The objective was to identify risk factors that are associated with a future CS diagnosis.

Research methods

The authors conducted the study using data from two large United States health insurance claims databases. These databases contain healthcare cost, use, and outcomes data for covering nearly 200 million insured Americans. We first identified patients newly diagnosed with gastrointestinal NETs between 2010 and 2014 and then compared patients with CS to those without CS. We performed statistical analyses to identify the risk factors associated with CS from one databased and then validated the results using the other database.

Research results

The authors identified 251 patients with CS and 753 without CS in one database, and 386 patients with CS and 1158 patients without CS in the other database. There were no significant differences in age, sex, and region between patients with CS and those without CS. In both databases, we found that CS patients were 2-4 times more likely than non-CS patients to have a diagnosis of liver disorder, enlargement of lymph nodes, or abdominal mass within 1 year prior to their CS diagnosis.

Research conclusions

This study suggests that diagnosis codes of liver disorder, enlargement of lymph nodes, or an abdominal mass are associated with higher risk of a future CS diagnosis. In a patient with NETs, even in the absence of typical symptoms, if one of these three conditions is noted, consideration should be given to ordering a test for CS. Such a practice could potentially aid physicians in speeding the diagnosis of CS.

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ORIGINAL ARTICLE

Retrospective Study

Clinical and pathological characterization of Epstein-Barr virus-associated gastric carcinomas in Portugal

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Data sharing statement: Data will be provided upon request to corresponding author.

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Abstract

AIM

To determine the prevalence of Epstein-Barr virus (EBV)-associated gastric carcinomas in the North Region of Portugal and to study its clinicopathological characteristics.

METHODS

We have performed a retrospective study including a total of 179 consecutive patients with gastric cancer (GC) submitted to gastrectomy during 2011 at the Portuguese Oncology Institute of Porto. Clinical and pathological data was collected from individual clinical records and inserted on a database with unique codification. Tumour tissues were collected from the institutional tumour bank. EBV was detected by *in situ* hybridization for the detection of EBV-encoded small RNAs (EBERs) and EBV latent proteins (LMP1 and LMP2A) were detected by immunohistochemistry.

RESULTS

The analysis showed that EBV-associated gastric carcinomas (EBVaGC) represents 8.4% (15/179) of all GC cases, with a significant differential distribution among histological types ($P < 0.001$): 100% (3/3) of medullary carcinomas, 100% (1/1) of adenosquamous carcinoma, 8.7% (8/92) of tubular adenocarcinomas, 8.0% (2/25) of mixed carcinomas and 2% (1/51) in poorly cohesive carcinomas. The analysis revealed a higher predominance of EBVaGC in the upper third and middle (cardia, fundus and body) of the stomach ($P = 0.041$), a significant lower number of regional lymph nodes invasion ($P = 0.025$) and a tendency for better prognosis ($P = 0.222$). EBV latent protein expression revealed that all EBVaGC cases were LMP1-negative, nevertheless 6 cases (40%) expressed LPM2A, which reveals that these cases show a distinct EBV-Latency profile (latency II-like).

CONCLUSION

EBVaGC represents 8.4% of all GC in the North Region of Portugal. The EBV-infected patients have specific clinic-pathological features that should be further explored to develop new strategies of management and treatment.

Key words: Gastric cancer; Epstein-Barr virus; Prevalencel; Epstein-Barr virus-associated gastric carcinomas

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Core tip: This is the first study to report the prevalence of Epstein-Barr virus (EBV)-associated gastric carcinomas (EBVaGC) in Portugal. The EBVaGCs were found in 8.4% of all gastric cancer cases, being more frequent in upper and middle regions of the stomach and among tubular and medullary carcinomas. Patients with EBV-positive tumours also have a significant lower number of regional lymph nodes with metastasis and a tendency for a better prognosis.

Ribeiro J, Oliveira A, Malta M, Oliveira C, Silva F, Galaghar A, Afonso LP, Neves MC, Medeiros R, Pimentel-Nunes P, Sousa H. Clinical and pathological characterization of Epstein-Barr virus-associated gastric carcinomas in Portugal. *World J Gastroenterol* 2017; 23(40): 7292-7302 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7292.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7292

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy worldwide with nearly one million new cases estimated in 2012 (952000 cases) and despite the significant reduction of its incidence, GC still remains the third leading cause of death by cancer[1,2]. Portugal is one of the European countries with the higher GC incidence and mortality rates, where GC represents the fifth most common cancer with about 3000 new cases per year and with 1387 deaths in men and 898 in women[1,2].

Epstein-Barr virus (EBV) is one of the most common viruses and has been associated with several malignancies including Burkitt's Lymphoma, Hodgkin Lymphoma, post-transplant lymphoproliferative disease, a subset of T/NK cell lymphomas and nasopharyngeal carcinoma[3]. Recently, EBV has been associated with the development of GC, with several reports pointing that E BV-associated GC (EBVaGC) might account for nearly 10% of all GC cases[4,5]. The mechanisms of EBVaGC carcinogenesis are not well understood although some authors have suggested that EBV infection can represent a late event after *Helicobacter pylori* (*H. pylori*) infection[6,7].

The presence of EBV in patients with gastric carcinoma was first reported in gastric lymphoepithelioma-like carcinomas (also classified as medullary

carcinomas, gastric carcinomas with lymphoid stroma or uncommon variant), nevertheless it has been detected in different histopathological subtypes of gastric carcinoma[8,9]. The majority of GC are adenocarcinomas and are classified according to the Lauren or World Health Organization (WHO) classification system[10]. These classification systems have a reduced clinical utility and recent studies suggested a new classification based on molecular features of tumours, where EBVaGC arises as a new subtype of gastric cancer[11,12].

Despite the high incidence of both GC and EBV infection prevalence in the Portuguese population, there are no studies reporting the prevalence of EBVaGC in Portugal[2,13]. We aimed to determine the prevalence and characteristics of EBVaGC and analyse the profile of EBV latent proteins expressed in gastric tumours from the North Region of Portugal.

MATERIALS AND METHODS

Study description

We have developed a retrospective study in a cohort of 179 consecutive patients diagnosed with gastric cancer and submitted to surgery at the Portuguese Institute of Oncology of Porto (IPO Porto FG EPE) in 2011. Inclusion criteria: (1) patients with histologically confirmed gastric cancer; (2) submitted to gastrectomy (total or partial); and (3) with representative tumour blocks for adequate evaluation of EBV presence.

All tumour samples were histologically examined by a senior pathologist and classified according to the WHO classification system[10,14]. All clinical and pathological data was collected from individual clinical records and inserted on a database with unique codification. TNM-staging was performed in accordance to the UICC/AJCC system 7th edition[14]. The clinical outcome of the patients was followed from the date of surgery to either the date of death or 1st August 2016. All procedures were approved by the Ethical Committee of IPO Porto (CES IPO 80/2014).

EBV detection

The presence of EBV infection was investigated in all patients using histological sections (3 μm slides) obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. EBV was identified using the *in situ* hybridization (ISH) for the detection of EBV-encoded small RNAs (EBERs) in FFPE tissue samples. The ISH was performed using Epstein-Barr virus Probe/Antibody ISH Kit (Leica, Newcastle upon Tyne, United Kingdom) in association with Ultra Vision Large Volume Detection System Anti-Polyvalent, HRP (THERMO SCIENTIFIC, Fremont, United States) according to the manufacturer's instructions. Detection of hybrids is achieved by enzymatic reaction using the *ImmPACT™ DAB* Peroxidase Substrate (VECTOR, Burlingame, CA, United States).

Only gastric tumours with presence of EBV infection in neoplastic cells were considered EBVaGC. For quality control, all EBV-positive cases and 10% of EBV-negative cases were retested and the results were 100% concordant. FFPE samples from a known EBV-positive post-transplant lymphoproliferative disorder (PTLD) tissue were used as positive controls and a sense probe for EBERs were used as the negative controls.

EBV latent proteins analysis

The presence of latent membrane protein 1 (LMP1) and latent membrane protein 2A (LMP2A) were evaluated with specific monoclonal antibodies in EBERs-positive gastric tissues using immunohistochemistry (IHC) (Table 1). IHC was performed using the UltraVision Large Volume Detection System Anti-Polyvalent, HRP kit (THERMO SCIENTIFIC, Fremont, United States) and the signal was detected using the *ImmPACT™ DAB* Peroxidase Substrate (VECTOR, Burlingame, CA, United States). All procedures were realized according to the manufacturer's instructions. PTLD samples with known positivity for LMP1 and LMP2A were used as positive controls. Gastric tumours were considered positive if 10% or more of the neoplastic cells were stained.

Statistical analysis

Statistical analysis was performed using the computer software IBM SPSS statistics for Macintosh, version 20.0 (IBM Corp, Armonk, NY, United States). χ^2 or Fisher Exact-test was used to compare categorical variables, with a significance level of 5%. Overall survival was defined as the time between the date of surgery to the date of last follow-up (censored) or the date of patient death (event). Cases lost to follow-up and those ending in death from any cause other than gastric cancer were regarded as censored data during the analysis of survival rates. The differences in survival times between subgroups were calculated using the log-rank test and the Kaplan-Meier method was used to calculate survival probabilities.

RESULTS

Characterization of the study population

The clinicopathological characteristics of all patients are described in (Table 2). A total of 179 patients (108 males and 71 females) with mean age of 64 ± 12 were included in this study; the majority were submitted to gastrectomy (n = 177), wherein 100 were total resections and 77 partial resections, and the remaining 2 patients were submitted to esophagogastrectomy. Regarding the tumour localisation in stomach: 26 (14.5%) were found in the upper third (cardia or fundus); 46 (25.7%) in the middle region (body); and 107 (59.8%) in lower third (antrum or pylorus).

Tumours were classified according to WHO (2010) classification system and the most common

histological type was tubular adenocarcinoma (n = 92, 51.4%) followed by poorly cohesive carcinoma (n = 51, 28.5%), mixed carcinoma (n = 25, 14%) and less frequent were mucinous adenocarcinoma (n = 5, 2.8%), papillary adenocarcinomas (n = 1, 0.6%), undifferentiated carcinoma (n = 1, 0.6%), adenosquamous carcinoma (n = 1, 0.6%) and medullary carcinomas with lymphoid stroma (n = 3, 1.7%) (Table 2). The correlation of histological type with anatomic localisation (data not shown), revealed that tubular type can be found in any stomach region, being more frequent in the lower third (56.5%) followed by upper and middle regions with 20.8% and 20.7%, respectively; poorly cohesive carcinomas were also more frequently located in the lower third (84.3%) and only 11.8% and 3.9% were in the middle and upper third, respectively; mixed adenocarcinomas were also more prevalent in lower third of the stomach with 72% of cases located in this region, with 16% in the middle and 12% in the upper region; and medullary carcinomas showed a similar distribution throughout the stomach with a percentage of 33.3% in all regions.

Regarding the characteristics of tumours, the most frequent invasion pattern was infiltrative (52.7% *vs* 46.1% expansive); the majority were poorly differentiated (54.5% *vs* 45.5% well or moderate differentiated); and lymphovascular invasion was extremely frequent (57.5% *vs* 42.5%) (Table 2). The tumour staging revealed that the majority of cases (63.8%) were at Stage III and IV and in only 6.7% of all cases presented distant metastasis.

Regarding survival analysis, the median overall survival was of 45 mo (mean 36 ± 20.5; range 1-63). Furthermore, we observed that lymph node metastasis (N), lymphovascular and perineural permeation and infiltrative tumours had a significantly reduced median overall survival (P < 0.001) (Figure 2A, B, C and D).

Characterization of EBVaGC

In our series, out of the 179 patients with GC, fifteen patients (8.4%) were positive for EBV - (Table 2). All EBV positive cases showed EBER positivity staining in > 90 of tumour cells while it was not observed in normal mucosa with or without atrophic gastric, intestinal metaplasia or in stromal cells (endothelial cells and fibroblasts) (Figure 1). In addition, one case showed EBER positivity only in the lymphocytic infiltration but not in tumour cells and therefore it was classified as EBV-negative GC.

Despite no statistical differences, it was observed that EBVaGC is more frequent in males (11.1% *vs* 4.1% in females) and among patients with more than 65 years old (10.1% *vs* 6.7% in younger patients) (Table 2). EBVaGC were not equally distributed among the stomach localisation (P = 0.087), being more often found in upper third and middle of the stomach than in the lower third; and regarding the anatomical site, it was more frequent (P = 0.041) in the body and fundus regions (40.0% and 13.0%, respectively) (Table 2).

Results have revealed statistical significant differences (P < 0.001) when comparing the distribution of EBV among different histological types according to WHO classification: EBV was detected in 8.7% (8/92) of tubular adenocarcinomas, 8.0% (2/25) of mixed carcinomas and only 2.0% (1/51) of poorly cohesive carcinomas. The results have also shown that all medullary (n = 3) and adenosquamous carcinomas (n = 1) were EBVaGC, while no EBV-positive case was identified in papillary adenocarcinomas and undifferentiated carcinomas (Table 2). The analysis of the invasion pattern of the GC, revealed that tumours with expansive patterns have a higher prevalence of EBV when compared with infiltrative patterns (13.0% *vs* 5.7%, respectively), despite not statistically significant (P = 0.237) (Table 2). No significant differences were also observed regarding the tumour differentiation and lymphovascular invasion (P > 0.050).

The results show that EBVaGCs were associated with a low number of regional lymph nodes invasion (P = 0.024) and that all cases had no evidence of distant metastasis, nevertheless there was no evidence of association with tumour stage (P = 0.289). Additionally, comparing the number of lymph nodes with metastasis between EBVaGCs and EBV negative cases, it was observed that EBVaGC cases have a significant lower number (mean difference of 3.7; 95%CI: 1.8-1.5; P < 0.001).

Table 3 resumes the individual characteristics of EBVaGC cases. Briefly, of the 15 EBVaGC cases 12 were males and 3 females with a mean age of 66 years old (range 44-82). Anatomically EBVaGCs were found in the body (n = 6), antrum (n = 4), fundus (n = 2), cardia (n = 2) and pylorus (n = 1). Regarding the histological subtypes, 8 were tubular adenocarcinoma, 3 were medullary carcinoma, 2 mixed carcinoma, 1 poorly cohesive carcinoma and 1 adenosquamous carcinoma. The invasion pattern showed that the majority of cases were expansive (n = 10/15) and regarding the differentiation, cases were equally distributed as poorly and moderately differentiated (n = 7 each). Moreover, there was no EBVaGC with distant metastasis.

The survival analysis revealed that despite not statistically significant, EBVaGC patients have a higher overall survival (41 ± 1.8 mo *vs* 46 ± 5.9 mo, P = 0.222) (Figure 3).

EBV latent proteins expression

Immunohistochemistry results revealed that all of EBVaGC cases were negative for LMP1 while 6 cases (40%) were positive for LMP2A and one case was inconclusive (Table 3). The positive cases for LPM2A expression were randomly distributed among histological subtypes: tubular adenocarcinomas (n = 2),

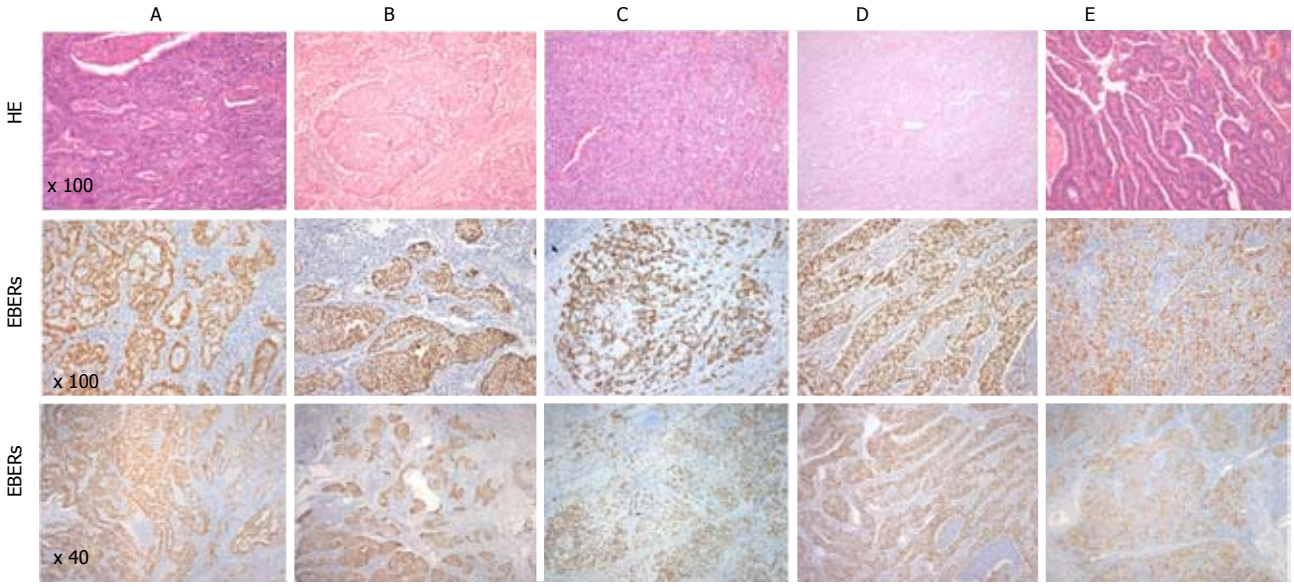


Figure 1 Representative images of EBER-ISH positive results in different histological types of gastric cancer and their respective HE staining. A: Adenocarcinoma tubular; B: Adenocarcinoma tubular; C: Adenocarcinoma Mixed; D: Adenosquamous Carcinoma; E: Medullary caricnoma. HE: Hematoxylin and eosin stain; EBERs: EBV-encoded small RNAs. Figure 1 Representative images of EBER-ISH positive results in different histological types of gastric cancer and their respective HE staining. A: Adenocarcinoma tubular; B: Adenocarcinoma tubular; C: Adenocarcinoma Mixed; D: Adenosquamous Carcinoma; E: Medullary caricnoma. HE: Hematoxylin and eosin stain; EBERs: EBV-encoded small RNAs.

Table 1 Antibodies used in immunohistochemistry

Protein	Primary Antibody	Dilution	Incubation	Retrieval method	Expression in gastric cells
LMP1	NCL-EBV-CS1-4, Leica, Newcastle upon Tyne, United Kingdom	1:100	3h, RT	15 min, microwave in *Vector*® Antigen Unmasking Solution	Cytoplasm
LMP2A	15F9, THERMO SCIENTIFIC, Fremont, United States	1:250	On, 4℃		Cytoplasm and membrane

LMP1: Latent membrane protein 1; LMP2A: Latent membrane protein 2A; RT: Room temperature; On: Overnight.

mixed carcinomas (n = 2), medullary carcinomas (n = 1) and adenosquamous carcinoma (n = 1) (Table 3).

DISCUSSION

Gastric carcinoma is a serious public health problem worldwide with high rates of mortality and Portugal has the highest gastric cancer mortality rates in Western European countries[2,15]. The impact of classification systems in GC clinical decisions has been reduced and more recently two studies suggested a new classification based on molecular features of gastric tumours[11,12]. In this new classification arises four new subtypes of gastric cancer: Epstein-Barr Virus positive tumours (EBVaGC); microsatellite unstable tumours; genomically stable tumours; and tumours with chromosomal instability (CIN)[11,12].

Over the past 30 years, EBV infection has been reported in gastric cancer tumours[16]. Currently, it is accepted that about 10% of all GC represents a specific subset of gastric carcinomas that are associated with EBV carcinogenesis[17]. The gold-standard method for the detection of EBV in tissues is EBER-ISH and the decision to accept one case as EBV-associated should be taken considering the presence of EBERs expression in tumour cells and its absence in the normal surrounding tissue. In our study we found that 8.4% of GC in our population are EBV-associated, based on the EBERs expression in > 90 of tumour cells and its absence in non-malignant or stromal cells. To the best of our knowledge, this is the first study reporting the prevalence of EBVaGC in the North Region of Portugal. The prevalence found in our population is in line with those reported in other studies, which have reported a prevalence ranging 2%-20%[5,8,18]. Similar data was found in other countries from Europe, including Netherlands (7.8%)[18], and Denmark (7.6%)[19] and also from Asiatic countries, such as South Korea (7.8%)[20] and Japan (8%)[21]. Geographic differences have been discussed as a possible reason for the variation of EBVaGC prevalence[8,22] and some studies suggest that EBVaGC prevalence might be inversely correlated with the background incidence of GC[4]. The fact that Portugal has a higher incidence of GC

Table 2 Patients characteristics and distribution of Epstein-Barr virus in gastric cancer patients *n* (%)

characteristics	All cases (*n* = 179)	EBV status		*P* value
		Negative (*n* = 164)	Positive (*n* = 15)	
Gender, *n* = 179				
Male	108 (60.3)	96 (88.9)	12 (11.1)	0.104
Female	71 (51.5)	68 (95.8)	3 (4.2)	
Age, *n* = 179 (64.38 ± 12 yr of age)				
< 65 yr of age	90 (50.3)	84 (93.3)	6 (6,7)	0.433
⩾ 65 yr of age	89 (49.7)	80 (89.9)	9 (10.1)	
Surgical procedure type, *n* = 179				
Total gastrectomy	100 (55.9)	89 (89.0)	11 (11.0)	0.351
Partial gastrectomy	77 (43.0)	73 (94.8)	4 (5.2)	
Esophagogastrectomy	2 (1.1)	2 (100)	-	
Tumour localization, *n* = 179				0.087
Upper third	26 (14.5)	22 (84.6)	4 (15.4)	
Middle	46 (25.7)	40 (87.0)	6 (13.0)	
Lower third	107 (59.8)	102 (95.3)	5 (4.7)	
Anatomical site, *n* = 179				
Cardia	21 (11.7)	19 (95.7)	2 (4.3)	0.041
Fundus	5 (2.8)	3 (60.0)	2 (40.0)	
Body	46 (25.7)	40 (87.0)	6 (13.0)	
Pylorus	13 (7.3)	12 (92.3)	1 (7.7)	
Antrum	94 (52.5)	90 (95.7)	4 (4.3)	
Invasion pattern, *n* = 167				
Infiltrative	88 (52.7)	83 (94.3)	5 (5.7)	0.237
Expansive	77 (46.1)	67 (87.0)	10 (13.0)	
Mixed	2 (1.1)	2 (100)	-	
Differentiation, *n* = 167				
Poor	91 (54.5)	84 (92.3)	7 (7.7)	0.724
Well+Moderate	76 (45.5)	69 (90.8)	7 (9.2)	
Lymphovascular invasion, *n* = 179				
Positive	103 (57.5)	94 (91.3)	9 (8.7)	0.841
Negative	76 (42.5)	70 (92.1)	6 (7.9)	
Histology WHO (2010), *n* = 179				
Papillary adenocarcinoma	1 (0.6)	1 (100)	-	<0.001
Tubular adenocarcinoma	92 (51.4)	84 (91,3)	8 (8.7)	
Mucinous adenocarcinoma	5 (2.8)	5 (100)	-	
Poorly cohesive carcinoma	51 (28.5)	50 (98.0)	1 (2.0)	
Mixed carcinoma	25 (14.0)	23 (92.0)	2 (8.0)	
Medullary carcinoma	3 (1.7)	-	3 (100)	
Undifferentiated carcinoma	1 (0.6)	1 (100)	-	
Adenosquamous carcinoma	1 (0.6)	-	1 (100)	
Primary tumour (T), *n* = 179				
T1a	22 (12.3)	22 (100)	-	0.289
T1b	18 (10.1)	15 (83.3)	3 (16.7)	
T2	25(14.0)	24 (96.0)	1 (4.0)	
T3	69 (38.5)	60 (87.0)	9 (13.0	
T4	7 (4.00)	7 (100)	-	
T4a	37 (20.7)	35 (94.6)	2 (5.4)	
T4b	1 (0.56)	1 (100)	-	
Regional lymph nodes (N), *n* = 179				
N0	73 (40.8)	67 (91.8)	6 (8.2)	0.024
N1	23 (12.9)	21 (91.3)	2 (8.7)	
N2	31 (17.3)	24 (77.4)	7 (22.6)	
N3	8 (4.5)	8 (100)	-	
N3a	23 (12.8)	23 (100)	-	
N3b	21 (11.7)	21 (100)	-	
Distant metastasis (M), *n* = 179				0.18
M0	148 (82.7)	133 (89.9)	15 (10.1)	
M1	12 (6.70)	12 (100)	-	
Mx	19 (10.6)	19 (100)	-	

$P < 0.05$ were considered statistical significant. EBV: Epstein-Barr virus; WHO: World Health Organization.

when compared to other European countries seems to indicate that other factors apart from geography may be influencing the distribution of EBVaGC. Indeed, data from different meta-analyses have failed to show this association[8,22].

We found that EBVaGC was more frequent in males

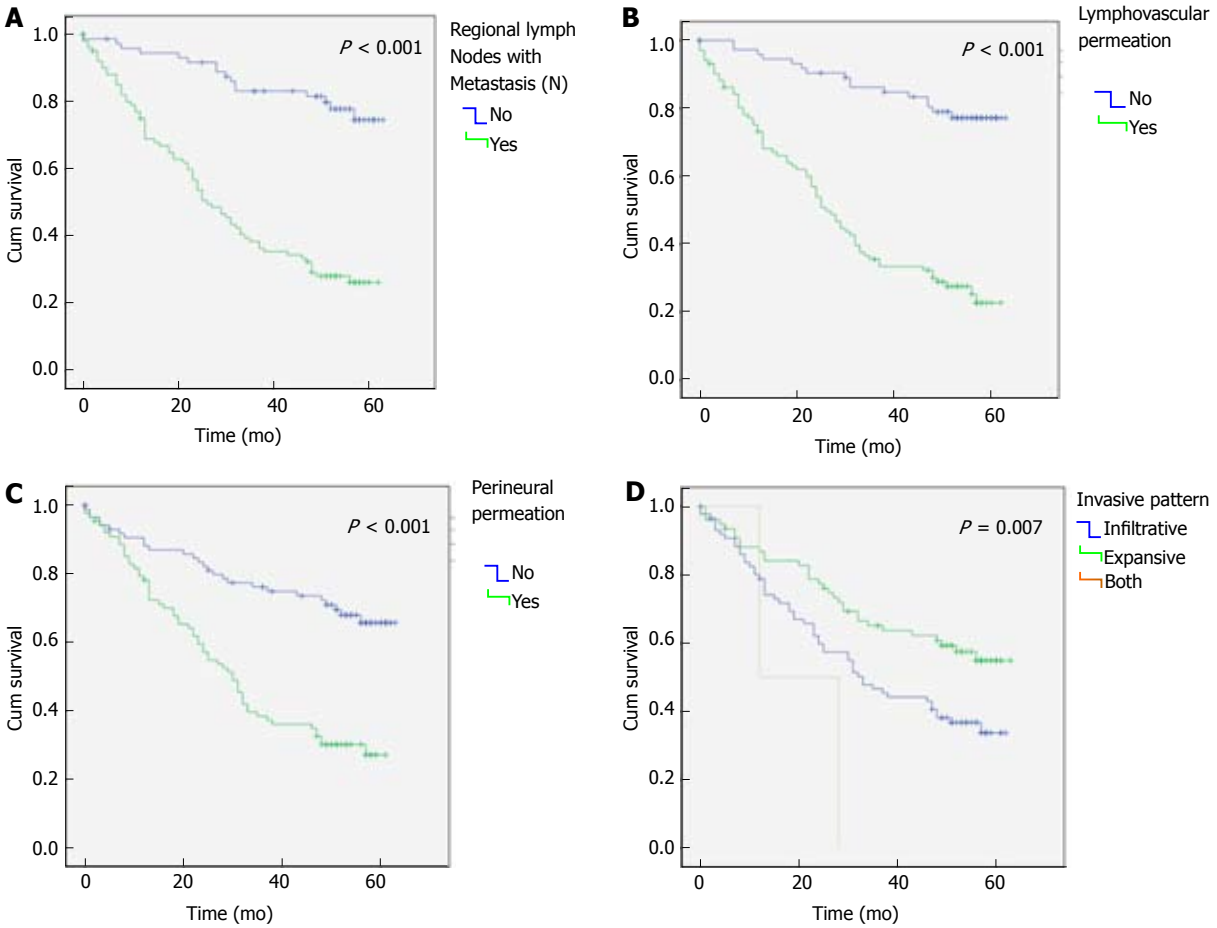


Figure 2 Kaplan-Meier survival curves for overall survival. Comparison of overall survival in gastric cancer patients considering: A: Regional lymph node metastasis; B: Lymphovascular permeation; C: Perineural permeation; and D: Invasive pattern.

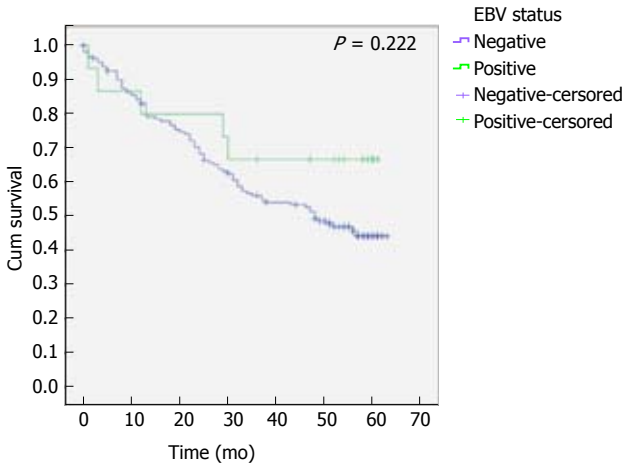


Figure 3 Comparison of overall survival in patients with Epstein-Barr virus-associated gastric carcinomas and Epstein-Barr virus-non associated gastric cancer. Kaplan-Meier curves with log-rank test estimate the overall survival of gastric cancer patients with and without Epstein-Barr virus (EBV)-associated gastric cancer.

than in females, which is in line with several studies and suggests an association with others factors, such as life style or hormonal risks[23-25]. In contrast with other reports, we have found no association between EBV status and patients' age[8,26]. Regarding the tumour location, our study revealed a higher predominance of EBVaGC in upper third and middle of stomach and considering that *H. pylori* preferentially colonises the antral region, these results suggest a possible antagonism of EBV and *H. pylori* in gastric mucosa[27-29]. In fact, Minoura-Etoh *et al*[30] described in an *in vitro* study that reactive products from *H. pylori* seem to induce EBV reactivation from latently in infected gastric epithelial cells, which would avoid EBV transformation of gastric cells in the same areas of *H. pylori* colonization. Despite this association remains unclear, two recent studies have suggested that *H. pylori* may contribute for EBV-associated gastric carcinogenesis by recruiting to gastric tissue the inflammatory cells that are already infected by EBV in the majority of adults[7,31]. This mechanism is not well understood and further studies should be made to establish if the recruitment of EBV-infected lymphoid cells might be the explanation for the infection and subsequent transformation of gastric epithelium. In our series, all EBVaGC cases had EBV only in tumour cells and not in normal tissues, nevertheless, we have

Table 3 Clinicopathological characteristics of Epstein-Barr virus-associated gastric cancers

ID	Gender	Age	Anatomic Site	WHO Classification	Invasion pattern	Differentiation	*n*	TNM Classification	LMP-1	LMP-2A
1	M	75	Body	Tubular Adenocarcinoma	Expansive	Poorly differentiated	6	T4aN2M0	-	-
2	M	80	Antrum	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N0M0	-	-
3	M	56	Fundus	Tubular Adenocarcinoma	Expansive	Moderately differentiated	2	T2N1M0	-	-
4	M	80	Antrum	Tubular Adenocarcinoma	Expansive	Moderately differentiated	2	T3N0M0	-	-
5	M	64	Body	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N2M0	-	(+)
6	M	56	Fundus	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N0M0	-	-
7	M	69	Body	Tubular Adenocarcinoma	Expansive	Moderately differentiated	3	T3N2M0	-	Inc.
8	M	82	Cardia	Tubular Adenocarcinoma	Infiltrative	Moderately differentiated	4	T3N2M0	-	(+)
9	M	55	Body	Mixed Carcinoma	Infiltrative	Poorly differentiated	3	T3N2M0	-	(+)
10	F	66	Antrum	Mixed Carcinoma	NA	Poorly differentiated	6	T1bN2M0	-	(+)
11	F	68	Body	Medullary Carcinoma	Expansive	Poorly differentiated	1	T1bN1M0	-	-
12	M	52	Cardia	Medullary Carcinoma	Expansive	Poorly differentiated	0	T1bN0M0	-	-
13	M	44	Antrum	Medullary Carcinoma	Infiltrative	Poorly differentiated	0	T3N0M0	-	(+)
14	F	65	Pylorus	Poorly cohesive carcinoma	Infiltrative	Poorly differentiated	0	T4aN0M0	-	-
15	M	76	Body	Adenosquamous carcinoma	Expansive	NA	6	T3N2M0	-	(+)

N: Number of regional lymph nodes with metastasis; NA: Not available; (-): Negative result; (+): Positive result; Inc.: Inconclusive result; LMP1: Latent Membrane Protein 1; Latent Membrane Protein.

found one case with EBV only in the lymphocytic infiltration, which we considered negative for EBV, and therefore more studies are required to understand if in such cases tumour cells will be later infected by EBV.

The association between EBV and gastric histological types remains controversial, indeed while one meta-analysis regarding EBV in gastric carcinoma has shown that EBVaGC was associated with diffuse histological type according to Lauren classification[5,8], two other meta-analyses did not find any association between EBV and histology subtypes[22,32]. Several studies demonstrated a strong EBV association with diffuse type while others have reported a similar prevalence between intestinal and diffuse types (classified in our study as poorly cohesive according to the WHO)[9,18,33,34]. Our study revealed that EBVaGC are more frequently associated with specific histologic subtypes. EBVaGC represent about 8% of tubular (intestinal type in the Lauren classification system) and mixed types while it was only found in 2% (n = 1/51) of poorly cohesive carcinomas (diffuse type in the Lauren classification system). These distinct features show that the differences in the GC histological distribution among different populations may impact on the prevalence of EBVaGC subtype, which should gather importance on the detection of EBV in GC. As expected, all medullary carcinomas, also known as gastric carcinoma with lymphoid stroma or lymphoepithelioma-like gastric carcinoma, were EBV positive[35]. In fact, EBV association with gastric cancer was first described in lymphoepithelioma-like gastric subtype and the literature shows that more than 80% of these cases are EBV-positive[5,22,36].

Some studies have revealed that EBVaGC is associated with poor or moderate differentiation of gastric cancer tissues[9,24]. In accordance with these reports, our study showed that 100% of cases were poorly or moderately differentiated and that these differentiation patterns seem to be associated with the histological types. This evidence may be a significant mark of EBVaGC tumours and may provide important information regarding the clinical approach to these tumours.

All these data support the evidence that recent studies have shown by considering EBVaGC as a distinct clinicopathological entity due to its individual features[11,12,18,37]. Interestingly, we found that EBVaGC had a significantly lower number of regional lymph nodes with metastasis and that none have a distant metastasis. Additionally, when analysing the overall survival of EBVaGC compared to all other cases it was observed that EBVaGC seems to have an improved survival, that despite not statistically significant may be explained by the low number of cases in our population. Several studies have reported a better overall survival of EBVaGC, especially in Asiatic populations suggesting that the explanation for this better overall survival could be due to the low

frequency of lymph node involvement and distant metastasis[18,23,24,38]. In Portugal, GC remains at high mortality rates due to the late diagnosis of this disease and these patients are frequently only treated with chemo/radiotherapy. In our study we have included only patients with available tissue sample from surgical procedures and therefore we had not included patients that had received only chemo/radiotherapy, therefore our findings should be applied in patients with recommendation for total or partial gastrectomy as according to the ESMO Clinical Practice Guidelines for Diagnosis, Follow-up[39]. Moreover, others factors may be influencing patients survival and this question should be explored with further large-scale studies.

To elucidate the role of EBV in the pathogenesis of gastric carcinoma several studies have focused on the study of EBV latent proteins expression in gastric tumour cells[40-42]. As expect, in our study LMP1 was not found in any of EBVaGC cases and, in fact, literature also shows that LMP1 is generally absent in EBVaGC except for the data reported in a few studies, which have detected low *albeit* detectable levels of LMP1 mRNA[41-43]. The absence of LMP1 expression in EBV positive cases suggests that LMP1 may not be needed for gastric carcinogenesis or at least not for sustaining the already established malignant tumours. Regarding LMP2A, our results demonstrated that EBV LMP2A is expressed in 40% of EBVaGC cases that is in line with previous studies[41,44]. Recent studies have pointed that LMP2A seems to have an important role contributing to malignant state in epithelial cells by inducing the genome hypermethylation through up-regulation of DNMT1 and phosphorylation of STAT3 and thus contributing for the carcinogenic potential of the cells[45]. A systematic review performed by our group has shown that EBVaGC are usually associated with a distinct latency pattern, characterized by the expression of EBERs, EBNA1, LMP2A and EBV microRNAs. Furthermore, literature also shows that EBV latency I pattern, which is characterized by the expression of EBERs, EBNA 1 and EBV microRNAs[46], has been also frequently found among EBVaGC tumours[47,48]. In line with this, our study demonstrates that the majority of our cases express EBERs with absence of LMP1 and LMP2A, however, 40% of our cases also expressed LMP2A, which does not fit into the standard EBV latency patterns. In fact, literature has described this distinct pattern as latency II-like and has also reported that lytic transcripts such as BARF0 and BARF1 can also be found within EBV-associated gastric neoplasias[44,49]. Our data reinforces that the clarification of which EBV transcripts (including lytic transcripts and EBV microRNAs) are expressed in gastric tumours assumes a great importance to understand the viral carcinogenesis in gastric cancer and may contribute for the identification of new molecular targets for treatment.

In our study, EBVaGC represents 8.4% of our population with gastric cancer and it is more frequent among tubular adenocarcinomas and medullary carcinomas rather than poorly cohesive carcinomas. EBVaGCs were more common in upper and middle regions of the stomach; were correlated with a lower number of regional lymph nodes with metastasis and with no distant metastasis. The results also revealed that EBVaGC patients seem to have an improved survival and better prognosis. These features suggest that EBVaGC is a distinct subtype of gastric cancer and that the mechanisms of carcinogenesis should be further investigated for particular therapeutic targets

COMMENTS

Background

Gastric cancer (GC) is the fifth most prevalent cancer and it remains the third most common cause of cancer-related death in the world. Epstein-Barr virus (EBV) is an oncogenic virus that has been associated with several malignancies, including B-cell lymphomas and nasopharyngeal carcinomas (NPC). Recently, EBV has been also associated with GC development with several studies suggesting that EBV-associated gastric carcinomas (EBVaGC) represent approximately 10% of all GC cases.

Research frontiers

Gastric carcinomas have been widely classified according to the Lauren or World Health Organization classification systems but these systems have a reduced clinical utility. A recent study has suggested a new classification based on molecular features of the tumours, where EBVaGC arises as a new subtype of gastric cancer. Despite the high incidence of both GC and EBV infection prevalence in the Portuguese population, there are no studies reporting the prevalence of EBVaGC in Portugal. The characterization of the EBVaGC in different populations is crucial to the development of new therapies and classification systems.

Innovations and breakthroughs

This is the first study in Portugal to characterize the EBV infection in gastric cancer. It demonstrated that EBV-associated carcinomas represent 8.4% of all gastric cancer cases and that EBVaGCs are a distinct subgroup of GC. Furthermore, it also showed that EBV displays a new latency profile in some of the EBVaGCs.

Applications

The understanding of clinical relevance and carcinogenesis of EBVaGC in different populations worldwide helps the improvement of preventive or management strategies as well as in the identification of more specific therapeutics.

Peer-review

This study showed that 8.4% of GC cases are EBV-associated being more frequent in upper and middle regions of the stomach, among tubular and medullary carcinomas, and having a lower number of regional lymph nodes invasion. Furthermore, this study confirms that EBVaGC may present a new latency-profile which may be useful to study to better characterize this GC subtype.

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ORIGINAL ARTICLE

Retrospective Study

Modified model for end-stage liver disease improves short-term prognosis of hepatitis B virus-related acute-on-chronic liver failure

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Abstract

AIM

To investigate whether the short-term prognosis of hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) could be improved by using a modified model for end-stage liver disease (MELD) including serum lactate.

METHODS

This clinical study was conducted at the First Affiliated Hospital, Fujian Medicine University, China. From 2009 to 2015, 236 patients diagnosed with HBV-related ACLF at our center were recruited for this 3-month follow-up study. Demographic data and serum lactate levels were collected from the patients. The MELD scores with or without serum lactate levels from survival and non-survival groups were recorded and compared.

RESULTS

Two hundred and thirty-six patients with HBV-ACLF were divided into two groups: survival group (S) and

non-survival group (NS). Compared with the NS group, the patients in survival the S group had a significantly lower level of serum lactate (3.11 ± 1.98 *vs* 4.67 ± 2.43, t = 5.43, P < 0.001) and MELD score (23.33 ± 5.42 *vs* 30.37 ± 6.58, t = 9.01, P = 0.023). Furthermore, serum lactate level was positively correlated with MELD score (r = 0.315, P < 0.001). Therefore, a modified MELD including serum lactate was developed by logistic regression analysis (0.314 × lactate + 0.172 × MELD - 5.923). In predicting 3-month mortality using the MELD-LAC model, the patients from the S group had significantly lower baseline scores (-0.930 ± 1.34) when compared with those from the NS group (0.771 ± 1.32, t = 9.735, P < 0.001). The area under the receiver operating characteristic curve (AUROC) was 0.859 calculated by using the MELD-LAC model, which was significantly higher than that calculated by using the lactate level (0.790) or MELD alone (0.818). When the cutoff value was set at -0.4741, the sensitivity, specificity, positive predictive value and negative predictive value for predicting short-term mortality were 91.5%, 80.10%, 94.34% and 74.62%, respectively. When the MELD-LAC scores at baseline level were set at -0.5561 and 0.6879, the corresponding mortality rates within three months were 75% and 90%, respectively.

CONCLUSION

The short-term prognosis of HBV-related ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study.

Key words: Hepatitis B virus; Liver failure; Model for end-stage liver disease score; Prognosis; Serum lactate level

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Core tip: This is a retrospective study to evaluate the short-term prognosis of hepatitis B virus (HBV)-acute-on-chronic liver failure (ACLF). Two hundred and thirty-six patients with HBV-ACLF were divided into two groups: survival group (S) and non-survival group (NS). In predicting 3-mo mortality using the model for MELD-LAC model, patients from the S group had significantly lower baseline scores compared with those from NS group using model for end-stage liver disease (MELD)-LAC model. AUROC was 0.859 calculated by using the MELD-LAC model, which was significantly higher than those calculated by using the lactate level (0.790) or MELD alone (0.818). The short-term prognosis of HBV-ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a severe public health problem all over the world. The prevalence of hepatitis B is particularly high in China. HBV-related acute-on-chronic liver failure (ACLF) is a rapidly progressive disease with a high mortality rate up to 60%-75%[1]. The accurate assessment of the disease severity is critical before clinicians can make decisions on potential treatments like medication or liver transplantation (LT). The model for end-stage liver disease (MELD)[2,3] is a well-accepted model for assessing the feasibility of LT; however, the accuracy of its prediction is still unsatisfying[4]. A more objective and quantitative model with higher repeatability to predict the short-term prognosis of HBV-related ACLF is urgently needed.

Lactate (LAC) is mainly metabolized in the liver and is widely used as an important indicator of organ failure or serious bacterial infection. Hyperlactatemia normally reflects both increased production and impaired clearance in patients with liver dysfunction[5], and a higher level of LAC always indicates a worse prognosis[6]. Although several potential hypoxic and non-hypoxic mechanisms have been implicated on the persistent hyperlactatemia and the high level of LAC, the exact role of these parameters has not been specifically addressed in clinical studies[7,8]. In this study, we investigated the serum LAC level in patients with HBV-related ACLF, and developed a modified MELD including serum lactate (MELD-LAC model) to predict the short-term prognosis (three months) of HBV-related ACLF.

MATERIALS AND METHODS

Patients

Three hundred and ninety-three patients, who were diagnosed with HBV-related ACLF and admitted in our center, were included in the present clinical study between September 2009 and October 2015. The subjects were aged from 18 to 65 years. The diagnosis of ACLF was conducted according to the American Association for the Study of Liver Failure: Update 2011[9]. Three months after diagnosis, these patients were followed by telephone. The patients were divided into two groups: survival group (S group) and non-survival group (NS group), based on whether they were surviving or not three months after admission. The clinical data were collected within the first 24 h after admission, including hepatic, renal, and coagulation functions, as well as the LAC level. The onset time of hepatic encephalopathy (HE) was also noted if it happened. MELD scores were calculated as follows: MELD = 3.8 × Ln(Tbil [mg/dL]) + 11.2 × Ln(INR) + 9.6 × Ln (Cr [mg/dL]) + 6.4 × cause. Since the ACLF was due to HBV infection, the cause was counted as 1 in this study.

On admission, each patient's baseline values of serum LAC level, total bilirubin, creatinine level and

prothrombin time-international normalized ratio were measured. The serum LAC level was measured using a commercial test kit (Johnson & Johnson Inc., United States) with a normal value of 0.9-2.0 mmol/L. Total bilirubin and creatinine levels were tested using an Olympus Chemistry Analyzer (AU2700). All reagents for bilirubin and creatinine tests were purchased from Olympus Corporation (Japan). PT test and INR calculation were performed using a Stago STA Compact coagulation analyzer (Diagnotica Stago, French).

Treatment

After HBV-related ACLF was diagnosed, all the patients were treated with standard protocols, including nutritional support, hepatocyte proliferation, prevention of infection, correction of anomalies in the coagulation system and improvement of cerebral edema. In addition, plasmapheresis was also performed when it was necessary. The survival data were collected after three months.

Statistical analysis

Data analyses were performed using SPSS 17.0 software. The significance of data was tested by the Student's test or the χ^2 test. The correlation between groups was analyzed by the Pearson's product-moment correlation coefficient test. *P*-values less than 0.05 were considered statistically significant. Logistic regression analysis was used to establish the prognosis model. The accuracies of the newly developed prognosis model (MELD-LAC model), LAC and MELD scores in predicting the short-term prognosis of HBV-related ACLF patients were assessed by the area under the receiver operating characteristic (AUROC) curve.

RESULTS

Demographic data

A total of 393 serum samples from patient with HBV-related ACLF were collected, 157 of which were excluded from the present study due to the complications of hepatocellular carcinoma, kidney injury, diabetes or alcoholic liver diseases. According to the follow-up results in the 236 enrolled cases, 130 (110 males and 20 females) were recruited into the S group, and 106 (87 males and 19 females) were recruited into the NS group. The two groups had no statistical difference in gender or age, but had significant differences in TBIL, Cr, INR, LAC levels and MELD scores ($P < 0.05$ for all) (Table 1). There were 68 patients in the S group and 57 patients in the NS group who had received plasmapheresis as an additional enhanced therapy, but it did not provide any positive outcome ($P = 0.896$). No patient in this study had received LT.

Approximately 82.3% (107 of 130) of patients in the S group and 95.2% (101 of 106) of patients in the NS group were found to have elevated LAC level of more than 2 mmol/L. A significant difference was found in the above percentages between the two groups ($\chi^2 = 9.40$, $P = 0.002$). Furthermore, 13.1% of patients (17 of 130) in the S group and 59.4% (63 of 106) in NS group were found to have elevated LAC level of more than 4 mmol/L. A significant difference between the percentages of the S group and the NS group was also found ($\chi^2 = 55.999$, $P < 0.001$). The mean LAC level in the S group (3.11 mmol/L ± 1.98 mmol/L) was significantly lower than that in the NS group (4.67 mmol/L ± 2.43 mmol/L) ($P < 0.001$). No significant difference was shown in the CTP scores between the S and NS groups ($P = 0.373$), but a significant difference was shown in the MELD scores between the two groups ($P = 0.023$).

Using the Spearman analysis method, the correlations between MELD score/serum LAC levels and the mortality in three months were analyzed (Table 2). The results from Pearson's analysis also showed that both serum LAC level and MELD score had a statistically significant correlation with the short-term prognosis of HBV-related ACLF ($r = 0.315$, $P < 0.001$).

Construction of a modified MELD including serum lactate

Based on single factor analysis, both MELD score and LAC level were correlated with the 3-mo mortality of HBV-related ACLF. The forward logistic regression method was used to establish the MELD-LAC model: 0.314 × LAC + 0.172 × MELD score - 5.923 (Table 3). The patients from the S group had significantly lower baseline MELD-LAC scores (-0.930 ± 1.34) compared with those from the NS group (0.771 ± 1.32, $t = 9.735$, $P < 0.001$).

The short-term prognosis of HBV-related ACLF was improved by using the modified MELD including serum lactate from the present 6-year clinical study.

Prediction of the short-term prognosis of HBV-related ACLF by using MELD-LAC model

To predict the 3-month mortality, the LAC model alone had a very similar positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity to MELD. Interestingly, the new MELD-LAC model by combining these two independent factors had a better predicting score than these two models alone. When the cutoff value of the MELD-LAC model was set at -0.4741, based on the best Yoden index, an AUROC curve of 0.859 (Table 4) was obtained when this equation was applied to evaluate the prognosis, with a sensitivity of 91.5% and a specificity of 80.10%, which were much greater than the other two models. Comparing the AUROCs obtained from the above three models, the prognosis performances were listed as follows: MELD-LAC > MELD > LAC (Figure 1). From our analysis, the patients with an MELD-LAC score of -0.5561, -0.4741 and 0.6879 had the three-month

mortality rate of 75%, 78.86% and 90%, respectively. The PPV and NPV of MELD-LAC were 94.34% and 74.62%, which were significantly improved compared with the scores obtained from the MELD and LAC model alone.

The patients were divided into two groups, when the cutoff value of the MELD-LAC model was set at -0.4741. The first group (MELD-LAC ≥ -0.4741) included 123 patients, and 78.86% were dead after three months. The second group (MELD-LAC < -0.4741) included 113 patients, and only 11.50% died after three months. A statistically significant difference was observed between these two groups (χ^2 = 107.35, P < 0.001). When compared with MELD with a cutoff value of 25, the MELD-LAC model with a cutoff value of -0.4741 apparently achieved better prediction of short-term prognosis.

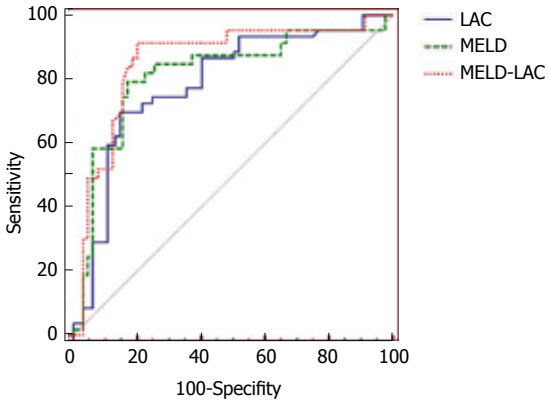


Figure 1 Area under the receiver operating characteristic curves of different models. MELD: Model for end-stage liver disease; LAC: The venous lactate.

DISCUSSION

ACLF is a severe health problem with a high mortality rate[10-12]. In clinical practice, predicting the progression of this disease is the biggest challenge for clinicians. Currently, three scoring systems, namely, King's College Hospital criteria, Child-Turcotte-Pugh (CTP) score and MELD score[4,13] systems, are commonly implemented to predict the prognosis of ACLF patients; CTP score has been used traditionally to assess the prognosis of cirrhosis instead of ACLF; the MELD scoring system has been developed to assess various liver diseases, including assessing the severity of ACLF of all causes to determine the ideal timing of liver transplantation, as well as providing direct information to support medical decision making[14]. The MELD scoring system is widely considered to be better than the CTP score; however, it does have several limitations, for example, the diagnostic sensitivity and specificity of MELD are not high enough.

In the MELD scoring system, several variables associated with poor prognosis of ACLF are not considered, including HE, infections, and hemorrhage. Therefore, modification for MELD is needed to improve its outcome in clinical practice. Addition of Na+ into the MELD (MELD-Na) has been proposed and has been successfully evaluated as a better score in patients with ascites, but only for those patients with sodium levels below normal. However, such a condition is only presented in 30% of patients with decompensated cirrhosis[15].

The elevation of lactate level in ACLF situation has been reported and might be attributed to several suggested mechanisms[16,17]. First, the liver is the organ primarily responsible for LAC clearance, and the LAC clearance may be impaired in the presence of severe liver dysfunction. Second, some ACLF-related complications, *e.g.*, bacterial infection, may also raise the LAC level. Last but not least, hypovolemia-related hypoperfusion is also very likely to induce an elevation of LAC level during the early pre-resuscitative phase. In fact, some previous studies have shown that the acutely injured liver may act as a source of evaluating LAC[18]. Taken together, we suggest that the LAC level could be used as a useful marker for assessing the severity of ACLF. As a simple measure widely available in current hospital system, early measurement of serum LAC can provide important prognostic information in patients with acute variceal hemorrhage requiring ICU admission[19-21]. But to the best of our knowledge, the LAC level had never been considered as a critical biomarker for prognosis prediction of HBV-related ACLF.

Our results showed that LAC could be used as a useful tool to assess the situation of HBV-related ACLF. The evaluated LAC level was found in 82.3% of patients in the S group, but 95.2% of those in the NS group (χ^2 = 9.40, P = 0.002). The mean LAC level in the S group (3.11 mmol/L ± 1.98 mmol/L) was lower than that in the NS group (4.67 mmol/L ± 2.43 mmol/L) (P < 0.001). Further analysis showed that the baseline LAC level was related with patients' prognosis with a rational sensitivity (86.80%). The AUROC for predicting the 3 mo mortality based on the LAC model was relatively lower than that from MELD (0.79 *vs* 0.818), which is consistent with previous studies[21].

In our study, a positive correlation between the LAC and MELD scores was demonstrated, suggesting that a combination of LAC and MELD scores was very likely to increase prediction accuracy for ACLF prognosis. Therefore, we developed a modified MELD including serum lactate level to improve the short-term prognosis prediction of HBV-related ACLF by combining two parameters together (LAC and MELD). By adopting the forward logistic regression method, LAC and MELD scores were subjected to the equation, to yield a new MELD-LAC model.

The new model showed a better (AUROC = 0.859) prediction score for the prognosis of HBV-related ACLF when compared with either LAC (0.790) or MELD (0.818) alone. When the cutoff value was set at -0.4741, the MELD-LAC model had a sensitivity

Table 1 Demographic data, biochemical factors, clinical and surgical characteristics for the S and NS groups

Group	S (n = 130)	NS (n = 106)	t or t'	P value
Age (yr)	43.51 ± 14.13	45.97 ± 10.69	1.480	0.140
Male/Female (n)	110/20	87/19	0.273	0.363
Liver cirrhosis, n (%)	63 (48.46)	68 (64.15)	5.820	0.011
TBil (μmol/L)	344.85 ± 160.82	456.63 ± 180.43	5.040	< 0.001
ALT	92.62 ± 82.80	101.84 ± 83.71	0.847	0.389
AST	92.81 ± 91.84	103.86 ± 79.20	0.978	0.329
GLO	27.72 ± 8.95	27.84 ± 8.85	0.100	0.920
WBC	8.27 ± 4.12	8.21 ± 4.29	-0.117	0.907
PCT	0.53 ± 0.29	0.58 ± 0.54	0.886	0.377
Ammonia	67.29 ± 35.55	69.03 ± 47.57	-0.312	0.755
INR	2.46 ± 0.97	3.11 ± 1.63	3.733	< 0.001
Cr (μmol/L)	79.31 ± 45.25	99.80 ± 88.74	2.293	< 0.001
CHE (U/L)	3956.30 ± 1377.54	3529.00 ± 1509.89	0.170	0.859
Alb (g/L)	31.22 ± 3.98	30.99 ± 3.96	-0.428	0.398
DNA	594275 ± 230160	425886 ± 110810	-0.691	0.491
LAC (mmol/L)	3.11 ± 1.98	4.67 ± 2.43	5.430	< 0.001
< 2.68	76	14	50.685	0
2.68-4	37	29	0.035	0.484
⩾ 4	17	63	55.999	< 0.001
CTP	10.54 ± 1.63	10.74 ± 1.73	0.892	0.373
MELD score	23.33 ± 5.42	30.37 ± 6.58	9.010	0.023
Plasmapheresis	68	57	0.05	0.896

TBil: Total bilirubin; Cr: Creatinine; INR: International normalized ratio for prothrombin time; MELD: Model for end-stage liver disease; LAC: The venous lactate; ALB: Albumin; GLO: Globulin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CHE: Cholinesterase; WBC: White blood cell; PCT: Procalcitonin; CTP: Child-Turcotte-Pugh score.

Table 2 Factors correlated with prognosis of hepatitis B virus-related acute-on-chronic liver failure

Factor	Correlation index	P value
MELD	0.548	< 0.001
LAC	0.499	< 0.001
TBiL	0.308	< 0.001
INR	0.289	< 0.001
Cr	0.014	0.828
Alb	0.071	0.273
CHE	0.058	0.373

TBil: Total bilirubin; Cr: Creatinine; INR: International normalized ratio for prothrombin time; MELD: Model for end-stage liver disease; LAC: The venous lactate; ALB: Albumin; CHE: Cholinesterase.

Table 3 The regression coefficients and indices of the model for end-stage liver disease and the venous lactate model

Index	β	SE	Wald	df	P value	Exp(β)
MELD	0.172	0.028	37.198	1	0.000	1.188
LAC	0.314	0.098	10.195	1	0.001	1.368
Constant	-5.923	0.824	51.724	1	0.000	0.003

MELD: Model for end-stage liver disease; LAC: The venous lactate.

of 91.50% and a specificity of 80.10% for predicting the mortality within three months. The patients were further divided into two groups according to the above cutoff value. The mortality was 78.86% in the higher score group (MELD-LAC ⩾ -0.4741) and 7.96% in the lower score group (MELD-LAC < -0.4741), showing a significant difference between these two groups (χ^2 = 107.35, P = 0.000). Under such a cutoff value setting (-0.4741), the MELD-LAC model seemed to provide a much more accurate prediction than normal MELD with a cutoff value of 25 (Table 5). According to our data, the three-month mortality rate of the patients was 75%, and 90% could be precisely predicted by the scores of MELD-LAC (-0.5561 or 0.6879) at baseline. Our results also showed no difference between the S group and NS group in CTP scores (Table 1), suggesting that CTP system is not a good tool in evaluating the outcome of HBV-related ACLF.

There are some limitations of this study. Our study indicated, for the first time, that a modified MELD including serum lactate was a better prediction model for the prognosis of HBV-related ACLF, with higher PPV and NPV. Further research is required to combine this model into the classic model or develop a more accurate prognostic model. Another limitation of this study is that the patient population came merely from a single center in this study and it was a retrospective study; further research and verification need to be done in larger multi-center studies.

Serum LAC test is a simple and mature clinical measurement in modern hospitals, and it reflects both direct liver injury and other organ dysfunction. Dynamic monitoring of LAC was able to provide a new opportunity for clinicians with more accurate disease assessment, prognosis predication and treatment guidance at the early stage of the liver disease. We concluded that the modified MELD including serum lactate is a better prediction model for the prognosis of HBV-related ACLF, with higher PPV and NPV.

Table 4 Prognostic value of the various models

model	AUROC	95%CI	Cut-off value	Sensitivity	Specificity	PPV	NPV
MELD	0.818	0.759-0.877	24.5	87.70 %	63.80%	85.85%	64.62%
LAC	0.79	0.730-0.850	2.68	86.80%	62.10%	86.79%	58.46%
MELD-LAC	0.859	0.807-0.911	-0.4741	91.50%	80.10%	94.34%	74.62%

AUROC: The area under the receiver operating characteristic; PPV: Positive predictive value; NPV: Negative predictive value. LAC: The venous lactate; MELD: Model for end-stage liver disease.

Table 5 Comparison of prediction performance of the model for end-stage liver disease and the modified model

Groups		Non-survival	Survival	χ^2, *P* value
MELD	MELD < 25	16	83	56.98, < 0.001
	MELD ≥ 25	90	47	
MELD-LAC	MELD-LAC < -0.4741	13	100	107.35, < 0.001
	MELD-LAC ⩾ -0.4741	97	26	

MELD: Model for end-stage liver disease; LAC: The venous lactate.

COMMENTS

Background

Hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) is a rapidly progressive disease with a high mortality rate up to 60%-75%. The accurate assessment of disease severity is critically needed, before clinicians make decisions on potential treatments such as medication or liver transplantation (LT). The model for end-stage liver disease (MELD) score model is a well-accepted model for assessing the feasibility of LT; however, the accuracy of prediction is still unsatisfying. A more objective and quantitative model with higher repeatability to predict the short-term prognosis of HBV-related ACLF is urgently needed.

Research frontiers

In the MELD scoring system, several variables associated with poor prognosis of ACLF are not considered, including hepatic encephalopathy (HE), infections, and hemorrhage. Therefore, modification of MELD is needed to improve its outcome in clinical practice. Addition of Na+ into MELD (MELD-Na) has been proposed and has been successfully evaluated as a better score in patients with ascites, but only for those patients with sodium levels below normal. However, such a condition is only presented in 30% of patients with decompensated cirrhosis.

Innovations and breakthroughs

Lactate (LAC) is mainly metabolized in the liver and is widely used as an important indicator in organ failure or serious bacterial infection. Hyperlactatemia normally reflects both increased production and impaired clearance in patients with liver dysfunction, and a higher level of LAC always indicates a worse prognosis. Although several potential hypoxic and non-hypoxic mechanisms have been implicated on the persistent hyperlactatemia and the high level of LAC, the exact role of these parameters has not been specifically addressed in clinical studies. In the present study, they investigated the serum LAC level in patients with HBV-related ACLF, and developed a modified MELD including serum lactate (MELD-LAC model) to predict the short-term prognosis (three months) of HBV-related ACLF.

Applications

This study developed a modified MELD model including serum lactate level to improve the short-term prognosis prediction of HBV-related ACLF by combining the two parameters together (LAC and MELD).

Peer-review

The authors of this paper found that the efficacy of the short-term prognosis of HBV-related ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study. The new model showed a better prediction score on the prognosis of HBV-related ACLF, when compared with either LAC or MELD alone.

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ORIGINAL ARTICLE

Observational Study

Chronic opioids in gastroparesis: Relationship with gastrointestinal symptoms, healthcare utilization and employment

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Abstract

AIM

To examine the relationship of chronic scheduled opioid use on symptoms, healthcare utilization and employment in gastroparesis (Gp) patients.

METHODS

Patients referred to our tertiary care academic center from May 2016 to July 2017, with established diagnosis or symptoms suggestive of Gp filled out the Patient Assessment of Upper GI Symptoms, abdominal pain and demographics questionnaires, and underwent gastric emptying and blood tests. They were asked about taking pain medicines and the types, doses, and duration. We used Mann Whitney *U* test, Analysis of Variance, Student's *t* test and χ^2 tests where appropriate for data analyses.

RESULTS

Of 223 patients with delayed gastric emptying, 158 (70.9%) patients were not taking opioids (GpNO), 22 (9.9%) were taking opioids only as needed, while 43 (19.3%) were on chronic (> 1 mo) scheduled opioids (GpCO), of which 18 were taking opioids for

reasons that included gastroparesis and/or stomach pain. Median morphine equivalent use was 60 mg per day. GpCO reported higher severities of many gastrointestinal symptoms compared to GpNO including nausea (mean ± SE of mean of 4.09 ± 0.12 *vs* 3.41 ± 0.12, $P = 0.011$), retching (2.86 ± 0.25 *vs* 1.98 ± 0.14, $P = 0.003$), vomiting (2.93 ± 0.24 *vs* 2.07 ± 0.15, $P = 0.011$), early satiety (4.17 ± 0.19 *vs* 3.57 ± 0.12, $P = 0.004$), post-prandial fullness (4.14 ± 0.18 *vs* 3.63 ± 0.11, $P = 0.022$), loss of appetite (3.64 ± 0.21 *vs* 3.04 ± 0.13, $P = 0.039$), upper abdominal pain (3.86 ± 0.20 *vs* 2.93 ± 0.13, $P = 0.001$), upper abdominal discomfort (3.74 ± 0.19 *vs* 3.09 ± 0.13, $P = 0.031$), heartburn during day (2.55 ± 0.27 *vs* 1.89 ± 0.13, $P = 0.032$), heartburn on lying down (2.76 ± 0.28 *vs* 1.94 ± 0.14, $P = 0.008$), chest discomfort during day (2.42 ± 0.20 *vs* 1.83 ± 0.12, $P = 0.018$), chest discomfort at night (2.40 ± 0.23 *vs* 1.61 ± 0.13, $P = 0.003$), regurgitation/reflux during day (2.77 ± 0.25 *vs* 2.18 ± 0.13, $P = 0.040$) and bitter/acid/sour taste in the mouth (2.79 ± 0.27 *vs* 2.11 ± 0.14, $P = 0.028$). GpCO had a longer duration of nausea per day (median of 7 h *vs* 4 h for GpNO, $P = 0.037$), and a higher number of vomiting episodes per day (median of 3 *vs* 2 for GpNO, $P = 0.002$). Their abdominal pain more frequently woke them up at night (78.1% *vs* 57.3%, $P = 0.031$). They had a lower employment rate (33.3% *vs* 54.2%, $P = 0.016$) and amongst those who were employed less number of working hours per week (median of 23 *vs* 40, $P = 0.005$). They reported higher number of hospitalizations in the last 1 year (mean ± SE of mean of 2.90 ± 0.77 *vs* 1.26 ± 0.23, $P = 0.047$).

CONCLUSION

GpCO had a higher severity of many gastrointestinal symptoms, compared to GpNO. Hospitalization rates were more than 2-fold higher in GpCO than GpNO. GpCO also had lower employment rate and working hours, when compared to GpNO.

Key words: Opioid; Gastroparesis; Symptoms; Hospitalizations; Employment

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Core tip: Chronic opioid use can cause gastrointestinal side effects and negatively influence the quality of life. The impact of chronic opioid use on symptoms, healthcare utilization, and employment of gastroparesis patients is not well studied. In our study, gastroparesis patients on chronic scheduled opioids had more severe gastrointestinal symptoms, less work productivity and more frequent hospitalizations compared to gastroparesis patients without opioid use. Whether opioid use is to treat a higher symptom severity from gastroparesis, or the opioid use worsens symptoms requires further study.

Jehangir A, Parkman HP. Chronic opioids in gastroparesis: Relationship with gastrointestinal symptoms, healthcare utilization and employment. *World J Gastroenterol* 2017; 23(40): 7310-7320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7310.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7310

INTRODUCTION

Opioid use has become a healthcare epidemic in United States with increasing prescription rates in the recent years, and over 3% of the adults are now chronically using opioids[1-3]. In 2013, the overall cost burden from opioids in United States was estimated to be $78.5 billion[4]. A recent systematic review identified mean costs to the payer (commercial or private insurance) of $23000-$25000 per year for the opioid misusers, approximately $15000 more than the non-opioid users[5].

Patients on chronic opioids are more likely to have bowel related issues[2]. The wide array of gastrointestinal symptoms that the patients on chronic opioids may experience, including constipation (38%-63%), gastroesophageal reflux disease (33%), nausea (20%-90%), vomiting (9%-84%), bloating (24%-75%) and delayed gastric emptying are known as opioid induced bowel dysfunction[2,6-13]. The actual incidence of some of these side effects may even be higher as many patients tend to underreport their symptoms, and may self-adjust their regimen to avoid side effects[12]. Approximately 6% of the chronic opioid patients develop worsening chronic or intermittent abdominal pain (AP) even with continuous or increasing doses of opioids, a condition termed narcotic bowel syndrome[8]. The opioid-induced gastrointestinal side effects are often not anticipated while prescribing these medications, and may lead to increase healthcare utilization[9].

Despite the known gastrointestinal side effects of opioids, they are used in some patients with gastroparesis (Gp). Previous studies reported that 30%-46% of the Gp patients regularly use opioids[10,14,15]. In addition to opioids causing constipation, opioids slow gastric emptying and also can cause nausea and vomiting by activating the chemoreceptor trigger zone in the area postrema at the floor of the fourth ventricle[9,16]. Moreover, it has been shown that Gp patients on opioids may have worse outcomes with medical treatment with prokinetics agents and after gastric pacemaker placement[14].

The aim of this study was to examine the relationship of chronic opioid use on symptoms, healthcare utilization and employment in patients referred for Gp. We studied gastroparesis patients; comparing those on chronic scheduled opiates (GpCO) to gastroparesis patients

not taking opiates (GpNO).

MATERIALS AND METHODS

Patients referred to our tertiary care academic center gastroenterology motility clinic at Temple University Hospital (TUH) with established diagnosis of gastroparesis, or patients who presented with symptoms suggestive of gastroparesis from May 2016 to July 2017 (n = 303) were studied. This observational study was reviewed and approved by the TUH Intuitional Review Board. Patients are often referred to our motility clinic for persistent or refractory symptoms of gastroparesis. Subjects were recruited at the end of their regularly scheduled appointments after obtaining informed consent. Inclusion criteria were the following: (1) adults aged 18 to 80 years old; (2) symptoms suggestive of gastroparesis; and (3) delayed gastric emptying on scintigraphy.

On their initial evaluation, patients were asked to fill out a questionnaire about their clinical condition. This questionnaire contained the following: Patient Assessment of Upper Gastrointestinal Symptom (PAGI-SYM), Abdominal Pain Questionnaire, and demographic questionnaire. Patients underwent 4-h gastric emptying scintigraphy (GES)[17] if had not already recently been performed. Blood tests were obtained (see below). A retrospective review of the questionnaires, gastric emptying scintigraphy and blood tests was subsequently performed.

Questionnaires

PAGI-SYM: This validated questionnaire for upper gastrointestinal symptoms of gastroparesis, dyspepsia, and gastroesophageal reflux disease asked patients to rate the severity of 20 common gastrointestinal symptoms over the past two weeks[18]. Patients were asked to rate symptoms over the prior two weeks as none (0), very mild (1), mild (2), moderate (3), severe (4), and very severe (5). They were also asked to rate symptoms of diarrhea and constipation on the same scale. In addition, they were asked about the duration of nausea per day in the past week, number of episodes of vomiting and bowel movements in the past week. We also asked the patients about the frequency of some of their gastrointestinal symptoms over the preceding 3 mo using the Rome IV questionnaires[19].

Abdominal pain questionnaire: This questionnaire was modified from previous studies assessing the presence and severity of abdominal pain in chronic pancreatitis[20,21]. If abdominal pain was present, patients filled out the remaining portion of the questionnaire asking about the duration, location of the most severe pain, relationship of pain to meals and nocturnal awakenings as a result of the pain.

Demographics questionnaire: This questionnaire asked patients to report information such as age, gender, race/ethnicity, height, weight, smoking history, alcohol usage, employment and working hours per week if employed. It also asked about the length of diagnosis with Gp, history of diabetes, and prior abdominal surgeries. Patients were asked about taking pain medicines and the types, doses, frequency, clinical indication and total duration of pain medicine use.

Laboratory analysis

Patients underwent blood drawing including hemoglobin A1c and thyroid stimulating hormone, as hyperglycemia and hypothyroidism are risk factors for delayed gastric emptying[22,23]. We also checked serum trypsinogen level as symptoms of Gp can resemble those of chronic pancreatitis (CP)[24]. Lastly, we ordered random serum cortisol levels as opioid-induced adrenal insufficiency has been reported in the literature[25-27].

Gastric emptying scintigraphy

Gastric emptying scintigraphy was performed using a low-fat, egg white meal with imaging at 0, 1, 2, 4 h after meal ingestion[17]. Patients were instructed to stop medications that could affect gastrointestinal motility (including prokinetics) for 48 h prior to the study and to come to the Nuclear Medicine Section in the morning after fasting overnight, that is, an 8 h fast. The patients on chronic opioids were advised to gradually taper off their opioids as tolerated prior to their scheduled GES to prevent opioid withdrawal. Diabetics have their glucose checked at the beginning of the study, with appropriate treatment measures being taken if low blood sugar (hypoglycemia) or high blood sugar (hyperglycemia > 250 mg/dL) is detected. Gastric emptying scintigraphy is performed using a standard low-fat, Eggbeaters® meal to measure solid emptying. The meal consists of the equivalent of two large eggs radiolabeled with 0.5-1 mCi Tc-99m sulfur colloid served with two pieces of white bread and jelly. Patients are given 120 mL water. Following ingestion of the meal, imaging is performed at 0, 1, 2 and 4 h with the patient standing upright for measuring gastric emptying of Tc-labeled solids. Gastric emptying is analyzed as percent of radioactivity retained in the stomach over time using the geometric center of the decay-corrected anterior and posterior counts for each time point. Gastric retention of Tc-99m > 60% at 2 h. and/or > 10% at 4 h is considered delayed gastric emptying of solids.

Statistical analysis

We used Kolmogorov-Smirnov test to determine the normal distribution of continuous variables; Student's t-test was used for variables with normal distribution, while Mann Whitney U Test was used for variables with skewed distribution and symptoms assessed on ordinal scale. These results are expressed as mean ± SE of mean, or median with interquartile range as

appropriate. χ^2 test was used for categorical data, with results expressed as percentages[28]. A two-tailed *P* value less than 0.05 was considered as statistically significant while comparing chronic opioid using Gp patients to non-opioid users; no adjustment for multiple comparisons was made. Analysis of Variance (ANOVA) was used for comparison of multiple groups, followed by Students; *t*-test with *p* value adjusted with Bonferroni correction for multiple comparisons. Unanswered questions were excluded from the analyses. Gp patients who were using opioids as needed (*n* = 22) were not included in the analyses. Statistical review of the study was performed by an epidemiologist/statistician of our department.

RESULTS

Patients

Of 303 patients referred for symptoms suggestive of gastroparesis in 15 mo, 80 patients had normal gastric emptying tests and were excluded from the analyses. Of the remaining 223 patients, the majority (*n* = 196, 87.9%) had previously been diagnosed with Gp. More than half (52%) of the Gp patients came from outside the catchment area of TUH that we defined as more than 50 miles from our center. The most frequent type of Gp was idiopathic Gp (*n* = 122; 54.7%), followed by diabetic Gp (*n* = 62, 27.8%), post-surgical Gp (*n* = 20; 9.0%), and atypical Gp (*n* = 19; 8.5%) (Table 1). The median age of these patients was 44 years (interquartile range of 32 to 56 years), and 80.7% were females. Amongst 210 patients who reported their race, the most common races were Whites (79.4), African Americans (7.6%) and Hispanics (6.7%). Over two third of patients with Gp (*n* = 158, 70.9%) were not taking opioids, 22 (9.9%) were taking opioids only as needed, while 43 (19.3%) were on chronic scheduled opioids (Table 1).

Gastroparesis patients on chronic scheduled opioids

Amongst the 43 patients on chronic scheduled opioids, 6 (14%) were taking opioids exclusively for Gp and/or stomach pain, while another 12 (27.9%) were taking opioids for reasons that included Gp and/or stomach pain. Other frequent causes of opioid use included back pain (*n* = 27, 62.8%), leg pain (*n* = 10), arthritis (5), fibromyalgia (4), Reflux Sympathetic Dystrophy (3), and neuropathy (2). Nearly one fourth of these patients (*n* =10, 23.3%) were taking more than 1 opioid. The opioids used chronically included oxycodone (18 patients), fentanyl (9), methadone (5), morphine (5), tramadol (5), hydromorphone (4), hydrocodone (3), and oxymorphone (2). Median duration of narcotic use was 2 years (interquartile range of 0.6-6.5 years). Median oral morphine equivalent dose for GpCO was 60 mg/d, with interquartile range of 22.5 mg to 112.5 mg per day.

Patients with diabetic gastroparesis were more likely to be on chronic scheduled opioids (24.2 % *vs* 18.9%), or as needed opioids (14.5% *vs* 4.9%), when compared to the patients with idiopathic gastroparesis who were more likely to be non-opioid users (76.2% *vs* 61.3%), *P* = 0.039. Thirty-three Gp patients on chronic opioids were able to recall the duration of their Gp symptoms as well as chronic opioid use. Out of these, about 1 in every 4 GpCO (27.3%) stated that they started using opioids chronically before their symptoms of Gp started. For another 21.2% of GpCO, the duration of opioid use was the same as the duration of their Gp symptoms. For 51.5% of GpCO, the Gp symptoms started before the use of chronic opioids. Of note however, we had to rely on patients' recall for the duration of their opioids use, and patients reported drug ingestion histories can often be inaccurate[29].

GpCO and GpNO did not have any statistically significant difference in their age, duration of symptoms, gender, body mass index, racial distribution, history of diabetes, and past surgeries on esophagus and stomach (Table 2). GpCO were more likely to be active smokers (31.7% *vs* 13.0%, *P* = 0.004). There was a trend towards higher prior and/or current alcohol use in GpNO compared to GpCO (37.4 % *vs* 23.8% respectively, *P* = 0.100), as well as current alcohol use (20.0% *vs* 7.3% respectively; *P* = 0.057).

Laboratory studies

GpCO were also more likely to have low trypsinogen levels compared to GpNO (23.1% *vs* 4.2% respectively, *P* = 0.004). However, only about half of these patients had their trypsinogen levels drawn (Table 2). Among GpCO, 16.2% had low random serum cortisol levels, *vs* 10.4% among GpNO, *P* = 0.345 (Table 2). We did not confirm the diagnosis of adrenal insufficiency by checking adrenocorticotrophic hormone or by performing stimulation test; this requires further study to determine the actual incidence of adrenal insufficiency in chronic opioid using Gp patients. There was no statistically significant difference in the other laboratory tests, including hemoglobin A1c, thyroid stimulating hormone.

Gastric emptying scintigraphy

On gastric emptying tests, there was no difference between GpCO and GpNO at 2 h (median of 62% *vs* 66%, normal $\leqslant$ 60%) and 4 h (median of 22% *vs* 24%, normal $\leqslant$ 10%) (*P* > 0.05) (Table 2). Amongst GpCO 11 patients (25.6%) had severe delays in gastric emptying (> 35% retention at 4 h), compared to 30 patients (19%) in GpNO (*P* = 0.341).

Opioids did not seem to have a dose-related effect on the delay in gastric emptying, as we did not find any difference in the four quartiles of GpCO based on morphine equivalents per day by comparing the gastric emptying results of these groups using ANOVA (Table 3). We also calculated Pearson correlation coefficient

Table 1 Patients with gastroparesis referred to a tertiary care center between May 2016 and July 2017

Classification	Total	Chronic scheduled opioids	PRN opioids	No opioids
Idiopathic	122	23	6	93
Diabetic	62	15	9	38
Post-surgical[1]	20	2	5	13
Atypical[2]	19	3	2	14
Total	223	43	22	158

[1]Post-surgical gastroparesis consisted of patients with history of duodenal-jejunostomy, esophagectomy, fundoplication, gastric sleeve, gastric banding, gastric bypass, hiatal hernia repair, and vagotomy with pyloroplasty. [2]Atypical gastroparesis consisted of gastroparesis patients with history of Bulimia, Complex Regional Pain Syndrome, Ehlers Danlos Syndrome, Lupus, Parkinson's Disease, Reflux Sympathetic Dystrophy, Scleroderma and Sjogren's Syndrome.

between opioid dose and delay in gastric emptying, and there was no significant correlation between morphine equivalents per day and gastric retention at 2 h ($r < 0.01$, $P = 0.988$) or 4 h ($r = 0.19$, $P = 0.356$).

Of note, 14 patients on chronic scheduled opioids were still taking opioids at the time of their GES, which may have resulted in the delay in their gastric emptying. However, when we compared these patients to GpCO who were able to taper off the opioids prior to the study, there was no difference in gastric retention at 2 h (median of 60% *vs* 70% respectively, $P = 0.461$) and 4 h (16% *vs* 20%, $P = 0.718$).

GI Symptoms

GpCO had higher symptom severities of many GI symptoms including nausea, retching, vomiting, early satiety, post-prandial fullness, loss of appetite, upper abdominal pain, upper abdominal discomfort, heartburn during day, heartburn on lying down, chest discomfort during day, chest discomfort at night, regurgitation/reflux during day, and bitter/acid/sour taste in the mouth compared to GpNO ($P < 0.05$) (Table 4). The severity of constipation was not statistically different between the two groups (2.92 ± 0.30 in GpCO, compared to 2.63 ± 0.14 in GpNO, $P = 0.296$). On PAGI-SYM questionnaire, the total symptom severity score in GpCO was also higher than GpNO (Table 4).

As stated previously the median morphine equivalent use in GpCO was 60 mg per day. When we compared GpCO taking more than 60 mg morphine equivalents per day to GpCO taking 60 mg per day or less, the patients taking more than 60 mg per day reported more severe heartburn during the day (3.30 ± 0.45 *vs* 2.0 ± 0.21, $P = 0.023$). There was also a trend towards more severe heartburn in recumbent position (3.30 ± 0.46 *vs* 2.30 ± 0.38, $P = 0.116$), and bitter, acid or sour taste in the mouth (3.50 ± 0.44 *vs* 2.30 ± 0.36, $P = 0.058$) amongst GpCO taking more than 60 mg morphine equivalents per day (results not shown).

When we compared GpCO with severe delay in gastric emptying (> 35% at 4 h) with GpCO with mild to moderate delay in gastric emptying, there was no difference in symptom severity on PAGI-SYM questionnaire, and we did not notice any differences in the impact of gastroparesis on their employment and healthcare utilization (results not shown).

GpCO had a longer duration of nausea per day in the past week (median of 7 h *vs* 4 h in GpNO, $P = 0.037$), as well as higher number of vomiting episodes per day (median of 3 *vs* 1 in GpNO, $P = 0.002$) but there was no statistically significant difference in the number bowel movements in the past week (Table 5).

AP was frequently present in GpCO and GpNO (84.1% and 85.2% respectively, $P = 0.861$). Epigastrium was the most common location of the most severe AP in GpNO (44.2%); while GpCO most commonly had their most severe AP in periumbilical area (41.7%) followed by epigastric area (38.9%), $P = 0.863$. AP got worse with meal intake in majority of patients (76.5% in GpCO *vs* 80.0% in GpNO, $P = 0.508$). There was no difference in the overall duration of AP (median of 2 years in GpCO *vs* 1.5 years in GpNO, $P = 0.526$). AP more frequently woke up GpCO at night compared to GpNO (78.1% *vs* 57.3%, $P = 0.031$).

Nausea and vomiting were more frequent in GpCO in the past 3 mo (Table 6); 92.5% of GpCO had nausea interfering with their activities at least once a week or more frequently *vs* 76% amongst GpNO ($P = 0.021$).

Employment

The employment rate was lower in GpCO compared to GpNO (33.3% *vs* 54.2%, $P = 0.016$). The average number of working hours per week in GpCO (who were employed) was also lower than GpNO (median of 23 h *vs* 40 h respectively, $P = 0.005$).

It is plausible that a higher severity of abdominal pain in GpCO compared to GpNO was affecting employment. However, when we studied only those patients who had moderate to very severe abdominal pain as one of the symptoms from gastroparesis, GpCO still had a lower rate of employment (35.9% *vs* 54.5%, $P = 0.037$), and lower number of working hours per week (median of 20 h *vs* 40 h, $P = 0.003$), compared to GpNO. In GpCO on more than 60 mg morphine equivalents per day, there was a trend towards less working hours per week compared to GpCO on 60 mg or less morphine equivalents per day (median of 17.5 h *vs* 26 h, $P = 0.071$).

Health care utilization

There were higher number of hospital admissions for GpCO in the past year compared to GpNO (2.90 ± 0.77 *vs* 1.26 ± 0.23, $P = 0.047$) (Table 7). There was a trend towards a higher number of emergency room

Table 2 Gastroparesis patients: Demographics, employment, social history, laboratory tests and gastric emptying test in chronic opioid using gastroparesis patients, and patients with no opioid use

	Chronic scheduled opioids	No opioids	*P* value
Age (median in years with IQR)	49.0 (34.0-56.0)	[4]41.0 (30.0-55.0)	0.091
Age symptoms started (median in years with IQR)	40.0 (24.3-52.0)	[4]33 (22.8-47.3)	0.281
Average duration of symptoms (median in years)	[4]3.0 (1.0-15.5)	[4]4.0 (1.0-10.0)	0.937
Previously established diagnosis of Gp (%)	90.7% (39/43)	86.7% (137/158)	0.482
Female (%)	74.4% (32/43)	81.0% (128/158)	0.341
Body mass Index (median in kg/m^2 with IQR)	[4]24..3 (22.1-29.3)	[4]24.7 (20.3-30.8)	0.983
Race (% White)	83.3% (35/42)	83.9% (125/149)	0.917
Residing outside catchment area (50 miles)	53.5% (23/43)	51.6% (80/155)	0.828
Diabetes (%)	35.7% (15/42)	25.9% (41/158)	0.055
Surgery on stomach/esophagus (%)	28.6% (12/42)	19.1% (28/147)	0.136
Employed (%)	33.3% (14/42)	54.2% (84/155)	0.016
Working hours per week (median with IQR)	23.0 (10.5-35.0)	[4]40 (24.5-40.0)	0.005
Smoking history, current or past (%)	46.3% (19/41)	31.2% (48/154)	0.069
Current smoker (%)	31.7% (13/41)	13.0% (20/154)	0.004
Alcohol history, current or past (%)	23.8% (10/42)	37.4% (58/155)	0.100
Current alcohol use (%)	7.3% (3/41)	20.0% (30/150)	0.057
Random cortisol (% with low cortisol[1])	16.2% (6/37)	10.4% (11/106)	0.345
Hemoglobin A1c (median with IQR)	[4]5.9% (5.3%-7.7%)	[4]5.7% (5.4%-6.4%)	0.377
Thyroid stimulating hormone (% with high TSH[2])	4.9% (2/41)	1.7% (2/116)	0.271
Trypsinogen (% with low trypsinogen[3])	23.1% (6/26)	4.2% (3/72)	0.004
History of chronic pancreatitis	7.0% (3/43)	1.3% (2/158)	0.033
Gastric emptying scintigraphy: Retention at 2 h (median with IQR)	62% (50%-80%)	[4]66% (50%-72%)	0.359
Gastric emptying scintigraphy: Retention at 4 h (median with IQR)	[4]22% (14%-42%)	[4]24% (15%-35%)	0.522

[1]Low cortisol: AM less than 6.2 μg/dL (171 nmol/L), PM less than 2.3 μg/dL (63.4 μg/dL); [2]High TSH: Greater than 4.50 μIU/mL; [3]Low trypsinogen: Less than 19 ng/mL. [4]Results with non-normal distribution. Results expressed as median ± interquartile range, or percentage as appropriate. Gp: Gastroparesis; IQR: Interquartile range; TSH: Thyroid stimulating hormone.

Table 3 Comparison of gastric emptying scintigraphy results at different morphine equivalents per day in gastroparesis patients on chronic opioids using analysis of variance

Gastric emptying scintigraphy	1st quartile (≤ 22.5 mg of morphine equivalents per day)	2nd quartile (> 22.5 mg/d, and ≤ 60 mg/d)	3rd quartile (> 60 mg/d, and ≤ 112.5 mg/d)	4th quartile (> 112.5 mg/d)	*P* value
Retention at 2 h (mean ± SEM)	68% ± 6%	54% ± 8%	84% ± 5%	60% ± 9%	0.157
Retention at 4 h (mean ± SEM)	31% ± 7%	21% ± 8%	37% ± 10%	36% ± 19%	0.678

SEM: Standard error of mean.

visits in the past year in GpCO compared to GpNO (5.13 ± 1.46 *vs* 3.74 ± 0.65) but this did not reach statistical significance ($P = 0.468$).

DISCUSSION

Our study shows that chronic opioid use is present in nearly one fifth of the gastroparesis patients referred for evaluation and treatment of their gastroparesis. Use of chronic opioids in these gastroparesis patients was associated with a higher severity of many gastrointestinal symptoms, especially the symptoms often attributable to gastroparesis. In addition, patients on chronic opioids had decreased work productivity, and more frequent hospitalizations.

The prevalence of chronic scheduled opioid use in our study (19.2%) is less than 30%-46% reported in other studies[10,14,15]. It is plausible that many of our patients on opioids as needed were also taking opioids at least once a day, and including these patients gives a much higher prevalence of opioid use (29.1%). A significantly higher proportion of the Gp patients use opioids compared to the general adult population, which is estimated to be 3%[30].

Our study shows that Gp patients on chronic scheduled opioids have a higher severity of many upper gastrointestinal symptoms especially in those symptoms often attributable to gastroparesis. A higher frequency of upper gastrointestinal symptoms in patients taking opioids for chronic non-cancer pain (CNCP) has previously been reported[2]. Females have been shown to have a 60% greater chance of experiencing nausea and vomiting after receiving opioid therapy, which is noteworthy as majority of patients who suffer from Gp are females[12]. The female preponderance in our study is consistent with the previous literature that shows a higher prevalence of Gp and other functional gastrointestinal disorders in females[31]. The most common types of Gp in our population (idiopathic and diabetic) were also similar

Table 4 Symptom Severity as assessed with Patient Assessment of Upper Gastrointestinal Symptoms questionnaire; comparison between gastroparesis patients on chronic opioids and patients with no opioid use

GI symptom	GpCO (n = 43)	GpNO (n = 158)	P value
Nausea	4.09 ± 0.12	3.41 ± 0.12	0.011
Retching	2.86 ± 0.25	1.98 ± 0.14	0.003
Vomiting	2.93 ± 0.24	2.07 ± 0.15	0.011
Stomach fullness	3.84 ± 0.18	3.59 ± 0.11	0.254
Early satiety	4.17 ± 0.19	3.57 ± 0.12	0.004
Post prandial fullness	4.14 ± 0.18	3.63 ± 0.11	0.022
Loss of appetite	3.64 ± 0.21	3.04 ± 0.13	0.039
Bloating	3.67 ± 0.19	3.36 ± 0.13	0.396
Abdominal distension	2.95 ± 0.25	3.01 ± 0.14	0.753
Upper AP	3.86 ± 0.20	2.93 ± 0.13	0.001
Upper abdominal discomfort	3.74 ± 0.19	3.09 ± 0.13	0.031
Lower AP	2.67 ± 0.27	2.38 ± 0.13	0.315
Lower abdominal discomfort	2.79 ± 0.25	2.38 ± 0.13	0.130
Heartburn during day	2.55 ± 0.27	1.89 ± 0.13	0.032
Heartburn on lying down	2.76 ± 0.28	1.94 ± 0.14	0.008
Chest discomfort during day	2.42 ± 0.20	1.83 ± 0.12	0.018
Chest discomfort at night	2.40 ± 0.23	1.61 ± 0.13	0.003
Regurgitation or reflux during day	2.77 ± 0.25	2.18 ± 0.13	0.040
Regurgitation or reflux on lying down	2.64 ± 0.28	2.21 ± 0.14	0.120
Bitter/acid/sour taste	2.79 ± 0.27	2.11 ± 0.14	0.028
Constipation	2.92 ± 0.30	2.63 ± 0.14	0.296
Diarrhea	1.80 ± 0.30	1.79 ± 0.14	0.891
Total Symptom Severity Score	68.40 ± 2.82	56.63 ± 1.77	0.001

[1]Results with non-normal distribution. Results expressed as median with interquartile range, and percentage as appropriate. AP: Abdominal pain; BMs: Bowel movements; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

Table 5 Comparison of gastrointestinal symptoms between gastroparesis patients on chronic opioids and patients with no opioid use trial[10,11,27]

	GpCO	GpNO	P value
Episodes of vomiting in last 1 wk	13.0 (1.0-7.0)	11.0 (0.0-3.0)	0.002
Hours of nausea/day in last 1 wk	17.0 (3.0-18.0)	14 (1.5-12.0)	0.037
Total number of BMs in last 1 wk	14.0 (2.0-7.0)	4.0 (2.0-7.0)	0.714
AP one of the symptoms (%)	84.1% (37/43)	85.2% (132/155)	0.861
Duration of AP (yr)	12.0 (0.5-4.0)	[1]1.5 (0.7-4.5)	0.526
Location of most severe AP	Umbilical 41.7% (15/36)	Epigastric 44.2% (57/129)	0.863
AP wakes up at night (%)	78.1% (7/32)	57.3% (71/124)	0.031
AP worse with meals (%)	76.5% (26/34)	80.0% (100/125)	0.508

[1]Results with non-normal distribution. Results expressed as median with interquartile range, and percentage as appropriate. AP: Abdominal pain; BMs: Bowel movements; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

to previous studies on Gp[10,31].

In our study, the higher prevalence of opioid use in patients with diabetic gastroparesis compared to patients with idiopathic gastroparesis is possibly related to other co-morbidities, as both diabetic and idiopathic gastroparesis patients were equally likely to have abdominal pain as one of their symptoms of Gp, and there was no statistically significant difference in the severity of their abdominal pain or discomfort on PAGI-SYM questionnaire (results not shown). GpCO with diabetes more frequently reported using opioids for leg pain and/or neuropathy (33.3%) *vs* GpCO with idiopathic gastroparesis (21.7%), though the difference was not significant (P > 0.05).

The prevalence of abdominal pain in our study groups (> 80%) is towards the higher end of the reported prevalence of abdominal pain in Gp (42%-89%)[15]. Epigastrium was the most common location of the most severe AP, and majority of the patients with AP experienced post-prandial worsening of pain, similar to a previous study performed at our center on a different cohort of Gp patients[32]. Every 3 in 4 GpCO in our study woke up at night from AP, higher than about 1 in 2 of our GpNO patients and significantly higher than the reported 1 in 4 patients on opioids for CNCP reported by Tuteja *et al*[2]. It is possible that the abdominal pain may be the reason these patients were taking opioids. Alternatively, there could be enhanced pain perception in disabling narcotic bowel syndrome.

In this cohort, GpCO did not have increased severity of constipation, which is historically one of

Table 6 Frequency of symptoms in the past 3 mo using Rome IV questionnaire: comparison between gastroparesis patients with chronic scheduled opioid use and patients with no opioid use (percentage of patients with symptoms once a week or more often)

Symptom	GpCO	GpNO	*P* value
Post-prandial fullness interfering with activities	81.6% (31/38)	78.7% (118/150)	0.692
Unable to finish regular sized meal due to fullness	84.6% (33/39)	79.1% (117/148)	0.438
Epigastric pain/burning interfering with activities	75.0% (30/40)	66.9% (103/154)	0.324
Nausea interfering with activities	92.5% (37/40)	76.0% (117/154)	0.021
Vomiting	70.7% (29/41)	50.6% (78/154)	0.022
Bloating or stomach distension	72.5% (29/40)	68.8% (106/154)	0.653
Belching interfering with activities	62.5% (25/40)	51.3% (79/154)	0.206

GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

the most commonly reported symptoms of opioid induced bowel dysfunction[2]. Fentanyl was the second most commonly used opioid in GpCO, and a randomized cross over trial showed a lower prevalence of constipation in patients taking transdermal fentanyl compared to sustained release oral morphine[7]. Even though our questionnaire did not ask specifically about usage of stool softeners or laxatives, some of these patients had stool softeners (n = 4), stimulant laxatives (2) and osmotic laxative (1) listed on their medication list, and it is plausible that many other patients were on these over-the-counter medications as well, as these medications are often taken prophylactically in patients on chronic opioids[6,33]. Future studies can look into the prevalence of laxative use in opioid using gastroparesis patients. Our finding of higher severity of many gastrointestinal symptoms in GpCO compared to opioid-naïve Gp patients, despite no statistically significant difference in the 2 and 4 h retention in gastric emptying tests between the two groups is not novel, as Karamanolis et al showed that the symptom pattern in Gp is not determined by the severity of delay in gastric emptying[34].

Patients with Gp may have symptoms that mimic clinical manifestations of CP. In fact, Chowdhury *et al*[24] reported that 44% of small duct CP patients may have concomitant Gp. While the prevalence of CP in the general population is only 41.76 per 100000[35], the prevalence of CP in patients with Gp is not known. In our study, GpCO (7%) were more likely to have a history of CP compared to GpNO (1.3%). In addition, nearly one fourth (23.1%) of GpCO who had their trypsinogen levels checked had low levels, compared to < 5% in GpNO, suggesting some of our Gp patients using opioids chronically possibly had severe calcific CP with associated Gp, which might have been causing them abdominal pain. While the sensitivity of serum trypsinogen level in diagnosing CP was only 28%, the specificity was 100% in a study by Pezzilli *et al*[36].

Gp patients have been reported to have the longest length of hospital stay (5 d) amongst the functional gastrointestinal disorders[31]. Recent studies suggest increasing hospitalization due to Gp over the past 20 years[37-39]. A Nationwide Inpatient Sample Study reported 17220 admissions from Gp in 2012, a more than 400% increase compared to 1997[39]. Some authors suggest that the increasing hospitalization due to Gp comes from better recognition of this disorder[40]. Our study suggests a higher number of hospitalizations in GpCO than in Gp patients not taking opiates.

Our finding that GpCO are more likely to be unemployed and work less is consistent with the multi-national questionnaire based study showing that the chronic use of opiates negatively influences the quality of life[41]. We found a higher prevalence of current smoking in GpCO, and a study by Young-Wolff *et al*[42] suggested a higher likelihood of opioid use disorder in current smokers *vs* non-smokers.

Our study has some limitations. Whether opioid use is to manage a higher severity of Gp symptoms, or is responsible for the higher severity of symptoms is unclear as we do not have symptoms of patients prior to starting opioids. Increased opioid use in Gp patients with moderate to severe abdominal pain has previously been reported[15], however nausea, vomiting and retching are frequently reported side effects of opioids and it is plausible that the opioid use itself explains the higher severity of these symptoms amongst GpCO in our study. Secondly, these patients were generally referred from community settings and over half of these patients were referred from outside the catchment area of our tertiary care center, with GpCO and GpNO equally likely to be referred from outside the catchment area. This was a questionnaire based study. Some of the questions were not answered by all the patients, and some patients did not go for the laboratory tests that we requested. These missed questions and laboratory tests were excluded from analyses, however their number were relatively small in most cases, and likely did not to affect the results. Lastly, since this is a questionnaire based study, there is a potential of recall bias as well. This would likely apply both to patients taking and those not taking opioid analgesics.

In conclusion, chronic regular opioid use is present in a significant number (19.3%) of gastroparesis patients. These patients have a higher severity of many gastrointestinal symptoms including those of Gp. They have decreased work productivity compared to non-opioid using Gp patients. Whether opioid use is to

Table 7 Comparison of healthcare utilization between chronic opioid using gastroparesis patients and patients with no opioid use

	GpCO (n = 43)	GpNO (n = 158)	P value
ER visits in last 1 yr from Gp	5.13 ± 1.46	3.74 ± 0.65	0.468
Hospital admissions in last 1 yr from Gp	2.90 ± 0.77	1.26 ± 0.23	0.047

Results expressed as mean ± SE of mean. ER: Emergency room; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

treat a higher symptom severity from Gp, or opioid use itself worsens symptoms in patients with Gp requires further study.

ARTICLE HIGHLIGHTS

Research frontiers

Despite the gastrointestinal side effects associated with opioid use, they are used in some patients with gastroparesis. The relationship of opioid use to the gastrointestinal symptoms, healthcare utilization and employment is not known.

Research motivation

As opioid use had become a healthcare epidemic in United States, studies on opioid use in gastroparesis would be useful for clinicians and researchers.

Research objectives

This objective was to study the relationship of chronic scheduled opioid use to gastrointestinal symptoms, healthcare utilization and employment in gastroparesis patients.

Research methods

The authors used Mann Whitney *U* Test, Student's *t*-test, Analysis of Variance, and χ^2 test as appropriate for data analysis.

Research results

This study shows higher severity of many gastrointestinal symptoms, and more frequent hospitalizations in gastroparesis patients on chronic scheduled opioids, compared to gastroparesis patients not using opioids. Chronic opioid using patients also reported their work being effected more frequently by their gastrointestinal symptoms. The prevalence of chronic pancreatitis is also higher in opioid using gastroparesis patients.

Research conclusions

This study confirmed the hypothesis that chronic opioid use in gastroparesis is related with more severe gastrointestinal symptoms, and hospitalizations. Whether opioid use is to manage a higher severity of gastroparesis symptoms, or is responsible for the higher severity of symptoms is not clear as we did not have symptoms of patients prior to starting opioids. In clinical practice, this study implicates that the opioids may need to be used with caution in gastroparesis patients.

Research perspectives

Opioid use is quite prevalent in patients with gastroparesis. Opioid-using gastroparesis patients have more severe gastrointestinal symptoms. These opioid-using patients are more frequently hospitalized, compared to the patients without opioid use. They also more commonly report their employment being affected due to their gastrointestinal symptoms. Patients with gastroparesis may have chronic pancreatitis, possibly contributing to their gastrointestinal symptoms.

Future studies can look into the trends of laxative-use in opioid-using gastroparesis patients. The noticeable prevalence of chronic pancreatitis in gastroparesis patients in this study can be further confirmed in studies with larger sample size. This study found more frequent hospitalizations in gastroparesis patients; future studies to evaluate opioid use during hospitalizations in gastroparesis patients will add useful information to the current literature on gastroparesis.

Future research can look into opioid use in gastroparesis through different perspectives, this could be not only in the tertiary care centers, but also in smaller community settings so that the results more accurately reflect the generalized population. Moreover, bigger databases using diagnosis codes and medication-lists can be used to get a larger sample size.

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ORIGINAL ARTICLE

Observational Study

Medication beliefs predict medication adherence in ambulatory patients with decompensated cirrhosis

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Abstract

AIM

To investigate the impact of medication beliefs, illness perceptions and quality of life on medication adherence in people with decompensated cirrhosis.

METHODS

One hundred adults with decompensated cirrhosis completed a structured questionnaire when they attended for routine outpatient hepatology review. Measures of self-reported medication adherence (Morisky Medication Adherence Scale), beliefs surrounding medications (Beliefs about Medicines Questionnaire), perceptions of illness and medicines (Brief Illness Perception Questionnaire), and quality of life (Chronic Liver Disease Questionnaire) were examined. Clinical data were obtained *via* patient history and review of medical records. Least absolute shrinkage and selection operator and stepwise backwards regression techniques were used to construct the multivariable logistic regression model. Statistical significance was set at alpha = 0.05.

RESULTS

Medication adherence was "High" in 42% of participants, "Medium" in 37%, and "Low" in 21%. Compared to patients with "High" adherence, those with "Medium" or "Low" adherence were more likely to report difficulty affording their medications (P < 0.001), lower perception of treatment helpfulness (P = 0.003) and stronger medication concerns relative to medication necessity beliefs (P = 0.003). People with "Low" adherence also experienced greater symptom burden and poorer quality of life, including more frequent abdominal pain (P = 0.023), shortness of breath (P = 0.030), and emotional disturbances (P = 0.050). Multivariable analysis identified having stronger medication concerns relative to necessity beliefs (Necessity-Concerns Differential $\leqslant$ 5, OR = 3.66, 95%CI: 1.18-11.40) and more frequent shortness of breath (shortness of breath score $\leqslant$ 3, OR = 3.87, 95%CI: 1.22-12.25) as independent predictors of "Low" adherence.

CONCLUSION

The association between "Low" adherence and patients having strong concerns or doubting the necessity or helpfulness of their medications should be explored further given the clinical relevance.

Key words: Medication adherence; Medication beliefs; Illness perceptions; Quality of life; Liver cirrhosis

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Core tip: Medication non-adherence is common in people with decompensated cirrhosis but the impact that patients' medication beliefs and illness perceptions have on adherence is under-recognised. Clinician engagment with non-adherent patients should include open discussion of medications and liver disease. Acknowledgement of patient concerns surrounding their medicines, with positive reinforcement of medication necessity in terms of disease management may improve adherence behaviour and patients' quality of life.

Hayward KL, Valery PC, Martin JH, Karmakar A, Patel PJ, Horsfall LU, Tallis CJ, Stuart KA, Wright PL, Smith DD, Irvine KM, Powell EE, Cottrell WN. Medication beliefs predict medication adherence in ambulatory patients with decompensated cirrhosis. *World J Gastroenterol* 2017; 23(40): 7321-7331 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7321.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7321

INTRODUCTION

People with decompensated cirrhosis require intensive inpatient and outpatient management, experience poor quality of life (QoL), and have a median survival of approximately two years. While liver transplantation is a viable treatment for end-stage liver disease, this is not an option for many patients. A complex regimen of medications is usually prescribed to manage complications of portal hypertension and liver insufficiency, however medication mismanagement and non-adherence is relatively common among patients with decompensated cirrhosis.

Medication adherence is defined by the World Health Organisation (WHO) as "the extent to which a person's behaviour - taking medication, following a diet, and/or executing lifestyle changes - corresponds with agreed recommendations from a health care provider"[1]. Similar to other chronic diseases where approximately 50% of patients are thought to be non-adherent, up to 70% of patients with cirrhosis identify

as having "Low" or "Medium" levels of medication adherence[2].

Non-adherence with medications has been associated with increased mortality in diabetes, coronary heart disease and heart failure[3,4]. It has been estimated that between 22% and 37% of 30-d readmissions among patients with decompensated cirrhosis may be potentially preventable with improved management of pharmacotherapy[5,6]. For example, non-adherence with lactulose, a non-absorbable disaccharide syrup used in the treatment of hepatic encephalopathy (HE), is reported to be as high as 69%[7], and has been associated with approximately 36% of potentially preventable 30-d readmissions[6]. Intentional non-adherence due to adverse effects (diarrhoea, flatulence and abdominal pain)[7] or misunderstanding of the indication[6] carries a 3-fold risk of HE recurrence[8]; a substantial, potentially preventable burden on patients, carers and the healthcare system[9]. The one-year survival probability following an episode of overt HE is 42%[10], and persisting cognitive impairment or covert HE[11] may in turn lead to unintentional non-adherence with other medications.

Non-adherence and mismanagement of diuretic therapy, which is prescribed in the management of abdominal ascites, peripheral oedema or pleural complications, contributes to 55% of potentially preventable 30-d readmissions in people with decompensated cirrhosis[6]. While prevalence has not been reported in cirrhosis, 30%-66% of patients prescribed loop diuretics in the management of heart failure are non-adherent with therapy[12,13]. Incorrect use of diuretics in cirrhosis can lead to severe electrolyte disturbances and renal impairment, and may contribute to a requirement for recurrent large volume paracentesis. Bacterial infections in people with decompensated cirrhosis are common and often precipitate further deterioration. Spontaneous bacterial peritonitis (SBP) carries a mortality rate of 31.5% at one month and 66.2% at one year[14], with a one-year recurrence rate of 61% without prophylaxis[15]. Prophylaxis with antibiotic therapy considerably reduces SBP recurrence, development of hepatorenal syndrome, and improves probability of one year survival[15], however adherence with long-term antibiotics is known to be low in other patient groups[16,17].

The WHO has identified non-adherence as an international priority in the prevention of patient harm and optimisation of limited health resources[1]. However, medication adherence is a complex health behaviour that is the result of numerous interacting dynamic variables including health literacy, self-efficacy, psychological perceptions of medicines and disease, quality of life, and other internal and external barriers[1]. Previous studies in liver disease patients have identified that side effects and changes to routine are the most commonly cited reasons for non-adherence[7,18]. Illness perceptions have been shown to influence patients' confidence to self-manage alcoholic liver disease and limit self-efficacy[19], while self-perceived disease stigma can affect medical-care seeking behaviours and impact on QoL[20]. However there is limited information available about how perceptions and beliefs surrounding medications and liver disease affect adherence behaviours in people with decompensated cirrhosis. Identifying potentially-modifiable factors that influence adherence behaviour is important, as strategies to support adherence can be tailored to individual patients' needs.

The aim of this study was to: (1) investigate medication non-adherence in a cohort of ambulatory patients with decompensated cirrhosis; and (2) to identify the effect of patients' medication beliefs, illness perceptions, quality of life and clinical and demographic factors on medication non-adherence.

MATERIALS AND METHODS

A convenience sample of adult patients with cirrhosis who had experienced a decompensating event (abdominal ascites, spontaneous bacterial peritonitis, hepatic hydrothorax, encephalopathy or variceal bleeding) within the preceding two years were invited to participate when they attended routine outpatient follow-up at the Princess Alexandra Hospital in Brisbane, Australia, between February and October 2016. Patients were excluded if they were less than 18 years of age, unable to provide informed consent, undergoing transplant workup, or receiving intensive management by the palliative care team.

A structured questionnaire was used to obtain measures of medication adherence, quality of life, medication beliefs and illness perceptions. Questionnaires were completed independently by the patient or with the assistance of a carer, family member, or study coordinator, according to patient preference. Clinical data was collected from patients and/or their medical records, including standard biochemical and serological assays and liver imaging to confirm the diagnosis of cirrhosis and decompensation history. Socio-demographic items included patient-reported individual-based measures of education level and employment status, and residential area-based measures (Index for Relative Socioeconomic Disadvantage[21] and the Accessibility/Remoteness Index of Australia[22] for classification of remoteness of residence).

Medication adherence

Self-reported medication adherence was examined using the 8-Question Morisky Medication Adherence Scale (MMAS-8) which contains seven questions with yes/no alternatives, and one question which features a 5-point Likert scale. The scores from completed

questionnaires are categorised into "High" (score = 8), "Medium" (score 6 to < 8) and "Low" (score < 6) adherence groups[23-25].

Medication beliefs

The Beliefs about Medications Questionnaires (BMQ-General and BMQ-Specific) were used to elicit patients' beliefs about medications. Participants responded to eighteen statements across four domains using a 5-point Likert scale, from "strongly disagree" (score = 1) to "strongly agree" (score = 5). The BMQ-General contains 4 items that examine beliefs about Overuse of medicines by doctors, and 4 items about medication Harms. The BMQ-Specific contains 5 items in the Necessity domain and 5 items in the Concerns domain, which elicit patients' respective necessity and concern beliefs about the medicines prescribed for their liver disease. Scores derived from the Concerns domain can be subtracted from the Necessity domain to give a Necessity-Concerns Differential (N-C differential)[26].

Illness perceptions

The Brief Illness Perceptions Questionnaire (Brief-IPQ) was used to examine the strength of patients' perceptions about liver cirrhosis. The Brief-IPQ contains eight items, including identity (severity of symptoms) and consequences of disease on daily life, personal control and treatment control over disease, timeline for disease duration, self-perceived coherence or understanding of the disease, in addition to concerns and emotional representation that are caused by the disease. Patients self-measure each item on a scale from 0 to 10, where one end of the scale represents a benign perception and the other represents a threatening perception of illness[27].

Quality of life

The Chronic Liver Disease Questionnaire (CLDQ) was chosen to measure health related quality of life as it comprises specific domains relevant to the study population[28]. The full CLDQ contains 29 items across six domains; however responses to the twenty-ninth item have been excluded from analysis due to inapplicability to the majority of study participants. The twenty-ninth item pertains to worry about the availability of a liver if the patient requires a liver transplant; however patients being actively assessed for liver transplant were excluded from the study.

The shortened CLDQ used for the present study therefore contained eight questions related to Emotional Function, five questions within the domains of Systemic Symptoms and Fatigue, three questions within the domains of Abdominal Symptoms and daily Activity, and four items related to Worry. Individual item scores range from 1 to 7 and domain scores are calculated by averaging item scores within each domain. Higher scores represent better perceived health-related quality of life.

Data analysis: Statistical review of the study was performed by a biomedical statistician. Data was analysed using IBM® SPSS© Version 20.0. Where a question was missed within a validated tool, but at least 85% of the tool had otherwise been correctly completed, the missing value was imputed based on the mean or median response to that question, according to data skew. Imputation was required for a single value for two participants: one Brief IPQ-timeline (imputed median score 10) and one CLDQ-anxiety (imputed mean value 4).

Continuous and normally-distributed variables are presented as mean ± SD. Differences between groups were analysed by one-way ANOVA. Non-normally distributed data are presented as median (range) and have been analysed using the Kruskal-Wallis *H* test. Categorical data are presented as proportional percent and analysed using Pearson's chi-squared (χ^2), Fisher's Exact test or Linear-by-Linear Associated test for trend as denoted. Statistical significance was set at alpha = 0.05.

The relationship between "Low" medication adherence and patients' clinical, demographic and self-reported medication beliefs, illness perceptions and QoL were determined by calculating the odds ratio (OR) and 95%CI. Continuous variables were systematically assessed to identify optimal cut-points. Least absolute shrinkage and selection operator and stepwise backwards regression techniques were used to construct the final multivariable logistic regression model. Both methodologies identified the same significant factors for inclusion in the final model. The Hosmer-Lemeshow test was used to assess goodness-of-fit. Interactions between individual variables were not found to be statistically significant.

RESULTS

Demographic and clinical

One-hundred and thirty-four eligible patients were invited to participate in the study (Figure 1). Fourteen patients declined. Five patients indicated that they did not take medicines for their liver disease and thus did not complete essential components of the questionnaires. Of these five patients, three were unaware that their current pharmacotherapy included treatment for hepatic decompensation events, and two had ceased their therapy without medical advice. An additional fifteen patients incompletely filled out the questionnaires for other reasons.

Of the 100 patients who completed the questionnaire tools, the majority (65.0%) were male and the mean age was 58.4 ± 10.2 years. Primary disease aetiology was alcoholic liver disease (ALD) in 49 patients, hepatitis C (HCV) in 35, non-alcoholic fatty liver disease in 9, primary sclerosing cholangitis in two, hepatitis B in one, autoimmune hepatitis in one, drug-induced liver injury in one, alpha-1-antitrypsin

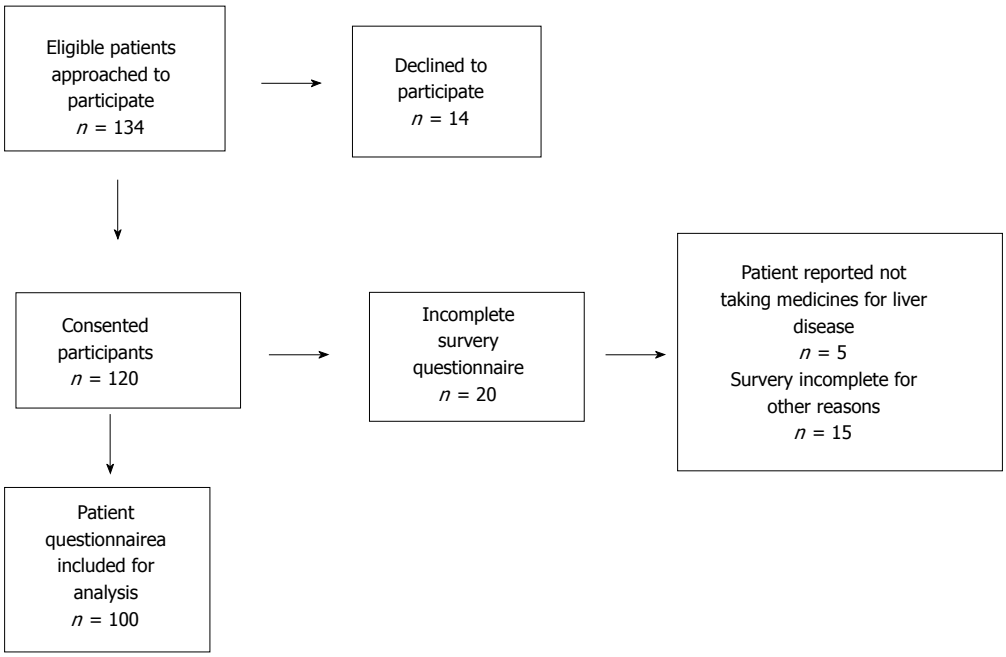


Figure 1 Participant recruitment flow diagram.

deficiency in one and unknown in one. Of the 51 patients who were not considered to have ALD, alcohol was a documented cofactor in 21 patients (41.2%).

Forty-two participants (42%) were categorised as having "High" medication adherence, 37% with "Medium" adherence, and 21% with "Low" adherence. Demographic and clinical characteristics of patients, and their association with medication adherence, are presented in Table 1. Male gender (P = 0.015) and inability to afford medications (P < 0.001) were associated with lower levels of medication adherence. Self-reported ability to afford medicines could not be predicted by employment status, relative sociodemographic disadvantage, or other sociodemographic factors (P > 0.05).

Medication beliefs

Compared to "Low" adherence, there was a non-significant increase in the strength of Necessity beliefs in "High" and "Medium" adherence groups, and a non-significant increase in the strength of Concerns, Harms and Overuse beliefs from "High" to "Low" adherence groups. Compared to patients with "Medium" and "High" medication adherence, patients with "Low" medication adherence reported a lower mean Necessity-Concerns Differential (P = 0.003; Table 2). Three patients had a negative Necessity-Concerns Differential and eight patients had a differential of zero, indicating that their Concerns about their liver disease medicines outweighed or were equal to the perceived Necessity of therapy respectively.

Patients were more likely to have a lower Necessity-Concerns Differential if they were male (6.6 ± 4.3 *vs* 8.5 ± 5.2, P = 0.046), reported inability to afford medications (4.8 ± 4.6 *vs* 7.8 ± 4.5, P = 0.011) or had fewer comorbidities (Pearson's r = 0.263, P = 0.008). Medication beliefs measured using the BMQ scales were not related to age, disease severity, education, sociodemographic status or other clinical and demographic variables.

Illness perceptions

Overall, participants had generally high levels of concern about their liver disease, felt they did not have much personal control over it, and perceived that it would persist for a long duration of time (Table 3). Patients with "Low" medication adherence reported lower perception of how much treatment could help their liver disease (treatment control, P = 0.003) and a lower self-perceived understanding of their liver disease (coherence, P = 0.014).

Patients with HCV reported experiencing more severe symptoms (identity score 6.5 ± 2.4 *vs* 4.9 ± 3.1, P = 0.009), greater impact of disease on daily life (consequences score 6.9 ± 2.4 *vs* 5.5 ± 3.3, P = 0.019) and perceived that their disease would persist for a shorter duration of time (timeline median score 8 [range 0-10] *vs* 10 [range 2-10], P = 0.010) compared to patients who did not have HCV. Female patients reported greater emotional impact of disease (emotional representation score 5.8 ± 3.3 *vs* 4.3 ± 3.4, P = 0.041) while patients with higher levels of education (completed high school, formal trade qualification, university degree *etc.*) perceived greater impact of disease on daily life (consequences score 6.7 ± 2.8 *vs* 5.3 ± 3.2, P = 0.024) compared to patients educated up to middle school. There was a negative correlation between the total Brief-IPQ and CLDQ scores (r = -0.707, P < 0.001), indicating that stronger threatening perceptions of illness are associated with

Table 1 Demographic and clinical factors in patients with high, medium and low medication adherence *n* (%)

Demographic and clinical variables		All patients	Medication adherence ranking			*P* value
			High (*n* = 42)	Medium (*n* = 37)	Low (*n* = 21)	
Age		58.4 ± 10.2	57.9 ± 10.9	58.2 ± 9.0	57.8 ± 10.8	0.787
Male gender		65 (65.0)	21 (50.0)	30 (81.1)	14 (66.7)	0.015
Primary aetiology	ALD	49 (48.0)	19 (45.2)	19 (51.4)	11 (52.4)	0.842
	HCV	35 (33.0)	14 (33.3)	13 (35.1)	8 (38.1)	0.963
	Other	16 (16.0)	9 (21.5)	5 (13.5)	2 (9.5)	0.383
[1]Child-Turcotte Pugh class	A	24 (24.0)	6 (14.3)	12 (32.4)	6 (28.6)	0.684
	B	59 (59.0)	29 (69.0)	21 (56.8)	9 (42.8)	
	C	17 (17.0)	7 (16.7)	4 (10.8)	6 (28.6)	
MELD score		14.4 ± 5.2	14.6 ± 4.6	14.2 ± 5.1	14.2 ± 6.7	0.936
Ascites at review (incl. suppressed by medication)		80 (80.0)	37 (88.1)	28 (75.7)	15 (71.4)	0.187
Encephalopathy at review (incl. suppressed by medication)		36 (36.0)	12 (28.6)	13 (35.1)	11 (52.4)	0.184
Hepatocellular carcinoma		8 (8.0)	3 (7.1)	3 (8.1)	2 (9.5)	1.00
Number of self-reported medicines		7.1 ± 3.5	7.2 ± 3.7	7.1 ± 3.6	6.9 ± 3.1	0.923
Number of comorbidities		5.5 ± 2.8	5.4 ± 2.8	5.8 ± 3.0	5.2 ± 2.5	0.703
[2]Unable to afford medicines		19 (20.2)	1 (2.5)	12 (36.4)	6 (28.6)	< 0.001
[3]Education	Nil, Primary, Middle school	39 (42.4)	14 (34.1)	13 (43.3)	12 (57.1)	0.215
	High school, Trade, University	53 (57.6)	27 (65.9)	17 (56.7)	9 (42.9)	
[4]Employment status	Employed	18 (19.1)	9 (22.0)	6 (18.2)	3 (14.3)	0.842
	Government welfare	72 (76.6)	30 (73.2)	25 (75.8)	18 (85.7)	0.602
ARIA	Highly accessible	89 (89.0)	36 (85.7)	34 (91.9)	19 (90.5)	0.713
	Accessible–remote	11 (11.0)	6 (14.3)	3 (8.1)	2 (9.5)	
IRSD	Most disadvantaged	32 (32.0)	16 (38.1)	7 (18.9)	9 (42.9)	0.093
	Low–moderate disadvantage	68 (68.0)	26 (68.9)	30 (81.1)	12 (57.1)	

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Categorical data presented as proportional percent (%) of column (adherence ranking) and analysed using Pearson's χ^2 or Fisher's Exact test unless otherwise noted. [1]Analysed using the Linear-by-Linear Exact Association test for trend; [2]Excluding 6 patients for whom data was not available (*n* = 40 High adherers, *n* = 33 Medium adherers and *n* = 21 Low adherers); [3]Excluding 8 patients for whom data was not available (*n* = 41 High adherers, *n* = 30 Medium adherers and *n* = 21 Low adherers); [4]Excluding 5 patients for whom data was not available (*n* = 41 High adherers, n = 33 Medium adherers and *n* = 21 Low adherers). *P*-values represent "Employed" *vs* "Unemployed" (data not shown), and "Government Welfare" *vs* "Not receiving Government Welfare" (data not shown) respectively. Two patients reported concurrent part-time employment and Government Welfare support and are represented twice. "Employed" includes full-time, part-time, casual and self-employment. "Government Welfare" includes disability support, aged pension, carer's pension, total permanent disability, Newstart allowance. Six patients reported no active income (living off savings or financially supported by family). ALD: Alcoholic liver disease; HCV: Hepatitis C virus ARIA: Accessibility/Remoteness Index of Australia; IRSD: Index for relative socioeconomic disadvantage.

Table 2 Responses to the beliefs about medications questionnaires in patients with high, medium and low medication adherence

Medication beliefs domains	All patients (*n* = 100)	Medication adherence ranking			*P* value
		High (*n* = 42)	Medium (*n* = 37)	Low (*n* = 21)	
Necessity	19.3 ± 3.8	19.6 ± 3.1	19.9 ± 4.1	17.6 ± 4.0	0.064
Concerns	12.0 ± 3.6	11.2 ± 3.3	12.2 ± 3.3	13.3 ± 4.2	0.074
Necessity-Concerns Differential	7.3 ± 4.7	8.4 ± 4.8	7.6 ± 4.2	4.3 ± 4.2	0.003
Harms	8.3 ± 2.5	7.9 ± 2.8	8.3 ± 2.0	8.8 ± 2.7	0.405
Overuse	10.3 ± 3.1	9.5 ± 3.0	10.7 ± 3.0	11.3 ± 2.9	0.053

Data presented as mean ± SD and analysed using one-way ANOVA. Higher scores indicate stronger medication beliefs within a given domain. Harms and Overuse scores range from 5 to 20. Necessity and Concerns scores range from 5 to 25. The Necessity-Concerns Differential (score range 0 to 20) is calculated by subtracting individual patients' Concern scores from their Necessity scores.

lower quality of life.

Quality of life

The greatest overall health-related QoL impacts reported by patients were in the domains of Fatigue and Worry. Impact of QoL on medication adherence is presented in Table 4. "Low" medication adherence was associated with lower QoL in terms of shortness of breath that impacted on daily activity (*P* = 0.030), greater emotional disturbances (*P* = 0.050), particularly irritability (*P* = 0.017) and mood swings (*P* = 0.031), and greater frequency of abdominal and bodily pain (*P* = 0.023 and *P* = 0.037, respectively). Patients with moderate or large ascites at the time of review reported greater impact of abdominal bloating (CLDQ-abdominal bloating score 3.47 ± 2.04 *vs* 4.69 ± 1.88, *P* = 0.014) and those with a history of HE reported more frequent irritability (CLDQ-irritability score 4.31 ± 1.57 *vs* 5.04 ± 1.72, *P* = 0.029), but these did not translate into an effect on medication

Table 3 Illness perceptions in patients with high, medium and low medication adherence

Brief illness perception questionnaire items	All patients (n = 100)	Medication adherence ranking			P value
		High (n = 42)	Medium (n = 37)	Low (n = 21)	
Consequences How much does your liver disease affect your life? 0 = No affect; 10 = Severely affects my life	6.0 ± 3.1	6.1 ± 3.0	6.0 ± 3.1	5.7 ± 3.2	0.846
Timeline How long do you think your liver disease will continue? 0 = Very short time; 10 = Forever	10.0 (0-10)	10 (3-10)	10 (0-10)	10 (3-10)	0.962
Personal Control How much control do you feel you have over your liver disease? 0 = Absolutely no control; 10 = Extreme amount of control	4.8 ± 3.0	4.6 ± 3.2	4.8 ± 2.9	5.0 ± 2.8	0.893
Treatment Control How much do you think your treatment can help your liver disease? 0 = Not at all; 10 = Extremely helpful	8.0 (0-10)	8 (2-10)	10 (4-10)	7 (0-10)	0.003
Identity How much do you experience symptoms from your liver disease? 0 = No symptoms; 10 = Many severe symptoms	5.5 ± 3.0	5.6 ± 3.0	5.5 ± 3.0	5.2 ± 2.9	0.905
Concern How concerned are you about your liver disease? 0 = Not at all; 10 = Extremely concerned	8.0 (0-10)	8 (0-10)	9 (0-10)	8 (0-10)	0.416
Coherence How well do you feel you understand your liver disease? 0 = Don't understand at all; 10 = Understand very clearly	8.0 (0-10)	8 (3-10)	8 (0-10)	7 (2-9)	0.014
Emotional Representation How much does your liver disease affect you emotionally? 0 = Not at all; 10 = Extremely affected emotionally	4.9 ± 3.4	4.7 ± 3.2	5.1 ± 3.9	4.7 ± 3.1	0.874

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Non-normally distributed data presented as median group score and (range) and analysed using the Kruskal-Wallis H Test.

adherence.

Patients who reported feeling hassled about sticking to their treatment plan (n = 10) and those that stated they did not take their medicines the preceding day (n = 9) reported lower overall QoL (average CLDQ score 2.96 ± 1.08 *vs* 4.30 ± 1.17, P = 0.001 and 2.97 ± 0.78 *vs* 4.28 ± 1.20, P = 0.002 respectively), especially within the domains of Activity, Emotion and Fatigue. Feeling hassled about sticking to the treatment plan was particularly associated with increased irritability (CLDQ-irritability score 3.50 ± 2.01 *vs* 4.81 ± 1.60, P = 0.019) and mood swings (CLDQ-mood swings score 3.33 ± 1.32 *vs* 4.81 ± 1.66, P = 0.003 respectively).

Factors associated with low medication adherence

Bivariate analysis indicated that patients with Necessity-Concerns Differential ⩽ 5, Brief Illness Perception Questionnaire "treatment control" score ⩽ 8 and "coherence" score ⩽ 8, or CLDQ score ⩽ 3 in the items of bodily pain, abdominal pain, shortness of breath or irritability, had higher odds of reporting "Low" medication adherence (Table 5). However, when included in the regression model having a Necessity-Concerns Differential ⩽ 5 (OR = 3.66, 95%CI: 1.18-11.40), Brief IPQ-coherence score ⩽ 8 (OR = 8.15, 95%CI: 0.98-67.78) or a CLDQ-shortness of breath score ⩽ 3 (OR = 3.87, 95%CI: 1.22-12.25) were the only independent predictors of "Low" medication adherence.

DISCUSSION

In our study, self-reported medication non-adherence in ambulatory patients with decompensated cirrhosis was prevalent, with over one-fifth of patients categorised with "Low" adherence and more than one-third categorised with "Medium" adherence. We have identified that lower levels of medication adherence in this group are associated with stronger patient Concerns about their medication relative to their belief in its Necessity, lower self-perceived understanding of liver disease, and lower QoL.

The relationship between medication beliefs and medication adherence behaviour has been explored in numerous chronic diseases, including asthma, cardiovascular disease and mental health disorders[29]. Perceptions of illness have also been shown to influence medication adherence in asthma, diabetes, hypertension and heart failure[30], though the impact of the different illness perception items on adherence appears to differ between diseases. In the present study, people with decompensated cirrhosis were more likely to have "Low" medication adherence if they had a lower Necessity-Concerns Differential, poorer self-perceived understanding of their hepatic disease (coherence), and had lower perceptions of the benefits of treatment for their hepatic disease (treatment control). It can therefore be inferred that decompensated cirrhosis patients who have a

Table 4 Health-related quality of life in patients with high, medium and low medication adherence

Quality of life domains	All patients (n = 100)	Medication adherence ranking			P value
		High (n = 42)	Medium (n = 37)	Low (n = 21)	
Abdominal symptoms	4.7 ± 1.5	4.6 ± 1.4	5.1 ± 1.4	4.2 ± 1.6	0.063
Abdominal bloating	4.5 ± 2.0	4.3 ± 2.0	4.9 ± 1.8	3.9 ± 2.1	0.109
Abdominal pain	4.9 ± 1.8	4.8 ± 1.7	5.5 ± 1.5	4.1 ± 2.2	0.023
Abdominal discomfort	4.4 ±1.8	4.7 ± 1.8	4.9 ± 1.7	4.6 ± 1.9	0.715
Activity	4.5 ± 1.5	4.6 ± 1.6	4.5 ± 1.3	4.1 ± 1.5	0.377
Not been able to eat as much as you would like	5.1 ± 1.9	5.0 ± 2.0	5.2 ± 1.6	5.0 ± 2.0	0.814
Trouble lifting or carrying heavy objects	3.4 ± 2.1	3.8 ± 2.3	3.3 ± 1.9	2.5 ± 1.9	0.067
Bothered by a limitation of your diet	5.0 ± 1.9	5.0 ± 1.9	5.0 ± 2.0	4.7 ± 2.1	0.824
Emotion	4.3 ± 1.5	4.7 ± 1.3	4.3 ± 1.7	3.7 ± 1.3	0.050
Anxiety	4.4 ± 1.9	4.8 ± 1.6	4.3 ± 2.1	3.8 ± 1.8	0.126
Unhappiness	4.5 ± 1.9	4.8 ± 1.9	4.6 ± 2.1	3.8 ± 1.3	0.125
Irritability	4.7 ± 1.7	5.0 ± 1.6	4.9 ± 1.8	3.8 ± 1.4	0.017
Difficulty sleeping at night	3.6 ± 2.2	4.0 ± 2.1	3.4 ± 2.3	3.1 ± 2.2	0.259
Mood swings	4.7 ± 1.9	5.1 ± 1.7	4.7 ± 2.1	3.8 ± 1.7	0.031
Unable to fall asleep at night	3.9 ± 2.3	4.1 ± 2.3	3.7 ± 2.3	3.7 ± 2.3	0.687
Felt depressed	4.6 ± 1.9	5.1 ± 1.8	4.5 ± 2.1	3.9 ± 1.6	0.058
Problems concentrating	4.3 ± 1.9	4.5 ± 2.0	4.5 ± 1.9	3.7 ± 1.9	0.232
Fatigue	3.2 ± 1.5	3.5 ± 1.6	3.3 ± 1.5	2.9 ± 1.3	0.340
Tired or fatigued	3.0 ± 1.6	3.0 ± 1.6	3.1 ± 1.7	2.7 ± 1.4	0.622
Sleepy during the day	3.2 ± 1.8	3.3 ± 1.9	3.5 ± 2.0	2.7 ± 1.2	0.255
Bothered by having decreased strength	3.6 ± 2.0	3.9 ± 2.0	3.4 ± 2.0	3.1 ± 2.0	0.281
Decreased level of energy	3.0 ± 1.8	3.2 ± 1.7	2.9 ± 1.8	2.6 ± 1.6	0.361
Drowsiness	3.9 ± 1.7	4.1 ± 1.8	3.8 ± 1.7	3.6 ± 1.6	0.466
Systemic symptoms	4.3 ± 1.3	4.3 ± 1.2	4.4 ± 1.4	4.0 ± 1.5	0.519
Bodily pain	4.2 ± 2.0	4.2 ± 1.8	4.7 ± 2.2	3.3 ± 2.0	0.037
Shortness of breath	4.5 ± 1.9	5.0 ± 1.8	4.4 ± 1.9	3.7 ± 1.8	0.030
Muscle cramps	4.2 ± 1.9	4.2 ± 2.0	4.1 ± 1.7	4.2 ± 2.2	0.957
Dry mouth	3.9 ± 2.0	4.0 ± 2.0	3.9 ± 2.0	3.7 ± 1.9	0.833
Itching	4.5 ± 2.1	4.2 ± 2.2	4.6 ± 2.0	5.0 ± 2.1	0.392
Worry	3.9 ± 1.8	4.1 ± 1.8	3.8 ± 2.0	3.6 ± 1.6	0.491
Worry about impact of liver disease has on family/friends	3.9 ± 2.1	4.0 ± 2.0	3.9 ± 2.2	3.7 ± 2.2	0.876
Worried that symptoms will develop into major problems	3.7 ± 2.1	4.0 ± 2.1	3.6 ± 2.2	3.1 ± 2.0	0.357
Worry about condition getting worse	3.9 ± 2.0	4.1 ± 2.0	3.7 ± 2.1	3.6 ± 2.0	0.569
Worry about never feeling any better	4.2 ± 2.2	4.5 ± 2.2	4.2 ± 2.3	3.8 ± 1.8	0.436

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Item scores range from 1 to 7 where a lower score indicates lower quality of life (*i.e.*, more frequent symptoms). Domain scores (range from 1 to 7) are calculated averages of items within the domain.

weaker belief in the necessity or helpfulness of their medications, and those who do not understand the consequences of cirrhosis, are less likely to perceive a need to take their medications and may therefore exhibit non-adherent behaviour.

Interestingly, perceptions of symptom frequency/severity (identity), concerns and consequences of disease on daily life were not associated with adherence behaviour. This was further confirmed using the CLDQ which identified that adherence was not influenced by the domains of Activity, Fatigue, Worry, Systemic or Abdominal Symptoms. This may be explained by changes to patients' priorities in the terminal stages of illness which may affect their decisions for self-care. For example, people with palliative diseases such as cancer and end-stage heart failure may exhibit non-adherent behaviour in an attempt to maintain control, reduce adverse events, or in response to social or financial circumstances[31-33]. Similar issues may affect people with decompensated cirrhosis, as this is a palliative condition for patients who are ineligible for transplantation. Most patients in the present study were aware of the incurable nature of their disease as evidenced by responses to the timeline item. The presence of hepatocellular carcinoma, considered to be an "imminently terminal" occurrence in people with decompensated cirrhosis who are ineligible for liver transplant, was not associated with adherence, which indicates that perceptions of palliation alone may not strongly influence adherence behaviour in this group.

Notably, people with HCV perceived that their liver disease would last for a shorter duration of time compared to people with other aetiologies of cirrhosis, possibly related to availability of new HCV direct-acting antiviral therapies. Unfortunately, this may be the result of false hope in this group as it is possible that many patients with HCV cirrhosis will have persisting complications despite a HCV "cure". People with HCV also perceived greater impact of symptom frequency/severity (identity) and consequences of disease on their daily lives compared to other aetiologies, but this was not associated with adherence behaviour. Where improved control over debilitating symptoms may be an incentive for better adherence for some

Table 5 Crude and multivariable predictors of low medication adherence in patients with decompensated cirrhosis

		Crude		[1]Multivariable		*P* value
		OR	95%CI	OR	95%CI	
Age ⩾ 60 yr		1.20	0.46-3.16	1.79	0.56-5.70	0.325
Gender, male		1.10	0.40-3.04	0.74	0.21-2.58	0.639
Unable to afford medicines		1.85	0.60-5.66	0.88	0.25-3.44	0.857
N-C Differential	⩽ 5	4.79	1.74-13.25	3.66	1.18-11.40	0.025
Overuse	⩾ 13	1.69	0.59-4.86	0.85	0.23-3.15	0.813
Brief IPQ- treatment control	⩽ 8	3.63	1.21-10.88	3.23	0.92-11.39	0.068
Brief IPQ- coherence	⩽ 8	13.62	1.74-106.62	8.15	0.98-67.78	0.052
CLDQ-bodily pain (3)	QoL score ⩽ 3	2.72	1.02-7.27	1.69	0.54-5.35	0.369
CLDQ-abdominal pain (5)	QoL score ⩽ 3	4.19	1.45-12.09	1.73	0.50-6.05	0.389
CLDQ-shortness of breath (6)	QoL score ⩽ 3	3.93	1.44-10.71	3.87	1.22-12.25	0.022
CLDQ-irritability (15)	QoL score ⩽ 3	3.12	1.08-9.04	1.70	0.47-6.11	0.416
CLDQ-mood swings (19)	QoL score ⩽ 3	2.09	0.75-5.82	0.93	0.24-3.58	0.917

[1]Odds ratio adjusted for N-C Differential ⩽ 5, Brief IPQ-treatment control score ⩽ 8, Brief IPQ-coherence score ⩽ 8 and CLDQ-shortness of breath score ⩽ 3 (indicative of experiencing the symptom "all the time", "most of the time" or "a good bit of the time") in the multivariable logistic regression model.

patients, others could perceive side effects of therapy to outweigh benefits. This may be evidenced by the increased likelihood for "Low" adherence in patients who had stronger Concerns (*i.e.,* side effects) relative to Necessity (*i.e.,* benefits) beliefs, and those with less understanding about cirrhosis (coherence) and the role of medicines in disease and symptom management (treatment control). Alternatively, studies in other chronic diseases with a heavy symptom burden like decompensated cirrhosis, such as asthma and heart failure, have suggested that many patients with good adherence report a more benign perception of illness identity, concerns and consequences[34,35]. This may be attributed to improved disease control or fewer episodes of exacerbation because of good adherence with treatment, and may partially explain the heterogeneity of responses to these Brief-IPQ items among people with decompensated cirrhosis.

A previous investigation by Polis *et al*[18] in cirrhosis patients has identified that medication adherence, defined as "never missing medications", was associated with having less abdominal symptoms and increased emotional well-being[18]. Similarly, we have identified that decompensated patients with "Low" medication adherence reported more frequent emotional disturbances, greater abdominal and bodily pain, and activity-limiting shortness of breath. Rather than a cause, these symptoms are postulated to be an effect of non-adherence. Conversely, irritability and mood swings were associated with patients reporting they felt "hassled about sticking to their treatment plan" and failure to take medications the day preceding the survey. Irritability and mood swings are thus postulated to impact on adherence in this group. While emotional disturbances are often associated with hepatic encephalopathy, irritability and mood swings were not necessarily more common in people with a history of HE, and these symptoms were not found to relate to non-adherence in patients prescribed lactulose. While the effect of adverse drug events experienced by individual patients on medication adherence was not specifically explored, the BMQ items designed to capture negative beliefs potentially related to adverse effects were not strongly related to nonadherence. Interestingly, disease severity measured using the Child-Turcotte Pugh classification was not related to QoL at either the domain or item level. This is contrary to other studies in cirrhosis patients[36,37], however by the nature of this study we aimed to recruit patients with a diverse decompensation history, which may explain the heterogeneity of responses to the CLDQ.

Strengths and limitations

The single study site, the General Hepatology Clinic at the Princess Alexandra Hospital, is one of the largest hepatology centres in Australia. The Clinic delivers ambulatory care to a substantial proportion of south-east Queensland hepatology patients, in addition to regional, remote and interstate patients who travel to access specialist services. Despite the broad representation of people with chronic liver diseases at our site, an element of selection bias may exist in the present study due to recruitment constraints. If two or more eligible patients were scheduled for hepatology review simultaneously (parallel hepatology clinics), patients who were taking more medicines for decompensation events were selectively approached. Thus, the patients included may represent the more severe end of the disease spectrum. Furthermore, participants could only be recruited if they were scheduled to attend clinic on a day the principal researcher (KH) was present and as such a convenience sample of patients was recruited.

As far as we are aware, this is the first study to use the BMQ and Brief-IPQ to measure beliefs and perceptions about medications and illness in people with decompensated cirrhosis. While these tools have not been previously validated in this cohort, our findings are comparable with studies in other chronic diseases. Non-equal questioning methodology (due

to patients' preference, need for carer/family member involvement, researcher assistance and feasibility) may have introduced an element of bias, however this was difficult to avoid given the study group of interest often requires assistance completing complex tasks and reflects a "real-world" clinic setting. To minimise potential bias, the study coordinator read survey questions aloud verbatim and did not actively seek additional information beyond what patients volunteered.

In conclusion, a large proportion of ambulatory patients with decompensated cirrhosis are non-adherent with prescribed medications. The association between "Low" medication adherence and patients having strong concerns or doubting the necessity of their medications should be explored further given the potential clinical relevance. Interventions that promote positive reinforcement of the value and necessity of medications in addition to education about disease and medication management tailored to individual patient needs may improve adherence.

COMMENTS

Background

Non-adherence and medication-mismanagement in people with decompensated cirrhosis have been associated with substantial potentially-preventable harm. The current study describes the relationship between patients' self-reported adherence and medication beliefs, illness perceptions and quality of life. The study was conducted in a cohort of patients receiving ambulatory care at a large tertiary government hospital.

Research frontiers

Existing data on medication-nonadherence behaviour in people with decompensated cirrhosis is largely qualitative. The present study intended to quantitatively measure these variables using validated tools, to correlate potentially-modifiable beliefs, perceptions and quality of life with medication adherence.

Innovations and breakthroughs

In this study, the authors have identified that patients with lower levels of medication adherence were more likely to have lower perception of treatment helpfulness, higher concerns relative to necessity beliefs surrounding their medicines, and poorer quality of life.

Applications

The authors findings have important implications for evolving clinical practice, as medication-management and disease education interventions can be developed to target these potentially-modifiable patient variables.

Terminology

Levels of adherence (High, Medium and Low) were assessed using a validated adherence tool, the Morisky Medication Adherence Scale-8 (MMAS-8). Patients' beliefs and perceptions towards their medicines were elicited using previously validated questionnaires (Beliefs about Medicines Questionnaire and Brief Illness Perception Questionnaire), which have been correlated with adherence behaviour. Quality of Life was measured using the Chronic Liver Disease Questionnaire.

Peer-review

This is a very interesting article dealing with the adherence to therapy by decompensated cirrhotic patients.

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CASE REPORT

Case of familial hyperlipoproteinemia type III hypertriglyceridemia induced acute pancreatitis: Role for outpatient apheresis maintenance therapy

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Author contributions: Abou Saleh M, Mansoor E and Cooper GS have all designed the case report, analyzed data, and written the manuscript.

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Abstract

Hypertriglyceridemic pancreatitis (HTGP) accounts for up to 10% of acute pancreatitis presentations in non-pregnant individuals and is the third most common cause of acute pancreatitis after alcohol and gallstones. There are a number of retrospective studies and case reports that have suggested a role for apheresis and insulin infusion in the acute inpatient setting. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis on the initial inpatient presentation followed by bi-monthly outpatient maintenance apheresis sessions for the prevention of recurrent HTGP. We also reviewed the literature for the different inpatient and outpatient management modalities of HTGP. Given that there are no guidelines or randomized clinical trials that evaluate the outpatient management of HTGP, this case report may provide insight into a possible role for outpatient apheresis maintenance therapy.

Key words: Apheresis; Pancreatitis; Plasmapheresis; Outpatient; Hypertriglyceridemia

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Core tip: There are a number of retrospective studies that have suggested a role for apheresis and insulin infusion in the acute management of hypertriglyceridemic pancreatitis (HTGP) but the post-discharge course and outpatient management of HTGP remain unclear. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis on the initial inpatient presentation followed by bi-monthly outpatient maintenance apheresis sessions for the prevention of recurrent HTGP. We also reviewed the literature for the different inpatient and outpatient management modalities of HTGP.

Abou Saleh M, Mansoor E, Cooper GS. Case of familial hyperlipoproteinemia type Ⅲ hypertriglyceridemia induced acute pancreatitis: Role for outpatient apheresis maintenance therapy. *World J Gastroenterol* 2017; 23(40): 7332-7336 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7332.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7332

INTRODUCTION

Hypertriglyceridemic pancreatitis (HTGP) accounts for up to 10% of acute pancreatitis presentations in non-pregnant individuals and is the third most common cause of acute pancreatitis after alcohol and gallstones[1,2]. Both genetic and secondary causes of lipoprotein metabolism have been implicated in HTGP. A serum triglyceride (TG) level of 10 g/L or greater is associated with acute pancreatitis. The risk of HTGP is approximately 5% with TG levels above 10 g/L and 10% to 20% with TG > 20 g/L[3]. The 2013 American College of Gastroenterology (ACG) guidelines in managing acute pancreatitis recommend obtaining a triglyceride level on all patients with acute pancreatitis without a known history of alcoholism or gallstones. While the ACG guidelines extensively explore the management of pancreatitis in general, there is a lack of data on the specific management of HTGP[4]. A number of retrospective studies and case reports in the past decade have suggested a role for insulin infusion with or without apheresis as an approach to rapidly lowering TG levels in an attempt to treat HTGP[1,5-18]. There are no randomized clinical trials evaluating the benefit of insulin infusion or apheresis in managing HTGP. Furthermore, there is a lack of characterization of post-discharge course and outpatient management of HTGP[19,20]. To our knowledge, there is only one case report of two patients with HTGP in 1996 who were managed with monthly plasmapheresis as maintenance therapy to prevent HTGP recurrence[21]. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis followed by bi-monthly maintenance apheresis sessions post-discharge as prevention of recurrent HTGP. This case report may provide insight into a possible role for outpatient apheresis maintenance therapy.

CASE REPORT

A 40-year-old Caucasian male with a past medical history significant for hyperlipoproteinemia type Ⅲ (on atorvastatin 80 mg daily, fenofibrate 200 mg daily and omega-3 polyunsaturated fatty acids), coronary artery disease status post 3-vessel coronary artery bypass graft, peripheral vascular disease, hypertension, diabetes mellitus (DM) type 2, and one reported episode of acute pancreatitis in the past presented with epigastric pain, nausea and decreased oral intake over 3 d. Physical exam was remarkable for tachycardia to 100 beats/min, localized epigastric tenderness, and xanthomas with striae palmaris. Labs were remarkable for mildly elevated lipase of 334 U/L (reference range 114-286 U/L) and TG levels of 45.3 g/L (reference range 0-149). Abdominal CT scan showed moderate fat stranding around the pancreas and stable pseudocyst. He was started on insulin drip at a rate of 1 unit/h. TG levels trended down after 3 d of insulin infusion to 9.57 g/L. The patient, however, continued to have severe abdominal pain and inability to tolerate oral intake. After 3 d of inpatient stay, patient received his first session of apheresis. After 24 h of apheresis, patient improved symptomatically and was able to tolerate oral intake. Triglyceride levels trended down to 4.61 g/L after 24 h of apheresis and were 6.75 g/L on the day of discharge.

Given that the patient had a complicated cardiac history and that this was his second HTGP presentation in one year, a decision was made to have the patient undergo maintenance apheresis sessions bi-monthly as an outpatient to prevent recurrent pancreatitis and cardiac complications. Patient was discharged on home cardiac medications which consisted of hydrochlorothiazide 12.5 mg daily, Plavix 75 mg daily, aspirin 325 mg daily, metoprolol tartrate 50 mg BID, atorvastatin 80 mg daily, fenofibrate 200 mg and omega-3 polyunsaturated fatty acids. Twelve mo post-discharge course was remarkable for a total of three admissions due to recurrent pancreatitis with one admission attributed to poor adherence to apheresis, fat-free diet and lipid lowering medications. His TG levels mostly remained otherwise successfully below 15 g/L. Repeat abdominal CT scans over 12 mo demonstrated resolution of previous pseudocyst and absence of local complications (Figure 1).

DISCUSSION

Patients with HTGP present with symptoms typical of

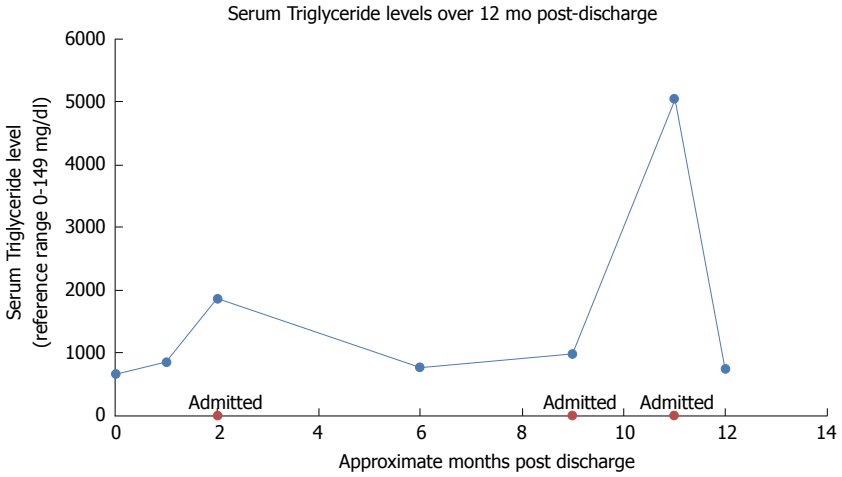


Figure 1 Serum triglyceride levels over 12 mo post-discharge. Hospital admissions are highlighted in red.

acute pancreatitis[1]. Specific features of HTGP that can help identify the etiology include xanthomas on extensor surfaces of arms and legs, lipemia retinalis and hepatosplenomegaly[22]. Lactescent serum is found in 45% of patients with mean Hypertriglyceridemia (HTG) level of 4537[2]. A serum TG level of 10 g/L or greater is associated with acute pancreatitis. The risk of HTGP is approximately 5% with TG levels above 10 g/L and 10% to 20% with TG > 20 g/L[23]. HTG by itself is not toxic to the pancreas, however, the breakdown of TG into free fatty acids (FFA) by pancreatic lipase causes lipotoxicity during acute pancreatitis, leading to a systemic inflammatory response[22]. Primary HTG that includes Frederick's phenotype Ⅰ-Ⅴ is associated with HTGP. Our patient was diagnosed with hyperlipoproteinemia type Ⅲ, an autosomal recessive trait, characterized by presence of Apo E2/E2[24]. Apo E ligand clears chylomicrons and Very low-density lipoprotein (VLDL) remnants from the circulation. Thus, this disorder leads to accumulation of VLDL and chylomicrons leading to HTG. Secondary HTG is caused by DM, alcoholism, hypothyroidism, pregnancy, and certain medications such as thiazides, beta blockers, corticosteroids, isotretinoin, immunosuppressants and antipsychotics[1].

A number of retrospective studies and case reports in the past decade suggested a role for insulin infusion with or without apheresis as an approach to rapidly lower TG levels in an attempt to treat HTGP[1,5-18]. There are no randomized clinical trials evaluating the benefit of insulin infusion, heparin or apheresis in managing HTGP to date. However, lowering of TG levels to below 5 g/L has been shown to advance clinical improvement in HTGP[25]. Apheresis is used to lower triglyceride level > 10 g/L and improve signs of severe inflammation such as hypocalcemia and lactic acidosis. One study reported (average TG of 14.06 g/L) a 41% decrease in TGs after one session of plasma exchange[18]. To our knowledge, there is only one case report of two patients with severe HTGP in 1996 who were managed with monthly plasmapheresis maintenance therapy[21]. Their 32-38 mo course was remarkable for only one episode of acute pancreatitis in one patient, suggesting a beneficial role for maintenance plasmapharesis[21].

With regard to anticoagulation during apheresis, studies have shown that citrate as opposed to heparin is associated with decreased mortality[18]. Intravenous insulin has been shown to be more effective than subcutaneous insulin in managing HTGP[26]. Insulin increases lipoprotein lipase which in turn accelerates chylomicron and VLDL metabolism to glycerol and FFA. It also inhibits lipase in adipocytes. Heparin is controversial in efficacy when used alone. It is thought to stimulate the release of endothelial lipoprotein lipase into the circulation, but the mechanism of lowering TG remains unclear[27].

Lipid lowering agents are indicated in the management of HTGP[25]. However, this is further complicated by the association between statin therapy and the development of acute pancreatitis. A series of case-control studies in Taiwan demonstrated that individuals using a statin therapy for the first time are more likely to develop an episode acute pancreatitis when compared to individuals who are not on statin therapy. These studies included Simvastatin (OR = 1.3, 95%CI: 1.02-1.73), Atrovastatin (OR = 1.67, 95%CI: 1.18-2.38), and Rosuvastatin (OR = 3.21, 95%CI: 1.70-6.06)[28-31]. Table 1 provides a summary of HTGP management strategies.

In summary, we presented a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis followed by outpatient bi-monthly maintenance apheresis sessions with a 12 mo post-discharge course remarkable for total of three admissions due to recurrent pancreatitis but with TG levels mostly remaining successfully below 15 g/L and repeat abdominal imaging demonstrating resolution of previous pseudocyst and absence of local

Table 1 Summary of hypertriglyceridemic pancreatitis management strategies

Management strategy	Description	Indication	Outcomes	Case report/Ref.
Diet restriction	Absolute restriction of fat intake	HTG, Primary prevention	Effective when combined with lipid lowering agents[15]	Tsuang *et al*[15], 2009
Lipid lowering agents	Fibrates (gemfibrozil 600 mg twice daily), niacin, N-3 fatty acids, statins	First line in HTG Adjuvant therapy in HTGP	Triglyceride level lowered about 60% by fibrates, about 50% by niacin, about 45% by omega-3 fatty acids[15]	Tsuang *et al*[15], 2009
Apheresis	Therapeutic Plasma Exchange which is removal of plasma and replacement with colloid solution (albumin, plasma). Citrate is used as an anticoagulant. Goal is TGH < 500	HTGP without contraindication to Apheresis such as inability to obtain central access or hemodynamic instability	Appears to be effective based on multiple case reports and case series. about 41% decrease in HTG levels. Apheresis within 48 h associated with better outcomes[16]	Furuya *et al*[16], 2002
Insulin	Intravenous regular insulin drip (0.1 to 0.3 units/kg/h). Goal is TGH < 500. Used alone or in combination with apheresis and/or heparin	Apheresis unavailable unable to tolerate apheresis hyperglycemia > 500	Intravenous insulin is more effective than subcutaneous[17] Effective in lowering triglyceride levels	Berger *et al*[17], 2001
Heparin	Combined with insulin. Subcutaneous heparin 500 units BID in 2 case reports	Controversial in HTGP	Controversial. Associated with increased mortality when compared to citrate (both combined with apheresis)[18].	Gubensek *et al*[18], 2014
Periodic apheresis	Described in 2 patients as monthly apheresis in 1996	Recurrence prevention especially in noncompliant patients	Reported success in one case report (2 patients in 1996)[21].	Piolot *et al*[21], 1996

HTGP: Hypertriglyceridemic pancreatitis; HTG: Hypertriglyceridemia; BID: Bis in die; TGH: Triacylglycerol hadrolase.

complications. This may suggest a beneficial role for outpatient apheresis as maintenance therapy in HTGP patients.

COMMENTS

Case characteristics

A 40-year-old Caucasian male with a past medical history significant for hyperlipoproteinemia type Ⅲ (on atorvastatin 80 mg daily, fenofibrate 200 mg daily and omega-3 polyunsaturated fatty acids), coronary artery disease status post 3-vessel coronary artery bypass graft, peripheral vascular disease, hypertension, diabetes mellitus type 2, and one reported episode of acute pancreatitis in the past presented with epigastric pain, nausea and decreased oral intake over 3 d.

Clinical diagnosis

Physical exam was remarkable for tachycardia to 100 beats/min, localized epigastric tenderness, and xanthomas with striae palmaris.

Differential diagnosis

Acute pancreatitis, gastritis, peptic ulcer disease, gastroenteritis, gastroesophageal reflux disease.

Laboratory diagnosis

Labs were remarkable for mildly elevated lipase of 334 U/L (reference range 114-286 U/L) and triglyceride levels of 45.3 g/L (reference range 0-149 g/L).

Imaging diagnosis

Abdominal CT scan showed moderate fat stranding around the pancreas and stable pseudocyst.

Treatment

Insulin, apheresis, atorvastatin 80 mg daily, fenofibrate 200 mg and omega-3 polyunsaturated fatty acids.

Related reports

There are a number of case reports in the literature that have demonstrated a benefit from apheresis and insulin therapy in the inpatient management of hypertriglyceridemic pancreatitis (HTGP). There is only one case report that have suggested a role for outpatient apheresis as a maintenance therapy.

Term explanation

HTGP is acute pancreatitis caused by high levels of serum triglycerides. Apheresis is the process of removal and separation of blood components and replacement with colloid solution (albumin, plasma).

Experiences and lessons

Multiple studies have demonstrated a role for apheresis in the acute management of HTGP. This case report suggests a role for outpatient apheresis in preventing recurrent attacks of HTGP.

Peer-review

This is a nice case report on a timely topic: hypertriglyceridemia-induced acute pancreatitis and role for outpatient apheresis maintenance therapy. This manuscript is generally of interest. The authors provided the complete review of this issue. The manuscript provides the updated evidence to the readers. It can be accepted for publication.

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CASE REPORT

Rescue case of low birth weight infant with acute hepatic failure

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Author contributions: Okada N contributed to the conception of the manuscript; Sanada Y, Urahashi T, Ihara Y, Yamada N, Hirata Y, Katano T, Ushijima K, Otomo S and Fujita S performed the treatment and collected data; Okada N drafted the manuscript; Mizuta K reviewed the manuscript; all authors read and approved the final manuscript.

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Abstract

We report a case involving a rescued low birth weight infant (LBWI) with acute liver failure. Case: The patient was 1594 g and $32^{3/7}$ gestational wk at birth. At the age of 11 d, she developed acute liver failure due to gestational alloimmune liver disease. Exchange transfusion and high-dose gamma globulin therapy were initiated, and body weight increased with enteral nutrition. Exchange transfusion was performed a total of 33 times prior to living donor liver transplantation (LDLT). Her liver dysfunction could not be treated by medications alone. At 55 d old and a body weight of 2946 g, she underwent LDLT using an S2 monosegment graft from her mother. Three years have passed with no reports of intellectual disability or liver dysfunction. LBWIs with acute liver failure may be rescued by LDLT

after body weight has increased to over 2500 g.

Key words: Liver transplantation; Acute liver failure; Low birth weight infant; Transplantable body weight; Monosegment graft

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Core tip: We report a case involving a rescued low birth weight infant (LBWI) with acute liver failure. The patient was 1594 g at birth. At the age of 11 d, she developed acute liver failure due to gestational alloimmune liver disease. Medications were initiated, and body weight increased with enteral nutrition. Her liver dysfunction could not be treated by medications alone. At 55 d old with a body weight of 2946 g, she underwent living-donor liver transplantation (LDLT) using an S2 monosegment graft. Conclusion: LBWIs with acute liver failure may be rescued by LDLT after body weight has increased to over 2500 g.

Okada N, Sanada Y, Urahashi T, Ihara Y, Yamada N, Hirata Y, Katano T, Ushijima K, Otomo S, Fujita S, Mizuta K. Rescue case of low birth weight infant with acute hepatic failure. *World J Gastroenterol* 2017; 23(40): 7337-7342 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7337.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7337

INTRODUCTION

Neonatal acute hepatic failure is a rare but serious disease[1]. Reports have indicated that the cause of neonatal acute hepatic failure is most frequently gestational alloimmune liver disease (GALD), but in rare cases, it can be metabolic disorder, viral infection, or mitochondrial disorder, among other possibilities[2]. The initial treatment for neonatal acute liver failure is apheresis and medication while the cause of hepatic failure is determined. If hepatic recovery has not been achieved with medication alone, liver transplantation is indicated[1,3]. However, the living donor liver transplantation (LDLT) procedure for neonatal recipients is challenging due to the size mismatch between the liver graft and the body of the recipient. Moreover, management during the perioperative period is also challenging[4]. LDLT is particularly difficult for low birth weight infants (LBWIs) for the reasons discussed above. In such cases, LDLT can result in recovery if the body weight of the infant can be increased by nutritional management[5]. However, few reports have discussed LDLT for neonatal recipients with low body weights[6]. Herein, we report a case involving a rescued LBWI with acute liver failure and discuss the limitation of body weight as an indicator for neonatal LDLT.

CASE REPORT

The patient was born at $32^{3/7}$ gestational wk because of fetal distress. Her birth weight was 1594 g (Figures 1 and 2). Immediately after birth, hypoglycemia and hypotension appeared. A daily administration of hydrocortisone and continuous administration of dopamine hydrochloride were started at the age of 1 day. At the age of 11 d, coagulation dysfunction and an elevated serum ferritin level due to acute liver failure were observed (ferritin 2865 ng/mL, total bilirubin 12.5 mg/dL, PT-INR 4.57). The patient's older sister was highly suspected of GALD by clinical course and pathological findings and underwent LDLT at 13 d of age. GALD was highly suspected in this case after excluding the possibility of metabolic disorder or infectious disease. The patient did not undergo magnetic resonance imaging or salivary grand biopsy for GALD diagnosis. We explained the high recurrence rate of GALD in siblings to her parents; however, the parents gave birth to a baby in another hospital without informing us. Starting at 11 d of age, exchange transfusion and medication therapy using high-dose gamma globulin and deferoxamine were initiated. The patient's body weight had been gradually increased with enteral nutrition using commercially available nutrients, administered 8 times per day *via* a gastric tube. She then recovered from hypotension, and the continuous administration of dopamine hydrochloride was finished. Exchange transfusion was performed a total of 33 times prior to LDLT. The patient was transported to our hospital at 44 d of age; at that time, her body weight was 2525 g. We speculated that her target body weight for LDLT would be over 2500 g based on the estimated graft volume. Computed tomography scan revealed a markedly atrophied liver (resected liver was 78 g). Laboratory data showed repeated coagulopathy and hyperbilirubinemia with daily exchange transfusion and a pediatric end-stage liver disease (PELD) score of 15.8 (T-bil 5.09 mg/dL, PT-INR 1.91, Albumin 3.4 g/dL). A liver biopsy revealed a marked loss of hepatocytes. The remaining hepatocytes were multinucleated, and there was widespread fibrosis around Glisson's sheath and in the parenchymal area (F3-4) (Figure 2F and G). Thus, her liver dysfunction could not be successfully treated *via* medications alone, and she was judged to be indicated for liver transplantation at that time. We tried to perform LDLT at 45 d of age, but before LDLT, she went into shock following hemothorax due to failure of catheter insertion with injury of the right subcla*via*n artery. After the recovery period, she underwent LDLT at 55 d of age using an S2 monosegment graft from her mother (107 g, graft-recipient weight ratio (GRWR) 3.6%; Figure 2 A). The body weight of the patient at LDLT was 2946 g. At the time of LDLT, the PELD score of the patient was 21.9 (T-bil 13.74 mg/dL, PT-INR

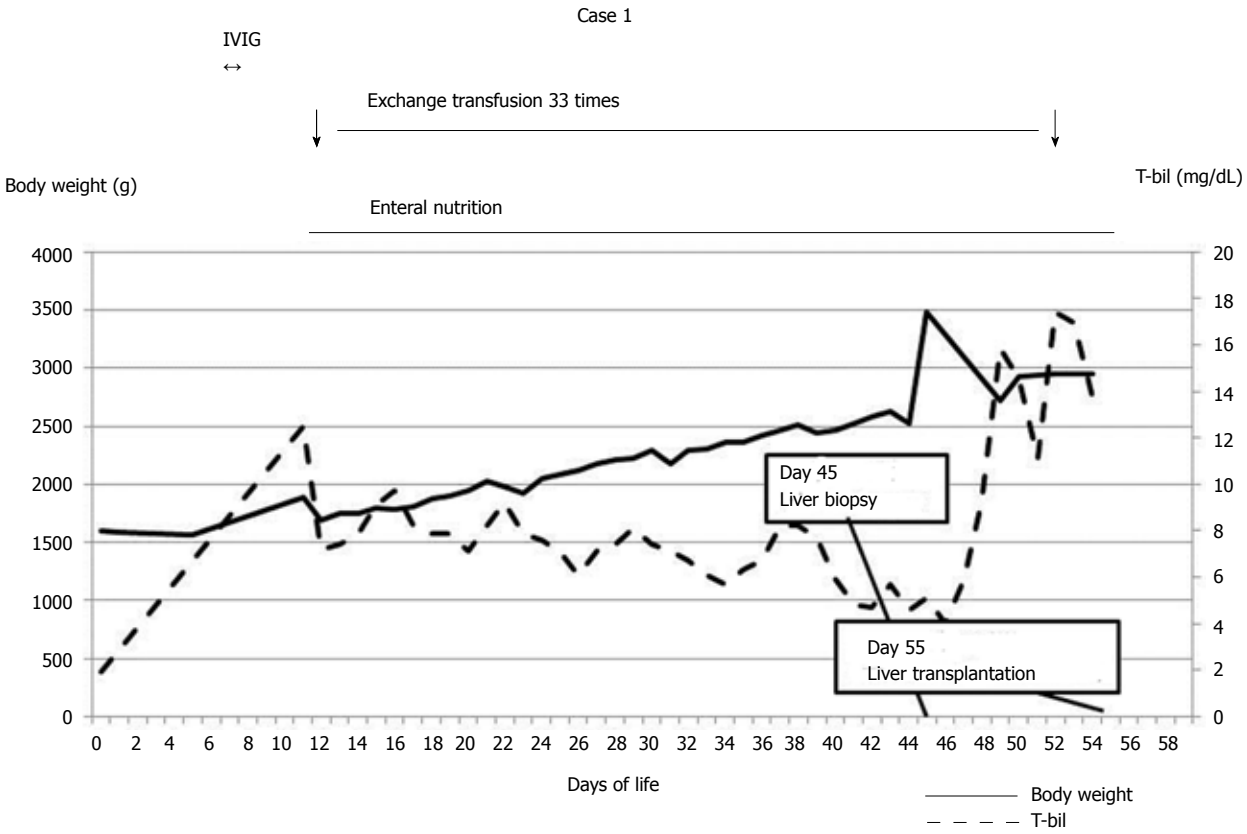


Figure 1 The preoperative treatment and changes in body weight and total bilirubin. The patient was able to gain weight due to the use of internal medication and enteral nutrition.

2.12, Albumin 3.7 g/dL). The operation duration was 13 h and 37 min and bleeding was 700 mL [238 mL/recipient body weight (kg)]. A transverse incision was created, and the liver was resected with temporary bypass of the portal vein. The resected liver was 78 g (Figure 2E). The recipient's right hepatic artery (2.0 mm) was anastomosed to the graft's left hepatic artery (2.5 mm) *via* a dorsal position of the portal vein anastomosis using a microsurgical technique. The graft-to-recipient distance ratio (GRDR) was 2.4 (58/24). Biliary reconstruction was performed using a Roux-en-Y hepaticojejunostomy. The abdominal wound could not be closed because respiratory failure occurred due to abdominal compartment syndrome (Figure2B and C). Intraoperative water balance was +1645 mL [558 mL/recipient body weight (kg)]. After LDLT, continuous hemodiafiltration (CHDF) had been performed for systemic edema, removing water as long as blood pressure and portal vein flow remained steady. Respiratory failure due to abdominal compartment syndrome and lung edema gradually improved. Thus, on postoperative day (POD) 5, the abdominal skin of the patient was closed without closing the abdominal fascia (Figure 2D). Tacrolimus and methylprednisolone were used as the standard postoperative immunosuppression therapy regimen. Acute rejection occurred on POD 17, so steroid pulse therapy was initiated. After steroid pulse therapy, liver enzyme, PT-INR and T-bil were nearly normalized. Respiratory failure due to large-for-size graft syndrome had been prolonged; however, it gradually improved, and she was extubated on POD 81. Cytomegalovirus infection and catheter infection occurred several times and were treated using antivirus or antibiotic drugs. The patient showed difficulty eating sufficient meals and required habilitation to eating. She was discharged on POD 225. Vessel complications did not occur. Three years have passed since LDLT, with no reports of intellectual disability or liver dysfunction.

DISCUSSION

Liver transplantation for a small recipient

We rescued a case involving an LBWI with acute liver failure; however, few reports to date describe such a case (Table 1). To our knowledge, the lightest LDLT recipient had a body weight of 2.4 kg at the time of LDLT[7], and the youngest LDLT recipient in Japan was 9 d of age[8-10]. For deceased donor liver transplantation, the smallest reported recipient was 2 kg, and the youngest was 7 d of age[11]. No consensus has been reached regarding the safe lower limit of body weight for small recipients. A recent UNOS database analysis of infants weighting less than 5 kg revealed one-year patient and graft survival rates of 77.7% and 66.1%, respectively[12]. A recent Japanese

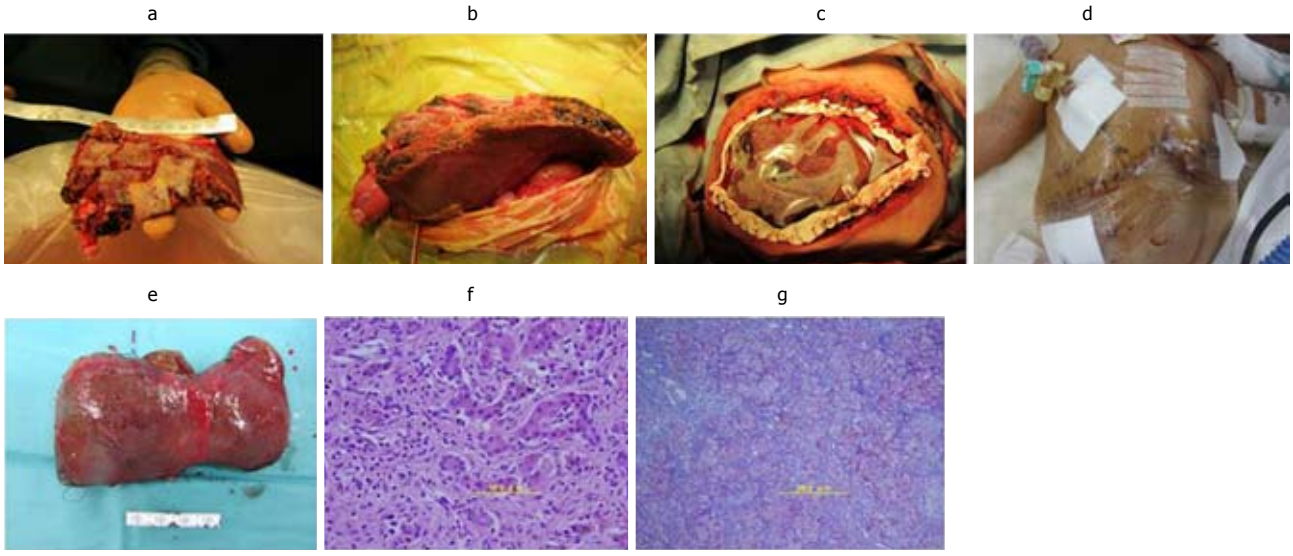


Figure 2 Images. A: The S2 monosegment graft (107 g) on the back table; B: An image obtained after reperfusion, the graft was too large to close the abdominal fascia; C: The abdominal fascia could not be closed at the time of living donor liver transplantation (LDLT), excess water was removed by continuous hemodiafiltration after LDLT; D: Secondary skin closure was performed on postoperative day 5; E: The resected liver was 78 g; F: Hematoxylin and eosin staining revealed a marked lack of hepatocytes and the presence of multinucleated hepatocytes; G: Azan staining revealed widespread fibrosis around Glisson's sheath and the parenchymal area (F3-4).

Table 1 Problems and management for low birth weight infant with acute liver failure

	Problems	Management
Pre-LT	Low body weight	Enteral nutrition targeting to over 2500 g
	Liver failure	Apheresis (exchange transfusion, plasmapheresis)
	Donor	Informed consent
LT	Large-for-size graft syndrome	Monosegment graft
	Hepatic artery reconstruction	Brunch patch, dorsal approach
	Abdominal compartment syndrome	Skin closure
		Open management→secondary skin closure
Post-LT	Fluid overload	Aggressive water removal using CHDF
	Respiratory failure	

CHDF: Continuous hemodiafiltration.

study reported an improved survival rate of 90.1% in an infant recipient within 3 mo[7]. Recipients who were within 3 mo of age also showed a higher rate of biliary complications[7]. However, our case did not have biliary complications. The problems we experienced in the described case were related to the following: (1) large-for-size graft syndrome[4]; (2) the transplantable body weight of the recipient; and (3) vessel reconstruction difficulties[13]. Challenges in managing transplantation for LBWIs with acute liver failure are nearly always due to the low body weight and fragility of the recipient.

The lower limit of the recipient's body weight for living donor liver transplantation

In liver transplantation, the transplantable body weight of a neonatal recipient is limited by graft size. If the GRWR is greater than 4.0%, the risk of abdominal compartment syndrome and insufficient blood supply in relation to a large-for-size graft are increased; the recipient abdomen cannot be closed, or massive hepatocyte necrosis may occur in the transplanted graft[8,10,14,15]. In pediatric LDLT, particularly in cases for which the recipient's body weight is less than 6 kg, monosegment grafts or hyper-reduced left lateral grafts have been transplanted[4]. A total of 268 pediatric patients underwent LDLT 275 times between May 2001 and December 2015 at Jichi Medical University Hospital. These transplantation procedures involved 196 left lateral segment grafts, with a median graft weight of 230 g (range, 138-382 g). Monosegment grafts were transplanted in 13 cases (including 9, 3, and 1 cases involving S2, reduced S2, and S3 monosegment grafts, respectively); in these cases, the median graft weight was 124 g (range, 93-180 g). A hyper-reduced left lateral segment graft was transplanted in 1 case, and the graft volume was 172 g. The smallest graft that we have transplanted was 93 g, and the median GRWR was 3.6% (2.4%-4.2%). A previous study reported hyper-reduced left lateral segment graft weights of 72-189 g,

with most graft weights being 100-150 g[7]. The lower limit of the smallest graft volume from a living donor is approximately 100 g[10]. Given this graft weight, transplantable recipient body weights of 2500 g or more will result in a GRWR of 4.0% or less. Based on this estimation, 2500 g is a reasonable body weight for such recipients.

The specific peritransplant management of living donor liver transplantation for low birth weight infant

In our experience, the body weights of the described patient were increased by enteral nutrition and became greater than 2500 g at 44 d of age. Another case of increased body weight by enteral nutrition was reported, and the body weight increased to greater than 2500 g at 41 d of age[5]. Despite the existence of acute liver failure, the body weights of the patients increased to over 2500 g *via* enteral nutrition while exchange transfusions were performed. Thus, in cases of acute liver failure, body weight may be increased with enteral nutrition if appropriate medication therapy that includes apheresis, such as exchange transfusion or plasmapheresis, is performed.

At times, it is impossible to close the abdominal fascia of the recipient during LDLT due to abdominal compartment syndrome. In this case, the abdominal fascia could not be closed during LDLT; instead, the case required secondary skin closure. In cases involving pediatric LDLT patients, apheresis and dialysis are reportedly effective[16]. Aggressive water removal using CHDF was required in this case. Thus, even if LDLT for an LBWI with acute liver failure is successfully performed after an increase in body weight, circulation and respiratory management are challenging due to volume overload. Under these circumstances, aggressive water removal using CHDF and secondary abdominal closure approaches are useful and important tools for successful postoperative management.

Hepatic arterial reconstruction during living donor liver transplantation

Vessel reconstruction in cases similar to the described case is difficult due to the high GRDR between the hepatic vein and the portal vein bifurcation and to diameter mismatch for the hepatic artery between the graft and the recipient[13]. Hepatic artery reconstruction by the dorsal approach of portal vein anastomosis has been reported to help reduce the risk of hepatic artery complications among small recipients if the GRDR is over 2.4[13]. At times, the hepatic arteries of such recipients are thin. The hepatic artery of this case was not thin; however, the GRDR was 2.4, and the hepatic artery was anastomosed by a dorsal approach. In our experience of 13 cases of LDLT using monosegment grafts, the thinnest artery among the recipients was 1.2 mm, with 3 cases involving hepatic arteries of less than 1.5 mm. The graft-to-recipient diameter ratio was 0.67: 1.67. In addition, the dorsal position was selected for hepatic artery anastomotic approaches in 7 cases (53.8%), and the branch patch technique was selected in 5 cases (38.5%). Hepatic artery complications after LDLT occurred in 3 cases, although interventional radiology was sufficient for achieving recovery in all 3 cases.

We rescued a case of LBWI with acute liver failure by performing LDLT. In cases involving LBWIs with acute liver failure, infants may be rescued by LDLT after their body weights have been increased to over 2500 g *via* repeated exchange transfusions and enteral nutrition.

COMMENTS

Case characteristics

The patient was 1594 g, $32^{3/7}$ gestational wk at birth and immediately after birth, hypoglycemia and hypotension appeared.

Clinical diagnosis

At the age of 11 d, coagulation dysfunction and an elevated serum ferritin level due to acute liver failure were observed.

Differential diagnosis

The case was highly suspected of gestational alloimmune liver disease and differential diagnosis was metabolic disorder, infectious disease and Neimann-Pick disease type C.

Laboratory diagnosis

Laboratory data showed repeated coagulopathy and hyperbilirubinemia with daily exchange transfusion and a pediatric end-stage liver disease score of 15.8.

Imaging diagnosis

Computed tomography scan revealed a markedly atrophied liver.

Pathological diagnosis

A liver biopsy revealed a marked loss of hepatocytes and the remaining hepatocytes were multinucleated, and there was widespread fibrosis around Glisson's sheath and in the parenchymal area (F3-4).

Treatment

The case underwent living donor liver transplantation at 55 d of age using an S2 monosegment graft from her mother (107 g).

Related reports

Kasahara M *et al* and Mizuta K *et al* reported the living donor liver transplantation for small recipient (*Exp Clin Transplant* 2014; 12 Suppl 1:1-4, *Am J Transplant* 2010; 10: 2547-2552).

Term explanation

Low birth weight infant (LBWI) is defined as a birth weight of a infant of 2499 g or less regarding of gestational age.

Experiences and lessons

In cases involving LBWI with acute liver failure, infants may be rescued by living donor liver transplantation after their body weights have been increased to over 2500 g.

Peer-review

The authors presented an interesting case with LBWIs (1594g, 323/7 wk) with acute liver failure.

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LETTERS TO THE EDITOR

S-Adenosyl-L-methionine towards hepatitis C virus expression: Need to consider *S*-Adenosyl-L-methionine's chemistry, physiology and pharmacokinetics

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Abstract

S-Adenosyl-L-methionine (SAM) is a cofactor serving as a methyl donor in numerous enzymatic reactions. It has been reported that SAM has the potential to modify antioxidant-enzymes, glutathione-biosynthesis and methionine adenosyltransferases-1/2 in hepatitis C virus -expressing cells at millimolar concentrations. The efficacy of SAM at micromolar concentrations and the underlying mechanisms remain to be demonstrated.

Key words: *S*-Adenosyl-L-methionine; Bioavailability; Concentration; Liver

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Core tip: *S*-Adenosyl-L-methionine (SAM) serves as a cofactor for enzymes that transfer its methyl group to nucleophilic functionalities of various biomolecules including DNA and RNA. Exogenous SAM has been shown to be a useful pharmacological agent in liver-associated diseases. SAM is a labile species, undergoes spontaneous decomposition in biological samples, and its oral bioavailability is only about 2%. Lozano-Sepulveda and colleagues observed that SAM modulates antioxidant enzymes, restores glutathione synthesis, and switches MAT1/MAT2 turnover in hepatitis C virus (HCV) expressing cells. The authors suggested that this may be a likely mechanism by which HCV expression is diminished by SAM. This SAM concentration range was chosen on the basis of cell viability experiments and is up to 1000 times higher than physiological intracellular. Other groups have used SAM in the concentration range 0 - 1000 nmol/L. The efficacy of SAM, its pharmacological effects towards

HCV and possibly adverse effects beyond cell viability need to be elaborated in further studies using SAM concentrations much lower than 1 mmol/L.

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TO THE EDITOR

S-Adenosyl-L-methionine (SAM) is the common cofactor of methylating enzymes, the methyl transferases. These enzymes catalyze the transfer of the methyl group of SAM to various nucleophilic functionalities of low-molecular-mass and high-molecular-mass biomolecules. Catechol amines, DNA, RNA, and proteins are well-investigated substrates of methyl transferases. SAM deficiency is associated with many different pathogenic conditions including liver diseases, depression and inherited methylation disorders. SAM supplementation in such diseases is a therapeutic means[1-5]. Lozano-Sepulveda and colleagues recently reported in the *World Journal of Gastroenterology* that SAM decreased hepatitis C virus (HCV) -RNA levels by 50% to 70% and induced a synergistic antiviral effect with standard IFN treatment[6]. The authors found that SAM modulated several antioxidant enzymes (*e.g.,* superoxide dismutase-1 and -2, thioredoxin), restored glutathione (GSH) synthesis, and switched methionine adenosyltransferase (MAT) turnover in HCV-expressing cells. The study by Lozano-Sepulveda and colleagues adds to the pleiotropic effects of SAM. However, this study by Lozano-Sepulveda and colleagues suffers from a major limitation, namely the use of very high SAM concentrations (range, 1 - 5 mmol/L)[6]. The choice of this SAM concentration range appears arbitrary. Another study limitation is that no SAM concentration/dose-response experiments have been performed.

SAM is a physiological substance and is widely distributed in extracellular and intracellular compartments of the human body[7-11]. The concentration of SAM in plasma of healthy subjects is of the order of 150 nmol/L, seemingly independent of the concentration of total homocysteine[7]. The intracellular SAM concentration in human lymphocytes has been reported to be about 5 nmol/10^6 cells; in mouse liver the SAM content was determined to be 0.5 nmol/mg protein[7]. The latter values are close to those reported by others using different analytical methods[12]. In freshly isolated human erythrocytes the concentration of SAM is of the order of 4 μmol/L[13]. This value agrees with more recently reported median SAM concentrations in erythrocytes of diabetic (3.8 μmol/L) and non-diabetic (3.5 μmol/L) male and female subjects[14].

The pharmacokinetics of SAM has been frequently investigated in animals as well as in healthy and diseased humans[15-17]. The oral bioavailability of SAM is of the order of 1% - 4%. Ingestion of 1000 mg SAM as tosylate disulfate salt resulted in maximum plasma SAM concentrations of about 2.5 μmol/L in men and women[3]. Intravenous injection of 1000 mg SAM resulted in maximum plasma SAM concentrations of about 211 μmol/L[15]. Another study found that oral administration of 10 mg SAM/kg body weight did not result in significant increases in systemic SAM concentration[16]. Thus, the SAM concentration range used in the Lozano-Sepulveda's study[6] is almost 1000-fold higher than physiological and pharmacologically used SAM concentrations (0-1000 nmol/L), and even 5 - 25 times higher than plasma SAM concentrations from intravenously injected SAM.

Use of very high SAM concentrations in *in vitro* experiments, even if not toxic[6], may lead to entirely different or contradictory results than the use of physiological and pharmacological SAM concentrations[18]. Oral administration of radiolabeled SAM (*i.e.,* [*methyl*-^{14}C]SAM) in mice resulted in radioactivity accumulation in the liver due to authentic [*methyl*-^{14}C]SAM and [*methyl*-^{14}C]phosphatidylcholine. The latter was found to be about 8 (after 60 min) and 25 (after 240 min) times higher concentrated than [*methyl*-^{14}C]SAM[16]. In aqueous solution, SAM is unstable and decomposes spontaneously to its components including *S*-methylthioadenosine, adenosine, adenine, and homoserine lactone[19]. Above pH 6, SAM is chemically very labile. Its inherent reactivity towards nucleophilic functionalities of biomolecules such as DNA and proteins is about 1000 times higher than that of methylated folates[19]. These observations suggest that SAM does not only function as an universal cofactor in methyltransferases-catalyzed reactions, but also undergoes both spontaneous methylation reactions with various biomolecules and decomposition to species such as *S*-methylthioadenosine and homoserine lactone[19]. Possibly, SAM decomposes to additional substances with not yet known biological activities, albeit not necessarily acutely cell toxic. The decrease in total glutathione concentration in the HCV-expressing cells upon incubation with SAM at 1 mmol/L for 1 and 2 h seen by Lozano-Sepulveda *et al*[6] may be an indication of a (spontaneous) reaction of SAM with reduced glutathione (GSH) to form *S*-methyl-glutathione which cannot be detected by the Ellman's method. At least in rat kidney proximal tubules, *S*-methyl-glutathione is rapidly degraded by gamma-glutamyl-transpeptidase[20]. Measurement of oxidized glutathione, *i.e.,* glutathione disulfide (GSSG), is a much more suitable and direct approach to assess oxidative stress. Yet, no GSSG data

were reported in the paper[6]. It is worth mentioning that SAM (at 4 mmol/L) can also inhibit thioredoxin-mediated protein disulfide reductase activity[20]. This and further reports[22] are supportive of the chemical lability of SAM that makes it a spontaneous unselective methylating agent. Spontaneous decomposition of SAM considerably contributes to *S*-adenosyl-homocysteine which is a potent inhibitor of methyltransferases including protein arginine methyltransferases[23].

Lozano-Sepulveda and colleagues reported in their article interesting results and proposed possible mechanisms for the explanation of the effects exerted by SAM in HCV-expressing cells seen in their study[6]. Yet, the SAM concentrations used in the study are difficult to be reached within cells even by intravenous injection of SAM salts. The high chemical reactivity of the *S*-methyl group of SAM towards biomolecules and its spontaneous decomposition is likely to bear potential adverse effects. The efficacy and the safety of SAM, especially its pharmacological effects towards HCV, need to be elaborated in further studies taken into consideration the pharmacokinetics of SAM. Use of SAM at mmol/L-concentrations may raise unrealizable expectations.

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