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Hepatic encephalopathy: Ever closer to its big bang

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Abstract

Hepatic encephalopathy (HE) is a neuropsychiatric

disorder that commonly complicates the course of patients with liver disease. Despite the fact that the syndrome was probably first recognized hundreds of years ago, the exact pathogenesis still remains unclear. Minimal hepatic encephalopathy (MHE) is the earliest form of HE and is estimated to affect more than 75% of patients with liver cirrhosis. It is characterized by cognitive impairment predominantly attention, reactivity and integrative function with very subtle clinical manifestations. The development of MHE is associated with worsen in driving skills, daily activities and the increase of overall mortality. Skeletal muscle has the ability to shift from ammonia producer to ammonia detoxifying organ. Due to its large size, becomes the main ammonia detoxifying organ in case of chronic liver failure and muscular glutamine-synthase becomes important due to the failing liver and brain metabolic activity. Gut is the major glutamine consumer and ammonia producer organ in the body. Hepatocellular dysfunction due to liver disease, results in an impaired clearance of ammonium and in its inter-organ trafficking. Intestinal bacteria, can also represent an extra source of ammonia production and in cirrhosis, small intestinal bacterial overgrowth and symbiosis can be observed. In the study of HE, to get close to MHE is to get closer to its big bang; and from here, to travel less transited roads such as skeletal muscle and intestine, is to go even closer. The aim of this editorial is to expose this road for further and deeper work.

Key words: Ammonia; Skeletal muscle; Minimal hepatic encephalopathy; Hyperammonemia

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Core tip: This work is a contribution to the current knowledge of hepatic encephalopathy (HE) and shows new important data about aspect less studied, as ammonia effect inducing morphological ultrastructural damage in skeletal muscle and gut. These alterations were observed in a Minimal HE model without liver damage, which suggests that the damage caused by

ammonia may occur before liver failure.

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INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric complex syndrome, ranging from subtle behavioral abnormalities to deep coma and death^[1]. HE is potentially reversible and emerges as the major complication of acute or chronic liver failure (ALF, CLF)^[2]. RF Butterworth in his recent review^[3] stressed that brain edema that accompanies ALF is the primarily cytotoxic phenomena, and moreover, this correlates with alterations in expression of genes coding for key astrocytic proteins^[4].

There are clinical conditions with a greater chance of developing cerebral edema, such as deep encephalopathy, hyperacute (ALF-acetaminophen induced) liver failure, severe hyperammonemia, younger age and infection^[5]. A controversial area is to establish conclusively whether the percentage of deaths could be attributable to a cerebral edema or to a multiple organ failure. Clemmensen reported in patients with ALF a correlation between arterial ammonemia and brain herniation. Therefore, hyperammonemia in ALF is a key factor for the understanding the pathophysiology of ALF^[3].

On the other hand, situation changes drastically in CLF. In the first diagnostic stage, patients studied with conventional clinical assessments could be considered normal from a neurological view, but its evaluation with neuro-psychometric tests, flicker, *etc.*, could possibly staged as a subclinical HE, or "minimal hepatic encephalopathy" (MHE)^[6,7].

MHE may be persistent or intermittent (spontaneous or precipitated for different circumstances), but the data indicate that between two clinical episodes, patients do not return to their previous neurological status^[8]. It becomes necessary to understand HE pathophysiology in early stages, in its big bang, to establish the mechanism that triggers HE and subsequent neurological consequences.

As seen in Figure 1, many paths, molecules, and biological unbalanced equations are involved^[1]. The first reference of a patient with chronic liver disease, most likely associated with HE, was documented by G. Morgagni in 1765. Ammonia stands on the scene of HE from the very beginning, and is considered the main culprit. However, no one could give a judgment without risk of injustice. Thus in CLF, the defendant, who is supposed to be under trial in this editorial is

ammonia^[9]. Although, there are others implicated in HE pathophysiology, such as edema and manganese, it is not possible, to explain HE without the implication of hyperammonemia.

This publication is an approach to MHE, providing in brief some new data that are usually not taken into account, in an attempt to reveal the initial steps of the HE pathophysiology. Also it is suggested that research should be strongly directed to MHE, in order to seek for still unknown mechanisms.

The diagnosis of MHE is still a challenge, and some experimental models, such as the portal vein ligation (PVL), develops two basic characteristics of CLF in its early stages, mild hyperammonemia and MHE^[10], thus being a useful tool for the study of MHE pathophysiology and how it finally develops HE without liver disease^[11].

Despite the difficulties, it is very important the early diagnose of MHE, because the experimental models of MHE are teaching that there are significant pathological changes, which should be take into account at this stage. We will not discuss here the recognized features such as Alzheimer type II Astrocytes, an important morphological feature of HE^[12]. This work will take into account some of the roads less traveled about MHE, skeletal muscle and intestine, which is the major consumer of glutamine (GLN) and the major ammonia producer^[13]. Skeletal muscle becomes the main mass of tissue able to metabolize ammonia when liver failure is established and its capacity of ammonia detoxification is reduced drastically. In this way, perhaps we could reconsider that MHE is not so minimal.

SKELETAL MUSCLE AND MHE

Sarcopenia is a common feature of cirrhosis, and the loss of muscle mass and function contributes to its morbidity and mortality. Its prevalence in patients with cirrhosis is estimated to be 40%-70%, well correlated with HE^[14,15].

The skeletal muscle damage in cirrhosis is underestimated by physicians although its prevalence is higher than other gastrointestinal complications. Perhaps the poorly understanding of its pathogenesis, the not so well established diagnostic criteria and that none of the proposed treatment options have been well explored in randomized clinical trials, helps to this clinical conduct.

Patients with chronic liver disease (CLD) concomitantly decrease the capability of detoxification of ammonia to urea, shifts ammonia metabolic pathway to the muscular system, to the astrocyte in the central nervous system (CNS) and to the kidney. In the case of liver failure, the first two tissues turn into main organs in detoxifying ammonia. Skeletal muscle tissue, due to its large size, becomes the main ammonia detoxifying organ^[16]. In hypoproteic diet which is a very common procedure in treating patients with liver failure, muscular

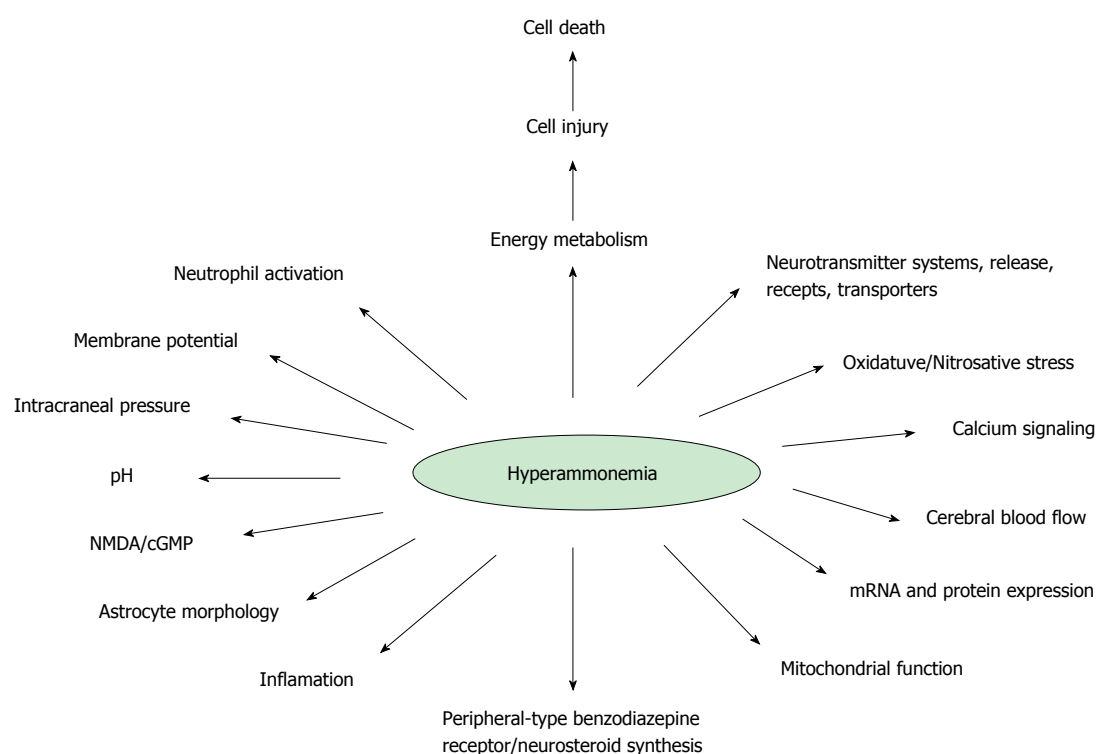


Figure 1 Hyperammonemia consequences (Modified from Bosoi 2009).

glutamine-synthase becomes an important enzyme in this metabolism, although a normal proteic diet may be metabolically more adequate and can be safely administered to the cirrhotic patient^[17]. But hypoproteic diet may decrease the muscular mass and therefore the ammonia detoxifying ability, therefore could be a controversial procedure in patients with liver disease and established hyperammonemia and MHE. On the other side, recently, diet supplementation with branched chain amino acids shown to decrease MHE and to increase muscle mass^[18]. This should be balanced because the astrocytic enzyme glutamate synthetase (GS) works at the limit of its possibilities to metabolize ammonia.

Sarcopenia becomes in a very useful tool and in the Model for End-Stage Liver Disease (MELD) should include this new item as a way to assess the nutritional and functional status of cirrhotic patients. Montano-Loza *et al.*^[19] used for skeletal muscle evaluation computed tomography and they conclude that sarcopenia is associated with improved prediction of mortality in patients with cirrhosis, primarily in patients with low MELD scores. In hospitalized cirrhotic patients there is a correlation between protein malnutrition and sepsis^[20].

Montano-Loza *et al.*^[19] sustain that the muscle mass and malnutrition is not taken into account in MELD and Child-Pugh scores. So, there is no possibilities to reflect these important features in prognosis of mortality parameters, risks associated with low muscle mass.

So it could be regarded that sarcopenia and cirrhosis has a very related pathogenesis so that simple dietary

interventions are insufficient. Efforts should focus on improved understanding of the multiple mechanisms triggered in chronic liver disease, the overlap of the different pathogenesis involved, to arrive to the development of more effective therapies. Sarcopenia is present in almost one third of patients with HCC^[21], and constitutes a strong and independent risk factor for mortality. Our results highlight the importance of body composition assessment in clinical practice.

Myostatin is a potent autocrine growth inhibitor produced by myocytes that inhibits skeletal muscle growth and reduces muscle mass in cirrhosis. Qiu *et al.*^[22] showed that exposure of mouse skeletal muscle myotubes in culture to ammonium acetate caused a time and concentration-dependent increase in myostatin mRNA and protein expression. They found that hyperammonemia-activated transcription factor p65 NF- κ B who binds to the myostatin promoter with transcriptional up regulation. They also found that myotube diameter was significantly greater in the NF- κ B knockdown cells compared with the control cells, further supporting their proposal that NF- κ B regulates myostatin expression during hyperammonemia. Their observations show that hyperammonemia induces myostatin expression in myotubes *via* an NF- κ B-dependent pathway^[16,22,23].

In our laboratory some new data was registered. PVL rats showed skeletal muscle structural/ultrastructural and functional changes (unpublished data). It has been suggested that ammonia uptake is increased in CLD and that the subsequent increase in GS capacity is a major

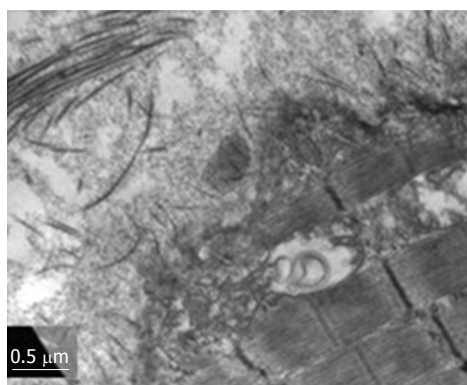


Figure 2 Portal vein ligation rat SM. MET, $\times 15000$, showing bands like Z with reinforced shortened and with ruptures. Vacuoles are enlarged and merged. Triad with pathological changes; mitochondrial damage, including loss of matrix density, loss of crests, swollen, fusion and breaking of the membrane in the cytosol and myofibrillogenesis. Collagen is seen in the upper right corner (unpublished data).

alternative pathway for ammonia detoxification^[24]. Desjardins *et al.*^[25] demonstrated in a porto-cava anastomosis model that GS activity was significantly increased as a result of a post-translational modification of the enzyme.

Our results in both, basic biochemical parameters in liver and skeletal muscle conventional microscopy observation, showed no differences when compared with the control group.

Despite these disappointing results, the fact is that the loss of skeletal muscle is nearly universal in cirrhosis and that adversely affects survival, inducing the development of other complications, and that negatively affects outcome after liver transplantation, decreasing quality of life. However, a reduction in skeletal muscle protein synthesis alone is not sufficient to account for continued reduction in muscle mass in cirrhosis, and an increase in proteolysis is necessary. On the other side cirrhosis is a state of accelerated starvation and the enhanced muscle metabolism may serve as a source of essential amino acids for critical cellular function.

When does skeletal muscle damage start?

There is not a complete answer, but to date the loss of skeletal muscle is associated with advanced stages cirrhotic.

The high-resolution optical microscopy (HROM) and transmission electron microscopy in the MHE showed surprising results. The skeletal muscle Triad showed in the dark cell, extensive convergent structure resembling the streets on a map. These membrane systems increased by altering the fibrillar surface structure generating a serious focal damage (Figure 2). Immunohistochemistry (PCNA, TUNEL, GS), ROS and mitochondrial respiration measure, supports the HROM and MET findings.

These results disaggregate the fact that it may not be absolutely necessary a damaged liver, perhaps

hyperammonemia alone could trigger the damage in skeletal muscle, somehow independent of liver damage (Figure 2).

GUT AND MHE

Is gut involved in MHE pathophysiology?

Gut is the major GLN consumer and ammonia producer organ in the body. Ammonia generation derives from the consumption of GLN and glutamate as the most important oxidative fuel for enterocytes and colonocytes^[26]. In Gut epithelial cells, these aminoacids have several roles, *i.e.*, represents substrates for another amino acids synthesis^[27], oxidative fuel^[28], nucleotide and nucleic acid synthesis^[29], glutathione production^[30], amino sugars, NAD⁺, N-acetylglucosamine and N-acetylgalactosamine synthesis, *etc.*^[31]. GLN administration in patients with different diseases, improved gut function and reduce bacterial translocation, reestablishing normal epithelial permeability^[32,33].

Hepatocellular dysfunction due to liver disease, results in an impaired clearance of ammonium and in its inter-organ trafficking^[34,35]. Besides acute or chronic liver failure, specific genetic disorders are also characterized by hyperammonemia accompanied by liver and brain disorders with different degrees of severity. Intestinal bacteria, also can represent and extra source of ammonia production. The most relevant bacteria are those with urease enzyme such as enterobacteriaceae. In cirrhosis, small intestinal bacterial overgrowth (SIBO)^[36] and dysbiosis^[37,38] can be observed. This modification in gut microbiota is produced for various circumstances. First, gut motility is reduced, and acid gastric secretion in stomach is reduced as a consequence of gastric enteropathy, both phenomena influence SIBO^[39]. Moreover, an impairment in bile acids production and secretion by the damaged liver was demonstrated^[37]. The amount and the profile modification of bile acids are due a reduction in secondary fecal bile acids, a bacteriostatic compound. Another factor involve on SIBO development in cirrhosis, is the alteration in local immunological system, mediated by a low cellular and humoral components of gut immune system^[39]. SIBO generates an increase in ammonia gut production, generating an active role of gut in the predisposition in HE development.

In addition of ammonia generation, luminal bacteria can generate other substances such as phenols, mercaptans, benzodiazepine-like compounds and short and medium chain fatty acids, also implicated in pathogenesis of HE^[40] exerting a synergic effect on CNS alteration, by modulating the synaptic processes. For example, the neurosteroid allopregnenolone is elevated in patients with HE. This steroid can enhance the effects of GABA on its specific receptors (mainly in GABA-A), increasing it neurodepressive function^[41]. On the other hand, endogenous benzodiazepines produced

by intestinal bacteria, can augment the opening time of GABA-A receptor after its interaction. The levels of this benzodiazepine like compounds are also elevated in blood of HE patients, exerting a potentiation effect with neurosteroids named before^[42-44].

When gut was evaluated in the PVL distal ileum showed impaired contractile response to acetylcholine and potassium chloride, and reduced expression of proteins markers of occlusive and adherents junctions, such as Zonulin 1 and B catenine, respectively. Also, a reduction in number of cells related with mucosal immune system was observed and altered L-Citrulline was recorded (data not published). All these findings suggest an active role of gut in the development of MHE in a PVL model.

CONCLUSION

This review tries to approach the early stages of the MHE, where even though there are no clinical manifestations, there is evidence of morphological ultrastructural alterations that are present even in the absence of hepatic failure. From this it can be hypothesized that it might not be necessary to have a clinically assessed liver damage to trigger skeletal muscle pathology and maybe it can be started by hyperammonemic condition by itself.

Regarding the gastrointestinal tract, an increase in gut epithelial permeability, alteration in motility and immunological local status were described, generating a predisposition for future complications. Concomitantly, an inespecific inflammatory infiltration is considered guilty of many of the gut impairments of this pathology, followed by mucosal atrophy, edema of lamina propia, fibromuscular proliferation and thickened muscularis mucosa.

In the study of HE, to get close to MHE is to get closer to its big bang; and from here, to travel less transited roads such as skeletal muscle and intestine, is to go even closer. The aim of this review is to expose these roads for further and deeper work.

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Factoring the intestinal microbiome into the pathogenesis of autoimmune hepatitis

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Abstract

The intestinal microbiome is a reservoir of microbial antigens and activated immune cells. The aims of this review were to describe the role of the intestinal microbiome in generating innate and adaptive immune responses, indicate how these responses contribute to the development of systemic immune-mediated diseases, and encourage investigations that improve the understanding and management of autoimmune hepatitis. Alterations in the composition of the intestinal microflora (dysbiosis) can disrupt intestinal and systemic immune tolerances for commensal bacteria. Toll-like receptors within the intestine can recognize microbe-associated molecular patterns and shape subsets of T helper lymphocytes that may cross-react with host antigens (molecular mimicry). Activated gut-derived lymphocytes can migrate to lymph nodes, and gut-derived microbial antigens can translocate to extra-intestinal sites. Inflammasomes can form within hepatocytes and hepatic stellate cells, and they can drive the pro-inflammatory, immune-mediated, and fibrotic responses. Diet, designer probiotics, vitamin supplements, re-colonization methods, antibiotics, drugs that decrease intestinal permeability, and molecular interventions that block signaling pathways may emerge as adjunctive regimens that complement conventional immunosuppressive management. In conclusion, investigations of the intestinal microbiome are warranted in autoimmune hepatitis and promise to clarify pathogenic mechanisms and suggest alternative management strategies.

Key words: Intestinal microbiome; Inflammasomes; Autoimmune hepatitis; Dysbiosis; Toll-like receptors

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Core tip: The intestinal microbiome is a reservoir of microbial antigens and activated immune cells that have

been implicated in the pathogenesis of diverse systemic immune-mediated diseases. Dysbiosis, increased intestinal permeability, and molecular mimicry between microbial and self-antigens may initiate or sustain autoimmune hepatitis. Multiple drug, molecular, dietary, and probiotic interventions can modify the intestinal microbiome and attenuate the immune response. The role of the intestinal microbiome in autoimmune hepatitis warrants rigorous study, and new therapies may emerge that strengthen current treatment regimens.

Czaja AJ. Factoring the intestinal microbiome into the pathogenesis of autoimmune hepatitis. *World J Gastroenterol* 2016; 22(42): 9257-9278 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9257.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9257>

BIOGRAPHY

Albert J Czaja, MD is Professor Emeritus of Medicine at the Mayo Clinic College of Medicine, Rochester, Minnesota, United States (Figure 1). He graduated from Dartmouth College in 1965, received a Bachelor of Medical Science at the Dartmouth Medical School in 1966, and MD from Harvard Medical School in 1968. He was trained in internal medicine at the Philadelphia General Hospital, University of Pennsylvania Division, from 1968-1972, and he was a major in the United States Army Medical Corps from 1972-1975 at the United States Army Institute of Surgical Research at Fort Sam Houston, Texas. Dr. Czaja was then an NIH Research Fellow in hepatology at the Mayo Clinic under the mentorship of W.H.J. Summerskill, MD from 1975-1977. He joined the staff of the Mayo Clinic Division of Gastroenterology and Hepatology in 1977, and he was appointed Professor of medicine in 1986. His research has focused on chronic hepatitis, especially autoimmune hepatitis, and he has contributed to the understanding of its diagnosis, treatment, prognosis, genetic predispositions, pathogenic mechanisms, and consequences. He has collaborated and published with investigators from 15 countries, and he has mentored physicians who have become leaders in their academic institutions and professional organizations. Dr. Czaja is a Fellow of the American College of Physicians, American College of Gastroenterology, American Gastroenterological Association, and American Association for the Study of Liver Diseases. He received the Meritorious Service Medal of the United States Army Medical Corps in 1976; the Fiterman award for distinguished clinical investigation in hepatology by the American Gastroenterological Association in 1997; the Henry S. Plummer Distinguished Physician Award by the Mayo Clinic Department of Medicine in 2006; the Distinguished Clinician Award of the American Gastroenterological Association in 2007; the Distinguished Clinician Educator Award (1st recipient) of the



Figure 1 Albert J Czaja, MD, Professor Emeritus of Medicine, Mayo Clinic College of Medicine, Rochester, Minnesota, United States.

American Association for the Study of Liver Diseases; the Gold Medal of the Canadian Association for the Study of the Liver and the Canadian Liver Foundation for the advancement of hepatology in autoimmune liver diseases and the education of young clinicians in 2013; and the Mayo Clinic Distinguished Alumnus Award in 2016. Dr. Czaja was a founding member of the International Autoimmune Hepatitis Group in 1992, and he continues to describe investigational pathways that promise to improve the treatment strategies of autoimmune liver disease. He has written over 540 articles, including 87 book chapters.

INTRODUCTION

Autoimmune hepatitis is a chronic immune-mediated inflammatory liver disease of uncertain cause^[1,2]. Population-based epidemiological studies have indicated that it is a rare chronic liver disease with an annual incidence of 0.67-1.9 cases per 100000 persons and a point prevalence of 4-42.9 cases per 100000 persons^[3-8]. Prevalence varies widely by geographical region (Singapore, 4 per 100000^[4]; Sweden, 10.7 per 100000^[9]; southern Israel, 11 per 100000^[8]; Spain, 11.6 per 100000^[6]; Norway, 16.9 per 100000^[3]; Netherlands, 18.3 per 100000^[10]; Denmark, 23.9 per 100000^[11]; New Zealand, 24.5 per 100000^[7]; and Alaska, 42.9 per 100000^[5]), and the incidence has been increasing in Denmark^[11] and the Netherlands^[10]. Rigorous epidemiological studies have not been performed in the adult population of the United States, but studies in the children of Utah have indicated an overall incidence of 0.4 cases per 100000 and a prevalence of 3 cases per 100000^[12]. The wide variability in the annual incidence and point prevalence of autoimmune hepatitis in different geographical regions and ethnic groups suggests that genetic and environmental factors contribute to its occurrence^[2,13,14].

Genetic factors within^[15-17] and outside^[18-22] the major histocompatibility complex (MHC) have been implicated as susceptibility factors for autoimmune hepatitis. Cytochrome P450 2D6 (CYP2D6) and formi-

minotransferase cytochrome c oxidase have been proposed as key antigenic targets in some patients^[23-25], and homologies between peptide sequences in CYP2D6 and hepatitis C virus (HCV), herpes simplex virus (HSV), and cytomegalovirus (CMV) have suggested that molecular mimicry between foreign and self-antigens initiates and sustains the disease^[2,23,26-28].

The principal target antigen in most white North American and northern European adults with autoimmune hepatitis is unknown, and as yet unrecognized self-antigens or foreign antigens that resemble self-antigens may trigger the disease or increase susceptibility to it, possibly by skewing components of the innate and adaptive immune responses toward a pro-inflammatory, autoreactive profile^[2,29]. The commensal bacteria of the intestine and their metabolic by-products constitute a reservoir of foreign antigens that can interact with mucosal immune cells and influence systemic immune responses^[30-34]. The human microbiome has already been implicated in the occurrence of multiple systemic immune-mediated diseases, including type 1 diabetes^[35-37], rheumatoid arthritis^[38-41], multiple sclerosis^[42], and inflammatory bowel disease^[43-45], and its role in the inflammatory liver diseases is also being scrutinized^[46-48].

Non-alcoholic steatohepatitis (NASH) may progress because of an influx of microbial products in the portal circulation, activation of toll-like receptors (TLRs) 4 and 9, and subsequent release of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β ^[49-51]. Primary sclerosing cholangitis (PSC) expresses high levels of TLR4 and TLR9 in biliary epithelial cells (BEC), produces IL-1 β , IL-8, and interferon (IFN)- γ in response to lipopolysaccharide (LPS), and commonly manifests atypical perinuclear anti-neutrophil cytoplasmic antibodies (pANCA)^[52-54]. These antibodies are directed against β -tubulin which cross-reacts with an antigen (FtsZ) present in all intestinal bacteria^[53]. Germ-free mice develop histologically more severe PSC than conventionally housed animals, and these findings suggest that commensal bacteria have a protective role against PSC^[55].

Similarly, primary biliary cholangitis (PBC) expresses TLR4 in BEC and periportal hepatocytes, produces pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and TNF- α) in response to intestinal bacterial components [LPS, flagellin, and cytosine-phosphorothioate-guanine oligonucleotide (CpG)], manifests BEC-destructive LPS-stimulated natural killer cells, and produces antimicrobial antibodies that target an antigen (pyruvate dehydrogenase complex-E2) which shares sequence homologies with intestinal *Escherichia coli*^[56-62].

Autoimmune hepatitis may also be influenced by the intestinal microbiome. Concurrent features of PBC or PSC occur in 7%-18% of patients ("overlap syndromes")^[63,64]; atypical pANCA are present in 49%-92% of individuals with autoimmune hepatitis^[65-68]; and alterations in the composition of the

intestinal microbiota (dysbiosis) have been found in experimental autoimmune hepatitis^[69]. The structural proteins binding intestinal epithelial cells (zona occludens 1 and occludin) are reduced in patients with autoimmune hepatitis compared to healthy volunteers; plasma LPS levels are increased; and the numbers of intestinal anaerobes (*Bifidobacterium* and *Lactobacillus*) are decreased^[70]. These findings support the concept that autoimmune hepatitis is associated with dysbiosis, increased permeability of the gastrointestinal mucosal barrier, and translocation of gut-derived microbial products into the systemic circulation.

The goals of this review are to describe the role of the intestinal microbiome in generating innate and adaptive immune responses, indicate how these responses may contribute to the development or maintenance of systemic autoimmune responses, and encourage investigations in autoimmune hepatitis that might improve understanding of its pathogenesis and results of its management.

LITERATURE SEARCH

Abstracts cited in PubMed were identified using the search words "intestinal microbiome", "intestinal microbiome and autoimmunity", and "intestinal microbiome and autoimmune hepatitis". Key aspects of the abstracts judged pertinent to the review were noted, and full-length articles were selected from the abstracts. A secondary bibliography was developed from the references cited in the selected full-length articles, and additional PubMed searches were performed to expand the concepts developed in these articles. The discovery process involving abstract review and the acquisition of full-length articles was repeated, and a tertiary bibliography was developed after reviewing these selected articles. The number of abstracts cited by PubMed and reviewed for pertinence to this topic during the primary, secondary and tertiary searches exceeded 3800. Those judged most pertinent to the topic exceeded 200, and the number of full-length articles reviewed was 66.

INTESTINAL MICROBIOME AND IMMUNE RESPONSES

The human intestinal tract contains 10-11 trillion bacteria comprising 500-1500 different species^[47,71-76]. *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* are the four phyla that predominate^[77-79], and a phylogenetic core of microbial species has been defined that is composed of 66 Operational Taxonomic Units (OTUs) present in most individuals^[80]. Luminal microbiota have greater diversity, and they are more tightly clustered than mucosal microbiota^[81]. *Firmicutes* and *Actinobacteria* are more abundant in the luminal populations, and *Proteobacteria* are more abundant in

the mucosal populations^[81].

The composition of the microbiome is influenced by diverse environmental factors, including community sanitation levels and vaccination programs, and by host-related variables, including method of obstetrical delivery, age, genetic predisposition, dietary habits, personal hygiene, and antibiotic exposures. Changes in the intestinal microbiome tend to be slow from late childhood through adulthood with marked changes occurring mainly with advanced age^[74,82-85]. The microbiome becomes less diverse and more variable over short intervals with aging, and the species of *Bacteroides*, *Clostridium* and *Escherichia coli* constitute a greater proportion of the microflora in individuals aged ≥ 65 years^[82-84].

The intestinal microbiome varies in diverse ethnic groups^[75,86-89], and this diversity may reflect genetic factors, demographic issues (age, gender, socioeconomic status), lifestyle features (alcohol use, smoking, adiposity), and long-term diet^[90-92]. Disparities in the intestinal microbiota have been recognized between ethnic groups in the same country (rural versus urbanized)^[88,89,93,94] and between countries (Africa versus Europe, cross-Europe, and cross-Asia)^[87,88,91], and socioeconomic variations at individual and neighborhood levels have been associated with many of these differences^[88,95]. The nature of the long-term diet may be the critical element affected by the socioeconomic status^[95].

Amongst the diversity within the intestinal microbiota, common functional and phylogenetic elements have also been described^[89,96-99]. These common elements may be indispensable for the well-being of the individual as they can produce short-chain fatty acids, synthesize vitamins, and aid in digestion, metabolism and immune defense^[89]. The functional and phylogenetic core components have been shared across heterogeneous healthy human populations, and they tend to co-exist^[89].

The intestinal microbiome is essential for development of the intestinal immune responses which in turn maintain tolerance of the microflora^[41,100,101]. Germ-free mice have fewer CD4⁺ T lymphocytes in the lamina propria of the intestine, hypoplastic Peyer's patches, less immunoglobulin A production, and disorganized zones of T and B lymphocytes in the spleen and lymph nodes compared to wild-type mice^[102,103]. These immune deficiencies are corrected by the introduction of *Bacteroides fragilis*^[104]. Colonization also induces the production by IL-10-secreting, regulatory T cells (Tregs), possibly in response to the secretion of polysaccharide A by the bacteria and direct activation of TLR2 on the Foxp3⁺ Tregs^[105-108]. The introduction of *Clostridium* species induces similar changes^[109,110].

Emerging evidence suggests that the intestinal microbiome can influence systemic immune responses by activating TLRs^[34,47,48] and promoting the formation of inflammasomes within the liver^[51,111-113] (Figure

2). Changes in the composition of the intestinal microbiome induced by antibiotics, genetic factors, or the disease (dysbiosis) may sustain or enhance the innate and adaptive immune responses by overcoming or circumventing normal tolerogenic responses to the commensal bacteria^[32,114-116]. Bacterial components may act as antigens that stimulate the systemic immune response^[30,31,34,47,48] or that prime immune cells within the intestine that subsequently access peripheral lymphoid tissue^[39,117,118]. Molecular mimicry between microbial and host-derived antigens and promiscuous targeting by antigen-sensitized lymphocytes may then initiate or strengthen the autoreactive response in genetically-predisposed individuals^[33,57,115]. Investigations in cell cultures, animal models, and patients with diverse systemic immune-mediated diseases have justified these hypotheses and warranted their consideration in autoimmune hepatitis^[46-48].

KEY REQUIREMENTS FOR A MICROBIOME-DERIVED SYSTEMIC IMMUNE RESPONSE

Activation of TLRs

TLRs are the key receptors within the intestine that recognize microbe-associated molecular patterns, pathogen-associated molecular patterns, and damage-associated molecular patterns^[34,47,48,119,120] (Table 1). They are instrumental in generating an innate immune response to pathogens and cellular distress signals, and they can shape subsets of T helper (Th) lymphocytes that recognize microbial components and have the potential to cross-react with host antigens^[41,121,122]. Ten TLRs have been described in humans^[123], and each responds preferentially to specific ligands which may be viral and bacterial proteins or endogenous ligands in the absence of infection^[34,120]. All stimulated TLRs except TLR3 activate a signaling pathway that is dependent on the myeloid differentiation factor 88 (MyD88)^[124,125]. Signaling through the MyD88 pathway in turn activates nuclear factor-kappa B (NF- κ B) and promotes the transcription of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6)^[123,125,126].

TLRs can also influence the adaptive immune response^[41] (Figure 2). TLRs expressed by dendritic cells and macrophages can upregulate class II molecules of the MHC and enhance antigen presentation to CD4⁺ helper T lymphocytes^[127] (Table 1). TLRs can also increase the expression of the co-stimulatory molecules, CD80, CD86 and CD40, on the antigen presenting cells and thereby favor T lymphocyte activation and differentiation^[41,127]. Kupffer cells express all TLRs except TLR5^[128], and they are the primary cells within the liver that respond to TLR ligands. The production of pro-inflammatory cytokines, chemokines, and reactive oxygen species by the Kupffer cells promotes liver inflammation^[129]

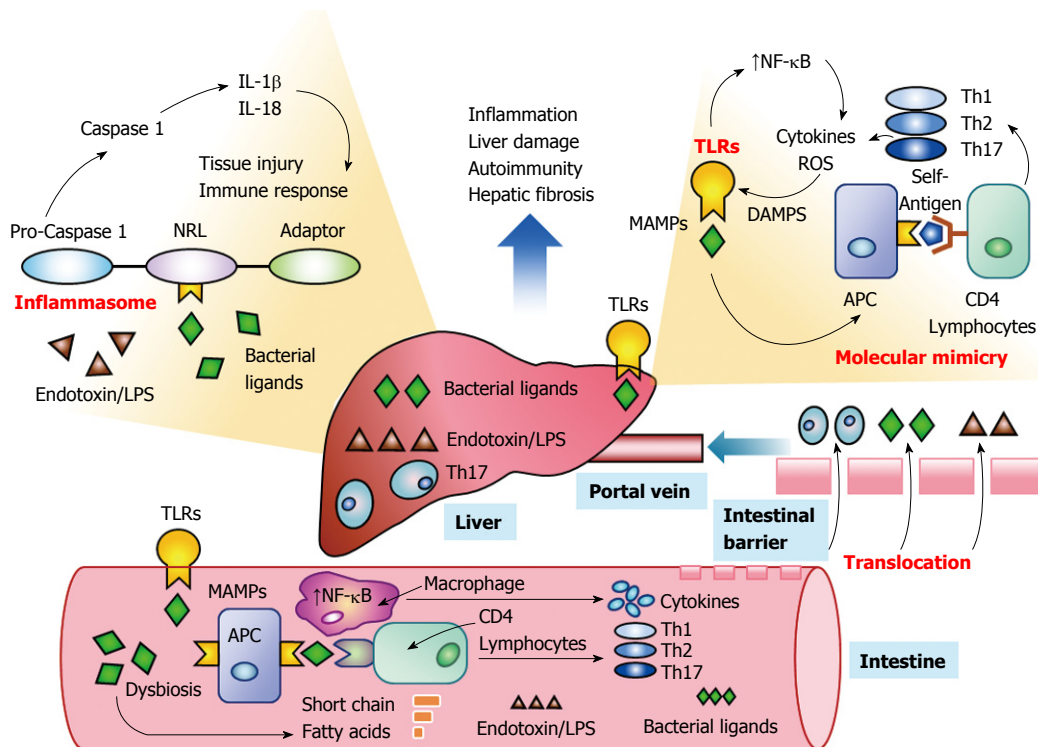


Figure 2 Interactions between the intestinal microbiome and the liver. Dysbiosis can generate microbe-associated molecular patterns (MAMPs) that activate toll-like receptors (TLRs) in the intestine. Activated TLRs can stimulate the transcription factor, nuclear factor-kappa B (NF- κ B), in macrophages and generate pro-inflammatory cytokines. They can also increase the expression of the major histocompatibility complex on antigen presenting cells (APCs) and sensitize CD4 lymphocytes to bacterial ligands. The activated lymphocytes can proliferate as T helper (Th) 1, Th2, and Th17 cells. The dysbiosis can also generate short chain fatty acids, endotoxin, lipopolysaccharide (LPS), and bacterial components that can serve as antigenic ligands. Tight junctions within the intestinal mucosa may weaken with the dysbiosis and allow paracellular translocation of lymphocytes, bacterial ligands and endotoxin. These gut-derived elements can then enter the portal vein and be delivered to the liver. The bacterial ligands within the liver can activate TLRs within hepatocytes, hepatic stellate cells, Kupffer cells, and sinusoidal epithelial cells and generate pro-inflammatory cytokines and reactive oxygen species (ROS) that can produce damage-associated molecular patterns (DAMPs) that activate TLRs in a self-amplification loop (upper right corner blow-up). The hepatic TLRs can also contribute to the sensitization of CD4 lymphocytes to bacterial ligands and self-antigens that resemble bacterial ligands (molecular mimicry). Concurrently, the bacterial ligands and gut-derived endotoxin can activate the non-obese diabetes-like receptor (NLR) of inflammasomes within hepatocytes and hepatic stellate cells (upper left corner blow-up). The release of caspase 1 can generate interleukin (IL) 1 β and IL-18 and promote tissue injury and the immune response. The net effect is to increase hepatic inflammation and liver damage and predispose to autoimmunity and hepatic fibrosis.

and the innate and adaptive immune responses^[41,127]. Hepatocytes, BEC, hepatic stellate cells (HSCs), and sinusoidal epithelial cells also express TLRs, but only HSCs express TLR1 through TLR9^[48,130].

The cytokine profile shapes the subsets of T lymphocytes that constitute the immune-mediated response, and it is influenced by the particular TLRs that are activated by ligands within the microenvironment (Table 1). Activation of TLR4 and TLR9 promotes the release of IL-12 and favors a type 1 cytokine pathway that is pro-inflammatory^[131]. TLR4 also induces the secretion of IL-23 and promotes the expansion and survival of pro-inflammatory Th 17 lymphocytes^[132]. In contrast, activation of TLR2 favors the production of IL-10 and IL-13 which promotes an anti-inflammatory type 2 cytokine response^[133].

LPS from gram-negative bacteria is the principal ligand activating TLR4^[123,134], and un-methylated CpG sequences in bacterial and viral genomes activate TLR9^[135] (Table 1). The viral proteins of HCV, CMV and HSV are key ligands that activate TLR2^[34,136,137]. TLR2, TLR5, TLR7, and TLR8 are expressed by CD4⁺

T lymphocytes, and ligands that activate these TLRs (viral proteins^[136], flagellin^[138], and single-stranded ribonucleic acid^[139]) can directly activate memory lymphocytes and stimulate their proliferation^[140]. Naturally occurring Tregs express TLR2, TLR5 and TLR8, and they can also be activated directly by viral and bacterial components^[141]. TLRs can also block the suppressive effect of Tregs by the recognition of microbial products that induce secretion of IL-6^[142]. Pathogen-specific adaptive immune responses can be favored, and defense mechanisms can be strengthened. Microbial elements can thereby modulate the innate and adaptive immune responses through the TLRs and affect immune homeostasis indirectly by modulating the cytokine profile or directly by affecting immune cell proliferation.

TLR4 is a crucial signaling pathway by which HSCs increase the extracellular matrix^[46] (Table 1). The production of chemokines and adhesion molecules is mediated by activated TLR4 in HSCs^[46], and the chemo-attraction of inflammatory and immune cells to the liver stimulates the fibrotic process^[143]. TLR4

Table 1 Key Requirements for gut-derived systemic immune response

Key requirement	Features	Mechanisms
Activation of TLRs	Intestinal receptors responsive to MAMPs and DAMPS ^[34,47,48,120] Signaling dependent on MyD88 ^[124,125] Activates NF- κ B ^[123,125,126] Favors T lymphocyte activation ^[41] Modulates actions of Tregs ^[141,142] Present in hepatocytes, HSCs, Kupffer cells, sinusoidal epithelial cells, BEC ^[48]	Increases pro-inflammatory cytokines ^[126] Upregulates class II MHC ^[127] Increases co-stimulatory molecules ^[41,127] Promotes pathogen-specific responses ^[142] LPS activates TLR4 ^[123,134] Sequences in bacteria activate TLR9 ^[135] TLR4 in HSCs promote fibrosis ^[46,144] Implicated in other liver diseases ^[58,148]
Stimulation of inflammasomes	Protein complexes that release pro-inflammatory IL-1 β and IL-18 ^[111-113] NLRs sense microbial products ^[156] Upregulated in Kupffer cells, hepatocytes, and sinusoidal epithelial cells ^[113] Activation by highly diverse ligands ^[112]	Upregulated in hepatocytes by LPS ^[113] Activates pro-caspase 1 ^[156] Promotes hepatic fibrosis ^[50] Shapes innate and adaptive immunity ^[112,160] Implicated in NAFLD ^[51] Activation separate from TLRs ^[112,155] Can activate TLRs and NLRs ^[116,173]
Emergence of dysbiosis	Microflora differ from commensals ^[116] Dysbiosis varies in specific diseases ^[116] Less bacterial diversity common ^[170] Antibiotics most frequent basis ^[165,175] Uncertain cause or effect of disease ^[116]	Genetic factors may affect composition ^[177] Gender-related compositional differences ^[179] May affect gender-related autoimmunity ^[180] Present in AIH and experimental NASH ^[47,69]
Molecular mimicry	Microbial and self-homologies ^[33,185] Cross-reacting antibodies ^[53,57,184] Promiscuous activity of effectors ^[186] Epitope spread ^[187]	pANCA react with bacterial antigen ^[53] AMA cross-reacts with <i>Escherichia coli</i> ^[56,57] Increasingly distant homologues targeted ^[187]
Breach of intestinal mucosal barrier	Gut-derived products enter system ^[195] Translocation prime basis ^[46,195] Active transport also possible ^[230]	Gut-derived lymphocytes in lymph nodes ^[118] Microbial components in peripheral blood ^[195] Activates TLRs and NLRs ^[123,130] Implicated in NASH and diabetes ^[197,198]

AMA: Antimitochondrial antibodies; BEC: Biliary epithelial cells; DAMPS: Damage-associated molecular patterns; HSCs: Hepatic stellate cells; IL: Interleukin; LPS: Lipopolysaccharide; MAMPs: Microbe-associated molecular patterns; MHC: Major histocompatibility complex; MyD88: Myeloid differentiation factor 88; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NF- κ B: Nuclear factor kappa B; NLRs: Non-obese diabetes-like receptors; pANCA: Atypical perinuclear anti-neutrophil cytoplasm antibodies; TLRs: Toll-like receptors; Tregs: Regulatory T cells. Superscripted numbers in brackets are references.

signaling also promotes activation of transforming growth factor-beta (TGF- β) by down-regulating production of an endogenous inhibitor of the TGF- β receptor^[144]. Furthermore, TLR4 signaling may down-regulate microRNA molecules that suppress the transcription of collagen^[46,145]. A polymorphism of the *TLR4* gene may impair the response of TLR4 to LPS. The LPS-induced signaling pathway that activates NF- κ B may thereby be disrupted and the production of the pro-inflammatory cytokines, TNF- α and interferon-beta, may be reduced^[146]. In this fashion, a genetic variation may affect the response of TLR4 to microbial ligands and the propensity for progressive hepatic fibrosis.

The signaling pathway involving TLR4, MyD88, and NF- κ B has been implicated in the progression of multiple liver diseases^[54,58,144,147,148]. Concentrations of gut-derived endotoxins have been increased in animal models of hepatic fibrosis^[149,150] and in the systemic and portal circulation of patients with cirrhosis^[151,152]. Other TLRs may respond to different microbial ligands and influence the subsets of lymphocytes that orchestrate the autoreactive response. TLR signaling pathways have not been evaluated in autoimmune hepatitis.

Stimulation of inflammasomes

Inflammasomes are protein complexes that form

within the cytoplasm of diverse cells, including macrophages, hepatocytes, and HSCs, in response to stimuli associated with cellular stress, damage or infection^[112,153,154] (Table 1). By releasing pro-inflammatory cytokines IL-1 β and IL-18, they drive the inflammatory response to tissue injury and influence cell death, inflammatory activity, and fibrosis^[111,113] (Figure 2). TLRs and inflammasomes have separate routes of activation^[112,155], but cooperation between them is pivotal in promoting communication between the intestinal microbiota and the systemic immune response^[34]. Factors that increase the expression of inflammasomes, such as saturated fatty acids and bacterial endotoxin, may increase activation of TLR4 and promote hepatic fibrosis^[50].

Inflammasomes consist of a sensor protein that is within the family of non-obese diabetes (NOD)-like receptors (NLRs), an adaptor molecule (apoptosis-associated speck-like CARD-domain containing protein), and pro-caspase 1^[156] (Table 1). The inflammasomes can sense microbial products^[157] and metabolic stress^[112], activate pro-caspase 1^[158,159], trigger the release of pro-inflammatory cytokines^[153], and shape the innate^[112] and adaptive immune responses^[160]. The expression of NLRP3 is upregulated in hepatocytes after stimulation with LPS, and Kupffer cells and sinusoidal epithelial cells also express high

levels of NLRP1 and NLRP3^[113].

The structural diversity of the ligands that activate NLRP3 is greater than the structural motifs that activate the TLRs, and the inflammasomes may be responsive to a broader range of activation signals than the TLRs^[161] (Table 1). Together the TLRs and NLRs provide receptors for signaling pathways that can respond to diverse endogenous and exogenous danger signals, including microbial components, and they each can generate pro-inflammatory responses that sustain and enhance the innate and adaptive immune responses to liver injury. TLRs may also have a counter-regulatory effect on the inflammasomes^[34]. Chronic stimulation of the TLRs by LPS induces the production of IL-10 and reduces the activation of NLRP3^[162]. Furthermore, activation of TLR2 or TLR4 can increase the autophagy of hepatocytes, the degradation of NLRP3, and the suppression of IL-1 β production^[163]. Inflammasomes have not been characterized in autoimmune hepatitis, and their interactions with TLRs have not been defined in this disease.

Emergence of immunogenic intestinal microbiota (dysbiosis)

Systemic inflammatory and immune-mediated diseases have been associated with intestinal microbiomes that distinguish them from normal or other disease-specific populations^[116] (Table 1). The intestinal microbiota have differed in patients with rheumatoid arthritis compared to patients with fibromyalgia^[164]. Decreased diversity in the microbiome has been associated with an increased risk of type 1 diabetes^[165], the occurrence of atopic diseases, including asthma^[166-169], and the presence of Crohn's disease^[170]. Patients with multiple sclerosis have reduced numbers of *Clostridia* and *Bacteroides* species compared to normal individuals^[42], and patients with type 1 diabetes have more colonies of *Bacteroides*^[37,171]. Compositional shifts in the intestinal microbiota, particularly the relative frequencies of certain bacterial taxa, have been associated with the phenotype and genotype of inflammatory bowel disease^[172].

These findings have suggested that alterations in the composition of the intestinal microflora (dysbiosis) may disrupt intestinal and systemic immune tolerances and contribute to immune-mediated diseases^[114,116]. Depletion of the commensal bacteria may allow intestinal populations of pathogenic or immunogenic organisms to proliferate and generate ligands that activate TLRs and NLRs^[116,173] (Figure 2). The major uncertainty has been whether the dysbiosis has been a cause or an effect of the disease.

Antibiotics are the principal agents promoting dysbiosis, and their use has been implicated in creating the dysbiosis associated with the occurrence of atopic diseases^[167,168,174], asthma^[166], type 1 diabetes^[165], and celiac disease^[175]. Twin studies have also indicated that genetic factors can shape the

intestinal microbiome^[176,177], and immune-mediated diseases with genetic predispositions have manifested dysbiosis^[30,47,116]. Importantly, the contribution of the 8.1 ancestral haplotype, which includes the DRB1 alleles commonly associated with systemic autoimmune diseases including autoimmune hepatitis, is probably small^[178], and dysbiosis rather than genetic factors has been implicated in the occurrence of experimental NASH^[51].

The composition of the intestinal microflora may also influence the gender bias for autoimmune disease^[179-181] (Table 1). Colonization by commensal microbes early in the life of NOD mice raises serum testosterone levels and protects male mice from developing type 1 diabetes^[179]. Furthermore, the transfer of the intestinal microbiota from mature male NOD mice to immature female NOD mice alters the intestinal microbiome of the females and protects them from developing diabetes^[179]. Blockade of the androgen receptor attenuates the microbiome-specific changes in the female mice and supports the concept that the commensal bacteria of the intestine can affect the propensity for autoimmune disease in genetically-susceptible animals by altering sex hormone levels or receptor sensitivities^[179].

Gender may also influence the composition of the intestinal microbiota and in turn the propensity to develop autoimmune disease^[180] (Table 1). The intestinal microbiota differ in male and female NOD mice, and this difference disappears after male castration. Furthermore, the greater frequency of female NOD mice to develop type 1 diabetes compared to male NOD mice is lost in germ-free animals^[180]. These findings suggest that the intestinal microbiome can influence sex hormone levels^[179] and also be influenced by them^[180], possibly in a self-amplification loop.

The increased female propensity for autoimmune disease may relate to estrogenic effects that modulate the autoreactive response directly by affecting the pro- and anti-inflammatory cytokine pathways of lymphocyte differentiation^[182] and indirectly by altering the intestinal microbiome to favor the translocation of sensitizing microbial antigens^[180]. Imbalances between blood estrogen and progesterone levels have affected the immune response during and immediately after pregnancy^[182], and the treatment of peripheral blood mononuclear cells with 17- β estradiol has increased their response to immunogens and the expression of TLR8^[183]. The relationship between sex hormone levels and the intestinal microbiome during pregnancy, menses, and menopause remains uncertain and important to clarify.

Multiple factors can promote dysbiosis, and a pathogenic or circumstantial relationship with autoimmune disease has not been established. Nevertheless, the association of dysbiosis with diverse systemic immune-

Table 2 Microbial mechanisms for breaching intestinal barrier

Microbial Effect	Features	Mechanisms
Translocation	Migration of gut-derived products ^[195,224] Tight junctions weakened ^[218] Increased intestinal permeability ^[195,218] Paracellular migration ^[37,224] Consequences ^[192] LPS and CpG delivered to liver ^[123,130,195] Activated immune cells translocate ^[118,193] Translocated microbial antigens activate peripheral immune cells ^[185] TLRs and NLRs activated ^[123,130]	Gut-derived SCFA affect tight junctions ^[200] Butyrate strengthens intestinal barrier ^[203] Induces mucin synthesis ^[201,203] Reduces bacterial translocation ^[204] Increases peripheral Tregs ^[205] Inhibits NF- κ B and inflammation ^[207] Lactate strengthens intestinal barrier ^[37] Fermented to butyrate ^[215,216] Low butyrate- and lactate- producing bacteria associated with weak barrier ^[217,218] TLRs affect molecular mediators ^[225,226] Signaling pathways disrupted ^[223] Junctional binding proteins dissociated ^[224] Paracellular migration routes formed ^[37,224] <i>E. coli</i> and <i>C. difficile</i> key effectors ^[37]
Increased mucosal permeability	Intestinal epithelial cells bound together by junctional complex of proteins ^[222,223] Occludin main component ^[222] Zona occludens couples cytoskeleton ^[222] Cingulin contacts cells ^[222] Actin and myosin anchor cells ^[222] Intermediate filaments bind cells ^[222] Signaling pathways seal junction ^[223] Protein kinase C modulates occludin ^[222]	TLRs affect molecular mediators ^[225,226] Signaling pathways disrupted ^[223] Junctional binding proteins dissociated ^[224] Paracellular migration routes formed ^[37,224] <i>E. coli</i> and <i>C. difficile</i> key effectors ^[37]
Active transport	Bacterial antigens actively transported across intestinal barrier ^[230]	M cells in Peyer's patches capable of active transport ^[230]

Superscripted numbers in brackets are references. Cpg: Un-methylated cytosine-phosphorothioate-guanine oligonucleotide; LPS: Lipopolysaccharide; NF- κ B: Nuclear factor kappa B; NLRs: Non-obese diabetes-like receptors; SCFA: Short chain fatty acids; TLRs: Toll-like receptors; Tregs: Regulatory T cells.

mediated diseases^[116], its recognition in autoimmune hepatitis^[69,70], its possible genetic associations^[176,177], and its gender bias^[179-181] suggest that dysbiosis may constitute an important antigenic or hormonal reservoir that can promote the autoreactive response in diverse systemic immune diseases, including autoimmune hepatitis.

Molecular homologies between microbial and self-antigens

Epitopes can be shared between microbial components and self-antigens, and this molecular mimicry can result in cross-reacting antibodies or the activation of T lymphocytes in genetically-predisposed individuals^[33,184,185] (Figure 2). The reactive T lymphocytes can in turn exhibit promiscuous activity^[186] and target epitopes that are distant homologues to the initial antigenic trigger (epitope spread)^[187] (Table 1). Bacterial components have generated autoantibodies found in systemic autoimmune diseases, such as systemic lupus erythematosus (SLE)^[188] and the antiphospholipid syndrome^[189], and they have been implicated in the progression of SLE^[190] and the exacerbation of Sjogren's syndrome, possibly through the activation of memory lymphocytes^[33,188,191]. The atypical pANCA found in PSC and autoimmune hepatitis target an antigen (β -tubulin) that cross-reacts with a bacterial antigen^[53], and the antibodies to pyruvate dehydrogenase complex-E2 found in PBC cross-react with intestinal *Escherichia coli*^[56,57]. The principal mechanism by which the intestinal microbiota may sustain or extend the autoreactive response is molecular mimicry, and experimental animal models of autoimmune hepatitis should evaluate this hypothesis.

Breach of the intestinal mucosal barrier

Interactions between the intestinal microenvironment and the systemic immune response imply that the natural barrier between the intestinal and systemic domains can be breached (Table 2). There is ample evidence to justify this supposition, but the actual mechanisms are uncertain. Reactive T lymphocytes expressing intestinal receptors can be found in the pancreatic islets and lymph nodes of patients and mice with type 1 diabetes^[117,118,192,193], and lymphocytes originating in the gut mucosa have been implicated in the autoreactive response in experimental autoimmune encephalomyelitis^[194]. Furthermore, microbial components have been detected in the plasma of patients with cirrhosis^[151,152] and portal circulation of animals with non-alcoholic fatty liver disease^[51].

The migration of gut-derived bacteria and bacterial products from the intestinal lumen to the liver, mesenteric lymph nodes, and other extra-intestinal sites may occur by translocation^[195] (Figure 2). Translocation implies that intestinal permeability has been increased, possibly because tight junctions within the intestinal mucosa have been weakened or the intestinal barrier has been overwhelmed by bacterial overgrowth^[46,195-197]. The translocated bacterial products, including LPS and unmethylated CpG, can then be delivered to the liver *via* the portal vein and activate TLRs and NLRs^[123,130]. Dysbiosis and a "leaky gut" have been implicated in the development of NASH, diabetes, and the metabolic syndrome^[197,198].

Bacteria produce short chain fatty acids (acetic acid, butyric acid, and propionic acid) which can affect the tight junctions within the intestinal mucosa^[197,199,200] (Table 2). Butyrate, which is the conjugate base of

butyric acid, induces mucin synthesis in the intestinal mucosa, strengthens tight junctions, and reduces bacterial transport across the stressed epithelium^[201-204]. Butyrate may also have an anti-inflammatory effect by promoting the extra-thymic differentiation of peripheral Tregs^[205,206] and inhibiting NF- κ B and the transcription of pro-inflammatory cytokines^[207]. Other short chain fatty acids (propionate) and bacterial by-products (succinate and acetate) do not induce the production of mucin and may increase gut permeability^[37,202].

Sodium butyrate acts in part by modulating the beta-catenin-dependent Wnt signaling pathway within cells^[208]. This pathway affects the transcription of genes that influence cell proliferation and differentiation. In colon carcinoma cell lines, the levels of beta-catenin transcriptional complexes within the cell influences its physiological response to butyrate. High levels of transcriptional complexes result in apoptosis of the cell and low levels result in the reversible limitation of cell growth after exposure to butyrate^[208]. The ability of butyrate to modulate cell proliferation and apoptosis may in turn influence cell viability and function, and these effects may help maintain the integrity of the gastrointestinal mucosal barrier.

Butyrate can also modulate cell responses to stress of the endoplasmic reticulum by promoting the apoptosis of the cell or its preservation through autophagy^[209-211]. Butyrate enhances the expression of peroxisome proliferator-activated receptor-gamma and the activation of caspases (especially caspase 3) that induce apoptosis in colorectal cell lines^[212]. It is also one of the short chain fatty acids, including propionate, that can induce autophagy in distressed cells and preserve their survival by generating energy and retarding the intrinsic (mitochondrial) pathway of apoptosis^[213,214]. Gut-derived short chain fatty acids, such as butyrate and propionate, may be important moderators of intestinal mucosal cell proliferation and function, and they may contribute to the prevention of systemic autoimmune responses and progressive colorectal cancer^[208].

Lactate, which is a bacterial byproduct of carbohydrate fermentation, also reduces intestinal permeability^[203] (Table 2). Lactate is fermented mainly to butyrate by intestinal microflora^[215]. The acetyl-coenzyme A pathway is the major route of butyrate production from lactate, and the intestinal microflora have considerable variability in lactate consumption^[215]. Furthermore, lactate-utilizing bacteria exhibit variable production of butyrate depending on the availability of other substrates^[216]. Patients with type 1 diabetes have a lower proportion of butyrate- and lactate-producing bacteria in their intestinal microbiome than case-control subjects, and the dysbiosis favoring increased intestinal permeability may contribute to the development of type 1 diabetes^[217,218].

Intestinal epithelial cells are bound together by

structural proteins^[37,219-221] that are organized into a tripartite junctional complex consisting of a tight junction, adherens junction, and desmosome^[222]. Occludin is the only known transmembrane protein with a domain in the paracellular space, and it is the principal component of the tight junction. Zona occludens 1 and 2 and cingulin are non-transmembrane proteins found in tight junctions at sites of cell-to-cell contact^[222]. They are bound to occludin, and they probably couple cells to the cytoskeleton^[222,223]. Actin and myosin filaments anchor cells together by calcium-dependent adhesion molecules (E-cadherins) in an adherens junction, and intermediate filaments are anchored to desmosomes and help bind cells^[222]. Multiple cellular signaling pathways affect the assembly and sealing of the junctions^[223], and they are cell-type specific with protein kinase C modulating occludin and zona occludens 1^[222].

Escherichia coli and *Clostridia difficile* can dissociate the binding proteins and increase intestinal permeability by opening a paracellular route^[37,224] (Table 2). The TLRs on the intestinal epithelial cells can modulate the integrity of the intestinal barrier, possibly by influencing the expression of molecular mediators that can affect the structure or function of the binding proteins^[225,226]. Activation of TLR2 increases the phosphorylation of isoforms of protein kinase C, and this action has been associated with enhanced expression of zona occludens and the sealing of tight junctions^[225]. Conversely, activation of TLR4 reduces the expression of phosphorylated occludin and increases intercellular permeability^[226]. Bacterial ligands derived from different microbial species may influence intestinal permeability through TLR signaling and the translocation of microbial products through a porous intestinal barrier may contribute to a systemic autoreactive response^[227-229].

Another mechanism by which the microbiome may influence the systemic immune response is by the active transport of bacterial antigens across the mucosal barrier by M cells within Peyer's patches^[230]. Whereas immune cells can be activated in the intestine and migrate to the liver or peripheral lymph tissue by translocation, they may also be activated in the systemic circulation by translocated or actively transported bacterial components that are presented by antigen presenting cells and recognized as foreign antigens by circulating naïve CD4⁺ T helper lymphocytes^[39,117,118,193].

IMPLICATIONS OF THE INTESTINAL MICROBIOME IN AUTOIMMUNE HEPATITIS

The intestinal microbiome has been implicated in the pathogenesis of diverse inflammatory liver diseases, including NASH, PSC and PBC^[48,51,54,59,62], and the pathogenic pathways of autoimmune hepatitis have

been incompletely defined^[2]. The principal target antigen remains unclear in most patients with autoimmune hepatitis, and conventional corticosteroid therapies have been unable to consistently induce sustained treatment-free remissions^[231,232]. Progressive hepatic fibrosis occurs in 25% of patients^[233], and emerging drug therapies and molecular interventions can suppress the immune response but not eliminate the disease^[234].

The intestinal microbiome is a reservoir of antigens and activated immune cells that could initiate, exacerbate, or perpetuate autoimmune hepatitis^[30-34]; dysbiosis has been demonstrated in experimental and human autoimmune hepatitis^[69,70]; atypical pANCA, manifested in most patients with autoimmune hepatitis, cross-react with an antigen found in intestinal bacteria^[53]; and the permeability of the intestinal mucosal barrier has been increased in patients with the disease^[70]. Investigations of the microbiome in autoimmune hepatitis may discover new antigens and suggest new therapies that might eliminate the primary antigen or the supplemental antigens that sustain or advance the disease.

EVALUATING THE INTESTINAL MICROBIOME IN AUTOIMMUNE HEPATITIS

Traditional stool culture techniques are limited in assessing the intestinal microbiome mainly because anaerobic organisms are difficult to culture^[235] and some microbial species may elude detection by conventional protocols^[235,236]. A common method for studying the diversity of the intestinal microflora has been to sequence the 16S ribosomal ribonucleic acid (rRNA) gene^[235,237]. The 16S rRNA gene is present in all prokaryotic cells; it has highly variable regions interspersed with highly conserved regions; and its sequences are unique to the major groups of prokaryotic organisms^[237]. These signature sequences can be used to reconstruct the phylogeny of the intestinal microbiome^[236].

Primers are designed that are complementary to the universally conserved regions that flank the variable regions, and the bacterial species and their proportions in the microbiome are determined^[237,238]. The variable regions are amplified by polymerase chain reaction (PCR), and the PCR products are purified for sequencing^[81]. Sequencing results are compared to established annotated datasets^[235]. The sequencing protocol misidentifies or omits a critical residue in only 1% of procedures^[237]. Ambiguities resulting from the sequencing determinations are the most common errors, and they may reflect inadequacies in the available datasets^[235,237]. Only known bacterial sequences or sequences closely homologous to known

bacterial sequences can be analyzed^[235].

Databases are available and they continue to evolve for analyses of 16S rRNA gene sequences^[236,239]. Quantitative Insights Into Microbial Ecology (QIIME)^[240] and Mothur, a comprehensive software package that integrates several algorithms from pre-existing software^[241], are open-source tools that can be used to describe and compare microbial communities. Short 16S rRNA sequences can be organized into OTUs, and a motif-based hierarchical method can analyze massive metagenomic datasets with high accuracy^[242]. The Human Microbiome Project provides a catalogue of bacterial species, and it will help define the nature of the intestinal microbiome, the factors that affect its composition, distribution and evolution, and the relationship of the intestinal microflora to human health and disease^[236,243,244].

Further advances in the techniques used to reconstruct the composition of the human intestinal microbiome include microarray technology, fingerprinting techniques such as determination of the terminal restriction fragment length polymorphisms, and next-generation sequencing (NGS)^[79,245-247]. Microarray hybridization of deoxyribonucleic acid provides a high throughput platform that consists of several thousand probes that can detect nucleic acid sequences simultaneously^[246-248]. Unknown microbial sequences and uncharacterized microbial populations are undetected by the microarray techniques, and uncertainties about the existence and importance of undiscovered microbial populations are the major limitations of this method^[247,248].

The Human Gut Chip has 4441 probes that includes 2442 probes specific for known microbes and 1919 probes that are explorative for unknown microbes^[247]. Probes with overlapping similarities are becoming more sensitive to microbial species that are less abundant^[249], and probes with explorative designs are being coupled to probes with microbial specificity in an effort to identify microorganisms with uncharacterized sequences^[247,250]. Essential for the design of useful explorative probes is the correct anticipation of genetic variations within the microbial community and the construction of probes with high sensitivity and specificity^[249].

Currently, intestinal ecosystems are being studied mainly by 16S rRNA sequencing^[236]. This technique is useful in identifying the microbial species that constitute the intestinal microbiome, determining the evolution and transition of the microbial community (phylogeny) and quantitating the microbial diversity^[236]. The challenge is to define the functions of the microbiome, and whole genome sequencing (WGS) may provide these insights^[236,251]. Array-based NGS can analyze the whole genome, exons, and regions of interest, and it may emerge as the lens by which to understand the function of the microflora^[79].

Table 3 Treatment considerations for investigation of gut-derived immune responses

Treatment Consideration	Nature	Findings
Dietary adjustments	Animal protein, saturated fats ^[90] High carbohydrate diets ^[90] Low fat high fiber diet ^[90]	<i>Bacteroides</i> , <i>Firmicutes</i> (including <i>Clostridia</i>), and <i>Prevotella</i> favored by different dietary regimens ^[37,90]
Probiotic preparations	<i>Bifidobacterium bifidum</i> ^[253] <i>Lactobacillus</i> strains ^[254,263,266] <i>Lactobacillus rhamnosus</i> ^[276] <i>Anaerostipes caccae</i> ^[277]	Expands Tregs in cell culture ^[278] Prevents diabetes in NOD mice ^[263] Improves liver tests in rat model ^[266] Increases tight junction proteins ^[276] Consumes lactate and produces butyrate ^[277]
Vitamin A and retinoic acid	Retinoic acid supplement ^[255] Dietary vitamin A ^[256]	Restores <i>Lactobacilli</i> in lupus model ^[255] Regulates cytokines in lupus model ^[256] Induces IL-10-producing Tregs ^[279] Reduces activity in RA ^[257]
Antibiotics	Tetracycline, minocycline ^[257] Vancomycin, metronidazole ^[269]	Improves tests and pruritus in PSC ^[269] Induces Tregs in colitis model ^[107,109,110]
Re-colonization	<i>Bacteroides fragilis</i> ^[107]	
Fecal transplantation	<i>Clostridia</i> species ^[109,110]	
Intestinal barrier protectors	Gelatin tannate ^[258-260]	Enhances mucus barrier ^[258,259] Reduces activity in murine colitis ^[259] Alters composition of microbiota ^[259] Limits inflammatory effects of LPS ^[260] Inhibits IL-8 and TNF- α in LPS cells ^[260]
TLR inhibitors	Oligodeoxynucleotides blocking TLR7 signaling ^[261]	Improves tests and reduces activity in murine model of lupus nephritis ^[261] Improves autoimmune lung injury ^[261]
Molecular interventions	Polysaccharide A ^[105,262]	Induces IL-10 producing Tregs ^[105,262] Protects against EAE in mice ^[105]
Short chain fatty acids	Acetate, propionate, butyrate ^[200]	Modulates gut signaling pathways ^[200] Inhibits histone deacetylases ^[200,264] Regulates gene expression ^[200] Enhances gut integrity ^[200]

Superscripted numbers in brackets are references. EAE: Experimental autoimmune encephalitis; IL: Interleukin; LPS: Lipopolysaccharide; NOD: Non-obese diabetes; PSC: Primary sclerosing cholangitis; RA: Rheumatoid arthritis; TLR: Toll-like receptor; TNF- α : Tumor necrosis factor- α ; Tregs: Regulatory T cells.

DEVELOPING TREATMENT STRATEGIES TO INVESTIGATE

The intestinal microbiome can be manipulated by dietary adjustments^[37,90,252], probiotic preparations^[197,253,254], supplements of vitamin A and retinoic acid^[255,256], antibiotics^[169,257], intestinal re-colonization^[33,107,109,110], pharmacological agents that decrease intestinal permeability^[258-260], molecular interventions that block TLR signaling and the production of pro-inflammatory cytokines^[261], molecular interventions (polysaccharide A) that stimulate anti-inflammatory responses^[105,262], and short chain fatty acids that modulate signaling pathways that affect gene expression, intestinal barrier integrity, and inflammatory responses^[200] (Table 3).

Antibiotics (tetracycline and minocycline) have reduced disease activity in rheumatoid arthritis, especially in seropositive patients with disease of short duration^[257]. Probiotic supplements containing *Bifidobacterium bifidum* have promoted the expansion of Tregs in cell cultures^[253], and probiotics enriched with strains of *Lactobacillus* alone or in combination with retinoic acid have prevented the development of type 1 diabetes in NOD mice^[254,263]. Gelatin tannate has been used in a murine model of acute colitis to protect the mucosal barrier, alter the composition of

the microbiota, and decrease inflammatory activity^[259]. Gelatin tannate has also been evaluated in LPS-stimulated cell cultures, and it has inhibited the expression of the intercellular adhesion molecule-1 and reduced the production of IL-8 and TNF- α in a dose-dependent fashion^[260]. Oligodeoxynucleotides designed to block TLR7 signaling have improved tests and reduced activity in a murine model of lupus nephritis and lung injury^[261]; polysaccharide A has induced IL-10 producing Tregs in experimental autoimmune encephalitis^[105,262]; and short chain fatty acids have modulated intestinal signaling pathways, inhibited histone deacetylases, regulated gene expression, and increased the integrity of the intestinal barrier^[200,264].

Manipulations of the intestinal microbiota have also shown promise in animal models and patients with liver disease. In rats with carbon tetrachloride-induced cirrhosis, antibiotic therapy and probiotic supplements have decreased systemic endotoxin levels and improved liver tests^[265], and in rats with ischemic/reperfusion liver injury, probiotic supplements with *Lactobacillus* have reduced the production of pro-inflammatory, pro-fibrotic cytokines and improved liver tests^[266] (Table 3). The intestinal microbiota have been implicated in the pathogenesis of PSC^[267,268], and a small randomized clinical trial has indicated that treatment with vancomycin or metronidazole can

improve serum alkaline phosphatase and bilirubin levels and decrease pruritus^[269]. Clarification of the role of the intestinal microbiome in autoimmune hepatitis is necessary to direct investigational strategies that would help develop ancillary interventions to improve the outcome of this disease.

Manipulations of the intestinal microbiota, especially with antibiotics, may have adverse consequences which must be defined in animal and human studies and counter-balanced against potential benefits (Table 3). The intestinal microbiome performs important digestive and detoxification functions, produces nutrients and short chain fatty acids that can affect intestinal integrity, defends against invading pathogens, and influences the innate and adaptive immune responses within and outside the intestine^[30,270,271]. Dysbiosis associated with antibiotics, especially in early age, may perturb immune tolerance for the microflora and predispose to other immune-mediated diseases (asthma, celiac disease, and type 1 diabetes)^[165,166,175,272]. Antibiotic manipulations may also favor the emergence of drug-resistant pathogenic or immunogenic microflora^[169]. The optimal nature and duration of the manipulations that might impact on the intestinal microbiome are uncertain, and the durability of the responses are unclear. Much work needs to be done to establish microbiome manipulation as a way forward in autoimmune hepatitis, but observations already made in diverse systemic immune-mediated diseases and the unmet needs in the management of autoimmune hepatitis justify rigorous evaluation of this possibility.

OVERVIEW

Dysbiosis has already been described in experimental and human autoimmune hepatitis^[69,70]; antibodies reactive to antigens homologous to bacterial antigens (atypical pANCA) have been commonly present in patients with the disease^[53,65]; and increased permeability of the intestinal mucosal barrier has been demonstrated^[70]. The intestinal microbiome is an available source of immune stimulatory antigens, products, and immune cells that already have been implicated in multiple systemic immune-mediated (rheumatoid arthritis, diabetes, multiple sclerosis, inflammatory bowel disease)^[35,38,40-43] and chronic liver diseases (NASH, PBC)^[51,54]. The role of the intestinal microbiome in the occurrence and behavior of autoimmune hepatitis warrants rigorous evaluation.

The sequencing of the 16S rRNA gene can be used to characterize the intestinal microbial population and determine disease-specific dysbioses^[236]. Microarrays comprised of thousands of microbial probes can be applied to enhance comprehension of the "pathological" components of the intestinal microbiome^[247,249]. WGS and NGS of genomic regions specific for the disease can define the disease-related metagenome^[79], and associations between gut-derived micro-organisms and

the immune-mediated mechanisms of autoimmune hepatitis can then be evaluated in experimental models.

Adjunctive forms of therapy may emerge to complement current immunosuppressive regimens. Individual bacterial species within the microbiome may be manipulated by diet, designer probiotics, re-colonization methods, antibiotics, selected vitamin supplements, and pharmacological agents that decrease intestinal permeability^[236]. Molecular interventions that block TLR signaling or modulate signaling pathways may also evolve to reduce pro-inflammatory cytokine production, limit unfavorable gene expression, and strengthen the integrity of the intestinal barrier^[200,261].

Major obstacles to the performance of these studies are the limited number of publicly available tools to analyze the microbial metagenome, especially in translational settings^[239], the multiplicity of environmental factors (diet, antibiotics, sanitation) that can affect variations of the microbiome among communities and between individuals^[273,274], and uncertainties about the roles of luminal and mucosal microbiota in directing the immune response^[81,275].

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Practice guidelines for the pathological diagnosis of primary liver cancer: 2015 update

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Abstract

In 2010, a panel of Chinese pathologists reported the first expert consensus for the pathological diagnosis of primary liver cancers to address the many contradictions and inconsistencies in the pathological characteristics and diagnostic criteria for PLC. Since then considerable clinicopathological studies have been conducted globally, prompting us to update the practice guidelines for the pathological diagnosis of PLC. In April 18, 2014, a Guideline Committee consisting of 40 specialists from seven Chinese Societies (including Chinese Society of Liver Cancer, Chinese Anti-Cancer Association; Liver Cancer Study Group, Chinese Society of Hepatology, Chinese Medical Association; Chinese Society of Pathology, Chinese Anti-Cancer Association; Digestive Disease Group, Chinese Society of Pathology, Chinese Medical Association; Chinese Society of Surgery, Chinese Medical Association; Chinese Society of Clinical Oncology, Chinese Anti-Cancer Association; Pathological Group of Hepatobiliary Tumor and Liver Transplantation, Chinese Society of Pathology, Chinese Medical Association) was created for the formulation of the first guidelines for the standardization of the pathological diagnosis of PLC, mainly focusing on the following topics: gross specimen sampling, concepts and diagnostic criteria of small hepatocellular carcinoma (SHCC), microvascular invasion (MVI), satellite nodules,

and immunohistochemical and molecular diagnosis. The present updated guidelines are reflective of current clinicopathological studies, and include a novel 7-point baseline sampling protocol, which stipulate that at least four tissue specimens should be sampled at the junction of the tumor and adjacent liver tissues in a 1:1 ratio at the 12, 3, 6 and 9 o'clock reference positions. For the purposes of molecular pathological examination, at least one specimen should be sampled at the intratumoral zone, but more specimens should be sampled for tumors harboring different textures or colors. Specimens should be sampled at both adjacent and distant peritumoral liver tissues or the tumor margin in order to observe MVI, satellite nodules and dysplastic foci/nodules distributed throughout the background liver tissues. Complete sampling of whole SHCC ≤ 3 cm should be performed to assess its biological behavior, and in clinical practice, therapeutic borders should be also preserved, even in SHCC. The diagnostic criteria of MVI and satellite nodules, immunohistochemical panels, as well as molecular diagnostic principles, such as clonal typing, for recurrent HCC and multinodule HCC were also proposed and recommended. The standardized process of pathological examination is aimed at ensuring the accuracy of pathological PLC diagnoses as well as providing a valuable frame of reference for the clinical assessment of tumor invasive potential, the risk of postoperative recurrence, long-term survival, and the development of individualized treatment regimens. The updated guidelines could ensure the accuracy of pathological diagnoses of PLC, and provide a valuable frame of reference for its clinical assessment.

Key words: Liver cancer; Hepatocellular carcinoma; Intrahepatic cholangiocarcinoma; Practice guidelines; Pathology; Diagnosis

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Core tip: Given the high prevalence of primary liver cancers in China, the present 2015 guidelines were formulated in response to the clinicopathological evidence amassed over the past 5 years. The guidelines included suggestions for a 7-point baseline sampling protocol, updated the definition of small hepatocellular carcinoma (HCC), described a grading system of microvascular invasion for routine pathological diagnosis, and included molecular diagnostic principles, such as the importance of clonal typing for determining the clonal original patterns and therapeutic strategy of postoperative recurrent and multinodule HCC.

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INTRODUCTION

Primary liver cancers mainly refer to malignancies that originate from hepatocytes hepatocellular carcinoma (HCC), which account for the majority of PLC, and intrahepatic cholangiocytes intrahepatic cholangiocarcinoma (ICC). PLC ranks as the second leading cause of cancer death worldwide, among which HCC is one of the most lethal malignancies because of its high morbidity and mortality, as well as aggressiveness. An estimated 782500 new PLC cases and 745500 deaths occurred worldwide during 2012, with China alone accounting for about 50% of the total number of cases and deaths^[1]. In China, current trends in the crude incidence and mortality of PLC are 28.71/100000 and 26.04/100000, respectively, making it the fourth most common cancer and the second leading cause of cancer related-death^[2].

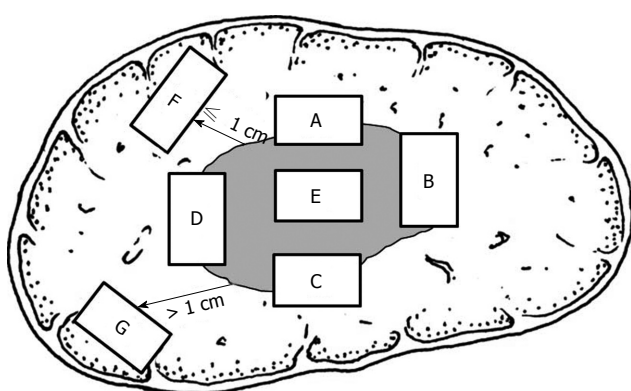
Hepatic pathology is a foundational subject in the field of hepatic surgery, the preferred first-line treatment for PLC. In an effort to ensure the accuracy of pathological diagnosis, a standardized process of pathological examination is required to provide a valuable frame of reference for the clinical assessment of the risk of postoperative recurrence, long-term prognosis, as well as individualized treatment regimens. However, most current practice guidelines for PLC focus on the clinical treatment^[3,4]. To our best knowledge, no consensus guidelines for the pathological diagnosis of PLC have ever been published. The lack of such guidelines has led to many contradictions and inconsistencies in the pathological characteristics and diagnostic criteria for PLC. To address this gap, Chinese pathologists developed an expert consensus on the pathological diagnosis of PLC in 2010^[5].

Since the development of these guidelines, much progress in the clinical management and pathological assessment of PLC has yielded many new concepts, such as tumor heterogeneity, pathobiological characteristics, molecular classification, personalized therapy and precision medicine, etc. The inclusion of these new concepts has become the cornerstone for the clinical management of PLC, placing greater demands on more stringent criteria and standards for hepatic pathological diagnosis. Therefore, in April 18, 2014, under the guidance of renowned academicians Prof. Wu Meng-Chao, Prof. Tang Zhao-You and Prof. Liu Tong-Hua, a Guideline Committee consisting of 40 specialists (supplementary materials) from Chinese Pathology, Surgery, Hepatology and Oncology Societies was created for the formulation of updated guidelines for the standardization of the pathological diagnosis of PLC.

The principal goals of the Guideline Committee include (1) incorporating the results of worldwide clinicopathological studies in PLC over the past 5 years according to the Evaluation Criteria of Grades of Evidence recommended by the American Association for the Study of Liver Diseases guideline (Table 1)^[6];

Table 1 Grades of evidence and classes of recommendations

Description	
Grade of evidence	
A	Data derived from multiple randomized, controlled trials or meta-analyses
B	Data derived from a single randomized trial or nonrandomized studies
C	Evidence based on clinical experience, descriptive studies, and opinion of respected authorities where further research is highly likely to impact confidence on the estimate of clinical effect
Class of evidence	
I	Conditions for which there is evidence and/or general agreement that a given diagnostic evaluation, procedure, or treatment is beneficial, useful, and effective
II	Conditions for which there is conflicting evidence and/or divergence of opinion about the usefulness/efficacy of a diagnostic evaluation, procedure, or treatment
II a	Weight of evidence/opinion is in favor of the usefulness/efficacy
II b	Usefulness/efficacy is less well-established by evidence/opinion
III	Conditions for which there is evidence and/or general agreement that a diagnostic evaluation/procedure/treatment is not useful/effective and in some cases, may be harmful


Figure 1 Specimen sampling sites.

(2) accepting the comments and suggestions of experts in hepatic pathology, surgery and oncology; (3) responding to the clinical concerns for improving the therapeutic efficacy for PLC; and (4) creating guidelines for the standardized pathological diagnosis of PLC. To meet these goals, the Guideline Committee organized several seminars for guideline formulation, mainly focusing on the following topics: gross specimen sampling, concepts and diagnostic criteria of small HCC (SHCC), microvascular invasion (MVI), satellite nodules, immunohistochemical and molecular diagnosis. The final version of the 2015 guidelines was approved at the last Guideline Committee meeting, which was held in February 1, 2015 in Shanghai, China.

GENERAL PATHOLOGY

Sample collection, fixation and processing

Peritumoral zones are representative of tumor heterogeneity in that they are rich in highly invasive cells, susceptible to the formation of MVI and satellite nodules and, therefore, more likely to impact liver cancer metastasis, postoperative recurrence and prognosis^[7,8]. Therefore, sampling around the periphery of tumor tissues is critical for objectively evaluating the biological behaviors of PLC. Specifically, a 7-point

baseline sample collection protocol is recommended (Figure 1). (1) At least four tissue specimens should be sampled at the junction of the tumor and adjacent liver tissues in a 1:1 ratio at the 12 (A), 3 (B), 6 (C) and 9 (D) o'clock positions; (2) for the purpose of molecular pathological examination, at least one specimen should be sampled at the intratumoral zone (E), but more specimens should be sampled for tumors harboring different textures or colors; (3) specimens should be sampled from both adjacent peritumoral liver tissues (F, ≤ 1 cm from the tumor capsule) and distant peritumoral liver tissues (G, > 1 cm from the tumor capsule) or the tumor margin in order to observe MVI, satellite nodules and dysplastic foci/nodules distributed throughout the background liver tissues; and (4) Tissue blocks should be approximately 1.5 cm - 2.0 cm \times 1.0 cm \times 0.3 cm in size and marked according to their sampling sites.

Regarding tissue fixation, the following recommendations were made to assure the quality of the tissues for pathological and immunopathological examination^[9]. (1) The surgeons should fill an Application Form of Pathological Examination describing the clinical diagnosis, location and type of lesions and number of tissues. The surgical margin, suspected lesions, important vessels and bile duct margin

should be marked with a dye or suture by surgeons. Small resected tissues, such as lymph nodes, should be placed into different containers and labeled with corresponding descriptions; (2) to maximally preserve the integrity of intracellular nucleic acids and proteins for avoiding autolysis, tumor specimens should be transferred to the Department of Pathology as soon as possible after resection, ideally within 30 min after surgical removal for sectioning and fixation^[10]; (3) the fresh specimens should be cut consecutively into 1-cm-thick multiple sections at the maximal diameter; a portion should remain unfixed fresh or cryopreserved for molecular examination; and (4) at room temperature, tissues should be fixed in a neutral formalin solution (v:v, 1:4-5) for 12-24 h and embedded in paraffin. Sections of 5- μ m thickness should be cut from each block and stained with hematoxylin and eosin for histological examination^[11].

Recommendations: (1) Hepatic tumor samples should be collected using the 7-point baseline sampling protocol; (2) the location and number of liver tissues collected should be determined as appropriate according to the size, shape and number of the liver tumors as well as the adjacent liver tissues; (3) because the detection rate of MVI and satellite nodules is related to the extent of adjacent liver tissues, it is necessary to describe the size of the adjacent liver tissues, and the suspected lesions should be sampled after reviewing several sections (C, I); and (4) when the tumor tissue is close to the surgical margin, sampling should be done at the region vertical to the margin closest to the cancer. When the tumor tissue is far away from the surgical margin, sampling should be done parallel to the surgical margin. The status of the surgical margin should be determined using the section with maximal area (C, I).

Description of macroscopic characteristics and clinical significance of SHCC

In the description of general hepatic tissue characteristics, pathologists should emphasize the size, number, color and texture of the tumor, its relationship with blood vessels and the bile duct, tumor capsule, tumor involvement, peripheral liver lesions, type of hepatic cirrhosis, the shortest distance between the tumor and surgical margin, and the status of the surgical margin. For tumor tissues with atypical morphology, the tissues should be photographed. Gross classification of HCC may reference the criteria developed by the Chinese Pathology Working Group for Liver Cancer^[12], and the Guidelines for the Diagnosis and Treatment of Primary Liver Cancer (2011 Edition) proposed by the Chinese Minister of Health^[13], in which a single tumor ≤ 1 cm in diameter is defined as microtumor, and a single tumor from > 1 cm to ≤ 3 cm in diameter is defined as SHCC. According to the classification system proposed by the World

Health Organization (WHO), ICCs are classified as mass-forming, periductal-infiltrating, and intraductal growth^[14].

Although some studies considered that patient outcomes might not be impacted as tumors reach > 5 cm in size^[15], discerning the presence of SHCC is important in the early diagnosis and therapy initiation for patients with liver cancer as it is a key step in the development and progression of HCC. However, the definition of SHCC varies greatly by international criteria - from 2 cm to 5 cm in diameter^[15]. Studies indicating that HCC growing near to or larger than 3 cm in diameter is an important turning point in the transformation of a tumor from having relatively benign features to more aggressive behaviors^[16,17]. Furthermore, the unique genetic changes in those SHCC ≤ 3 cm in diameter during the early stage have been reported^[18,19]. More data indicated that patients with tumors > 3 cm have an increased risk for MVI, satellite nodules, as well as poor prognosis^[17,20]. Specifically, the overall postoperative 5-year survival and recurrence-free survival of patients with SHCC ≤ 3 cm are 67.8% and 52%, respectively, which are significantly higher than that of 42.3% and 29.3% in patients with HCC > 3 cm, respectively ($P < 0.001$)^[17,21]. Moreover, up to now, most studies on patients with SHCC ≤ 2 cm are based on multi-center joint studies with long-term data collection because too few surgical cases in a single center exist. At present, there are almost no systematic studies or knowledge based on a large series of cases that describe the pathobiological characteristics of SHCC ≤ 2 cm^[16,19,22].

Recommendations: (1) SHCC of ≤ 3 cm is frequently well-differentiated with expansive growth, and has a low risk for MVI and satellite nodules^[17], which is suggestive of a relatively benign biological behavior in the progression to malignancy and is the basis of radical therapy. Thus, radical therapy should be initiated at an early stage before the tumor becomes highly invasive (B, I); and (2) in a small number of cases, SHCC may be poorly differentiated, invasive, or containing MVI and satellite nodules, which is indicative of highly malignant behavior. Given the heterogeneity of HCC, complete sampling of SHCC ≤ 3 cm should be performed to assess its biological behavior, and in surgical practice, therapeutic borders should also be preserved, even in SHCC (B, I).

Description of microscopic characteristics

Previous studies have described the analysis of microscopic tissue characteristics that include the following^[14,23]: (1) histological types of HCC, including common histological types (e.g., thin trabecular type, thick trabecular type, pseudoglandular type, compact type and fibrolamellar type, etc); (2) HCC cell type (e.g., clear cell type, lipid-rich type, spindle cell type and undifferentiated type, etc); (3) differentiation state

of HCC as assessed by the Edmondson-Steiner four-grading system; (4) the area and severity of tumor necrosis (e.g., interventional therapy), lymphocyte infiltration and interstitial fibrosis; (5) adenocarcinoma is the most common histological type of ICC, although it may also present in other special histological and cell types and its differentiation degree can be classified as well, intermediate and poor; (6) tumor growth patterns, including peritumoral invasion, capsule invasion, MVI and satellite nodules; and (7) the presence of chronic liver disease, such as chronic hepatitis or hepatic cirrhosis. Although there are many systems for grading and staging chronic viral hepatitis^[24-29], a simple histologic scoring system is recommended for routine pathological diagnosis, such as the Scheuer scoring system, *etc.* Furthermore, Masson's trichrome staining and reticular fiber staining can be routinely undertaken to assess the degrees of hepatic fibrosis and lobule reconstruction, respectively^[30].

Description of precancerous lesions

The main types of precancerous HCC lesions include the following^[23,31,32]: liver cell dysplasia, dysplastic foci, low-grade dysplastic nodule (LGDN), and high-grade dysplastic nodule (HGDN). Liver cell dysplasia refers to either large cellular changes, including increased cellular and nuclear volumes, nuclear pleomorphism, hyperchromatic chromatin and multinucleation, and small cellular changes, including decreased cell volume, increased nuclear-to-cytoplasm ratio with mild pleomorphism and hyperchromasia, but showing crowded nuclei. Dysplastic foci are lesions that are ≤ 1.0 mm in diameter and commonly composed of hepatocytes with small cell changes. LGDN is a nodule mainly comprising large cellular changes without obvious atypia, isolated interstitial arteries, or expansive growth patterns. In contrast, HGDN is composed of small cellular changes with increased atypia, isolated interstitial arteries, and expansive growth. "Nodule-in-nodule" is used to describe a focal malignant lesion occurring within a HGDN. According to the WHO classification system, hepatocellular adenoma (HCA) can be classified into four molecular pathological subtypes, including hepatocyte nuclear factor 1 α -inactivated HCA, β -catenin-activated HCA, inflammatory HCA and unclassified HCA, among which, β -catenin-activated HCA may have a higher risk of malignant transformation^[23,31,32].

The main types of precancerous lesions in ICC include biliary intraepithelial neoplasia (BilIN) and intraductal papillary biliary neoplasm (IPBN), as well as others^[14]. BilIN is usually graded as BilIN-1 (low-grade lesions), BilIN-2 (intermediate-grade lesions), and BilIN-3 (high-grade lesions or carcinoma *in situ*), according to the degree of nuclear atypia observed in biliary epithelial cells. IPBN refers to tubular papillary tumors with growth confined to the bile duct lumen. In

addition, IPBN may have BilIN with different grades. Along with BilIN and IPBN, other types of precancerous lesions in ICC include mucinous cystic neoplasms and biliary hamartomas harboring a high degree of BilIN, which may also correlate with increased risk of ICC.

Recommendations: It is important to conduct a differential diagnosis between HGDN and well-differentiated SHCC, the latter of which may manifest morphologically as varying degrees of increased cellular density and nuclear-to-cytoplasmic ratio, widened trabecular space, pseudoglandular structures, infiltrative growth, increased MVD as assessed by CD34 staining, higher Ki-67 index, and positive expression of p53 and glypican-3 (GPC-3), *etc* (B, I).

PATHOLOGICAL DIAGNOSIS OF MVI

MVI is also known as microvascular cancer embolus and refers to the cancer cell nest in vessels lined with endothelial cells. The incidence of MVI in liver cancer patients ranges from 15% to 57.1%^[33], which may be partly ascribed to differences in the sample collection protocol and diagnostic criteria between studies. MVI is most frequently found in the small branches of the portal vein in the adjacent liver tissues (including vessels of the cancer capsule) because these vessels are the major ones exiting the tumor, as the portal vein shows disordered hemodynamics^[34,35]. Branches of the hepatic vein are the secondary vessels exiting the tumor and may also develop MVI. Occasionally, the hepatic tumor may invade the hepatic artery, bile duct and lymphatic vessels, which should be reported independently^[33,36]. To differentiate the vascular nature of the tumor, immunohistochemistry may be performed to examine the expression of CD34 (vascular endothelium), smooth muscle α -actin (vascular smooth muscle), elastic fibers (elastic fiber layer of tiny blood vessel wall) and D2-40 (lymphatic endothelium).

Clinical studies indicate that MVI is related to poor prognosis in patients with HCC, including increased risk for postoperative recurrence and reduced long-term survival. In patients with HCC, a correlation between higher MVI grade and shorter disease-specific survival and recurrence-free survival has been noted^[37]. In a systemic review that included 20 observational studies of patients undergoing liver transplantation (LT), the presence of MVI shortened their 3-year disease-free survival [RR = 3.41 (2.05-5.7)] and 3-year overall survival [RR = 2.41 (1.72-3.37)]^[33]. A similar correlation between MVI and poor prognosis was also observed in patients with SHCC of ≤ 3 cm^[38]. Furthermore, Pawlik *et al.*^[39] found that the occurrence of MVI was positively correlated with the size of HCC, suggesting that the size and number of tumors are important predictive indices for MVI. Furthermore, Roayaie *et al.*^[40] found that vascular smooth muscle involvement of MVI and > 5 MVIs were closely related

to postoperative recurrence, and MVI located > 1 cm away from the adjacent liver tissues was associated with postoperative survival^[41]. There is also evidence showing that the presence of ≥ 50 loosely suspended cancer cells in MVI is closely related to the prognosis of patients with PLC. In contrast, the presence of < 50 loosely suspended cells in the lumen should be described in the report sheet and may be indicative of a low risk for recurrence^[34].

Recommendations

MVI is an independent prognostic marker for HCC patients (A, I); therefore, its presence should be evaluated in all tissue sections and graded according to the risk stratification based upon the number and distribution as follows: M0: no MVI; M1 (low-risk): MVI of < 5 and at ≤ 1 cm away from the adjacent liver tissues; and M2 (high-risk): MVI of > 5 or at > 1 cm away from the adjacent liver tissues (B, I).

PATHOLOGICAL DIAGNOSIS OF SATELLITE NODULES

Satellite nodules refer to the macroscopic or microscopic tumor cell nests located around or near, but separated from the main tumor with similar histological features as observed in the primary tumor. Generally, satellite nodules are derived from MVI. While difficult to distinguish from each other histologically, a diagnosis of satellite nodules is appropriate.

Studies show that the maximum micrometastasis spread distance (MMSD) in the distal area was < 3 cm in 92.3% of HCCs, and the MMSD in the proximal edge was < 1.5 cm in 91.7% of HCCs, suggesting that this area is important for pathological diagnosis and therapy^[42]. Lim *et al.*^[15] found that the incidence of satellite nodules was 7% and 23% in patients with HCC of < 5 cm and > 5 cm, respectively, indicating that satellite nodules were a factor predicting poor overall survival. Moreover, the presence of satellite nodules is also an important predictor of postoperative recurrence^[42]. The presence of MVI and satellite nodules may also provide a reference for the selection of clinical therapeutic modules. For example, Meniconi *et al.*^[43] found that in the absence of MVI and satellite nodules in the first resected HCC, a second hepatectomy or radiofrequency ablation for early intrahepatic recurrence predicted a better overall survival as compared to hepatic arterial chemoembolization.

Recommendations

Pathological diagnosis of satellite nodules should include the following pathological parameters^[44]: (1) number; (2) distribution and extent; and (3) presence of cancerous nodes in the distant adjacent liver tissues, including multinodular HCC (MNHCC), which may represent either intrahepatic metastases or *de novo* HCC arising from a polycentric origin. Molecular

cloning detection may then be helpful to elucidate the origin of the satellite nodules (B, I).

PROCESSING OF LIVER TISSUES

COLLECTED BY LIVER BIOPSY

Regarding the diagnosis of hepatic space-occupying lesions, a 16-gauge puncture needle is usually used to obtain a biopsy specimen containing junctional areas between the tumor and peritumoral zones or one from each zone. A relatively longer biopsy sample is required for the assessment of the degree of hepatic fibrosis or cirrhosis in the setting of chronic viral hepatitis. The appropriate tissue length should be longer than 1.5 cm and fixed with 10% neutral buffered formalin for 1-2 h, and ≥ 6 intermittent and consecutive sliced tissue sections should be placed on each slide for pathological evaluation^[45,46].

IMMUNOHISTOCHEMICAL DIAGNOSIS

For HCC, commonly used immunohistochemical markers for diagnosis include hepatocyte paraffin-1 (Hep Par-1), GPC-3, CD34, polyclonal carcinoembryonic antigen (pCEA), CD10, arginase-1, heat shock protein-70, and glutamine synthetase^[31,47,48]. Although Hep Par-1, CD10, arginase-1 and pCEA are hepatocyte-specific antigens, they cannot be used to distinguish benign and malignant hepatocellular tumors^[47]. For the immunohistochemical diagnosis of ICC, staining with antibodies specific for biliary cytokeratins, such as CK19, CK7 and mucin-1, is commonly employed. The diagnosis of dual phenotype HCC (DPHCC), a new highly aggressive subtype of HCC, is generally characterized by the expression of both HCC and ICC biomarkers^[7,49], and the diagnosis of DPHCC can only be made by immunohistochemical detection.

Although some reported biomarkers may aid in the evaluation and prediction of certain biological features of liver cancer, including risk for invasiveness, recurrence and long-term survival^[50,51], but further studies are required to confirm their clinical importance in multiple patient populations.

Recommendations

(1) Currently used biomarkers for liver cancer are somewhat imperfect in their diagnostic specificity and sensitivity; thus, a biomarker panel in combination with other tissue-specific markers could represent a useful tool for diagnosis and differential diagnosis between benign and malignant hepatocellular tumors, HCC and ICC, other specific types of hepatic tumors, and primary and metastatic liver cancer (B, I); and (2) although immunohistochemical staining for CD34 does not directly label hepatic parenchymal cells, it is valuable for determining the extent of MVD and examining its unique distribution pattern in different liver tumors. For instance, a diffuse staining pattern

is indicative for HCC, a scattered staining pattern for ICC, a patchy staining pattern for HCA, and a cord-like staining pattern for focal nodular hyperplasia, etc (B, I).

MOLECULAR PATHOLOGICAL DIAGNOSIS

Development of molecular classification techniques, including the detection of molecular targets and assessment of clonal origin, represents a promising new development in the field. Although many new systems based on molecular typing and predictive biomarkers have been reported in the literature^[52], validation of their clinical significance is still required through the use of controlled studies across multiple centers with large sample sizes. Specifically, the selection, detection and clinical significance of molecular targets for targeted drug therapy for PLC are still under investigation, but the results of preliminary clinical trials are worthy of high expectations^[53,54]. Regarding risk evaluation of precancerous lesions, molecular identification may be better suited than histopathological evaluation to detect the genomic instability for hepatocarcinogenesis and impact of clinical treatment modalities for patients with precancerous lesions, such as HGDN and HCA^[55]. Molecular pathology detection is conducive to optimization and choice of clinical treatment modalities^[56].

Postoperative recurrence of HCC (RHCC) seriously restricts the long-term curative effect of HCC treatments. Based on the clonal origin theory, a RHCC may originate from either a monocentric (monoclonal) origin or multicentric (polyclonal) origin. Theoretically, interventional therapy and targeted drug therapy are more suitable for RHCC that originate from residual cancer cells (monoclonal origin) after initial tumor resection, while repeated resections or liver transplantation are more appropriate for RHCC of multicentric origin that resembles a new primary tumor arising from a *de novo* tumor clone^[57]. However, due to long-term "latency" and "dormancy" of residual cancer cells left in the liver after resection, monoclonal recurrence may occur even in the so-called "late period" (> 2 years) after surgical resection, clinically coinciding with a special period for RHCC derived from multicentric origins^[58]. Although other researchers have proposed histological criteria for the clonal evaluation of RHCC^[59], the accuracy of such morphological criteria requires further validation of the molecular detection.

The clonal origin theory of RHCC is also applicable to MNHCC. Finkelstein *et al*^[60] reported that postoperative survival after LT was significantly better in patients with multicentric MNHCC than in those with monocentric MNHCC, indicating that genotyping has the potential to serve as a reference for LT recipient screening and prognostic evaluation. Gehrau *et al*^[61] also recommended a diagnostic and therapeutic roadmap based on the clonal detection of MNHCC that

would assist with patient selection for LT. Specifically, patients with MNHCC derived from multicentric origins could be ranked in the waiting lists to receive a LT, while those patients with monocentric MNHCC would be better suited for interventional treatment or targeted drug therapy using sorafenib^[61].

Recommendation

Evaluating the clonal origins of RHCC and MNHCC is vital to developing individualized therapeutic regimens and subsequently improving long-term clinical outcomes. Therefore, assessing the clonal origins of RHCC and MNHCC using molecular cloning methods may provide objective references for the formulation of individualized therapy plans (B, I).

CONCLUSION

A standardized pathological diagnostic process is the first precondition required for a correct pathological diagnosis, scientific clinical decision-making and precision treatment of PLC from the origin. Considering the high prevalence of PLC worldwide, especially in China, we report updated guidelines for pathological diagnosis of PLC, which includes standardized guidelines for specimen fixation, 7-point baseline sampling protocol and examination, a grading system for MVI in a routine pathology diagnosis, and immunohistochemical diagnostic panels, as well as molecular diagnostic principles, such as the importance of clonal typing of RHCC and MNHCC for determining therapeutic strategy and evaluating clinical prognosis.

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Glycoproteins and glycoproteomics in pancreatic cancer

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Abstract

Aberrations in protein glycosylation and polysaccharides play a pivotal role in pancreatic tumorigenesis, influencing cancer progression, metastasis, immunoresponse and chemoresistance. Abnormal expression in sugar moieties can impact the function of various glycoproteins, including mucins, surface receptors, adhesive proteins, proteoglycans, as well as their

effectors and binding ligands, resulting in an increase in pancreatic cancer invasiveness and a cancer-favored microenvironment. Recent advance in glycoproteomics, glycomics and other chemical biology techniques have been employed to better understand the complex mechanism of glycosylation events and how they orchestrate molecular activities in genomics, proteomics and metabolomics implicated in pancreatic adenocarcinoma. A variety of strategies have been demonstrated targeting protein glycosylation and polysaccharides for diagnostic and therapeutic development.

Key words: Glycoproteins; Glycosylation; Proteomics; Glycoproteomics; Pancreatic cancer; Pancreatic ductal adenocarcinoma

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Core tip: Protein glycosylation plays an important role in pancreatic tumorigenesis. Malignance induced changes in protein glycosylation can profoundly impact the function of a protein in multiple ways. One approach for developing better diagnostic and therapeutic strategies in pancreatic cancer involves targeting cancer-associated aberrant glycosylation. This review discusses the recent discoveries in glycoproteomics study of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is one of the most deadly cancers, in part because detection of pancreatic cancer is difficult

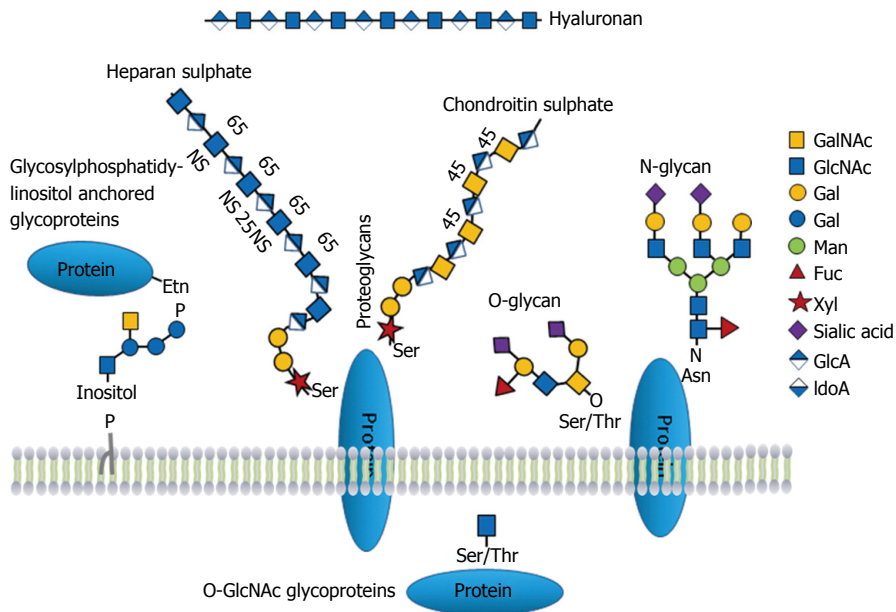


Figure 1 Illustration of protein glycosylation and polysaccharides.

at its early stages when surgical and other treatments are most effective^[1,2]. In addition, innate or adapted drug-resistance has been a major hurdle in pancreatic cancer chemotherapy^[3,4]. Malignance induced changes in protein glycosylation, such N-glycosylation and O-glycosylation, can profoundly impact the function of a protein in multiple ways, including protein maturation, expression, localization, as well as post-translational modifications, influencing a wide spectrum of glycoproteins and their binding ligands. One approach for developing better diagnostic and therapeutic strategies in pancreatic cancer involves targeting cancer-associated aberrant glycosylation. Recent developments of technology in proteomics and chemical biology have thus stimulated growing interest in elucidating the complex glycosylation events involved in pancreatic adenocarcinoma.

PROTEIN GLYCOSYLATION AND ITS IMPLICATION IN CANCER

Glycosylation is one of the most complex and common forms of protein post-translational modifications^[5,6]. It plays a pivotal role in many biological processes, such as protein folding, cell adhesion and trafficking, cell signaling, pathogen recognition and immune response^[7-11]. Protein glycosylation occurs in the endoplasmic reticulum and Golgi apparatus in multiple enzymatic steps. As illustrated in Figure 1, the most common protein glycosylations are N-linked and O-linked glycosylation. N-linked glycans are attached to the amide group of asparagine residues in a consensus Asn-X-Ser/Thr sequence (X can be any amino acid except proline)^[12]. O-linked glycans are linked to the hydroxyl group on serine or threonine

residues^[13]. One unique subclass of O-glycosylation is the phosphorylation-like, reversible O-GlcNAcylation^[14]. Less common forms of glycosylation include glycosylphosphatidylinositol anchors attached to protein carboxyl terminus, C-glycosylation that occurs on tryptophan residues^[15] and S-linked glycosylation through a sulfur atom on cysteine or methionine^[16]. In addition to protein glycosylation, proteoglycans and hyaluronan are major components of the extracellular matrix (ECM), which are implicated in cell proliferation and migration.

Most secretory and membrane-bound proteins produced by mammalian cells contain covalently linked sugar chains with diverse structures. The glycosylation form and density of glycans on a protein can be altered significantly in association with changes in cellular pathways and processes resulted from diseases, such as malignancy. In fact, altered glycosylation patterns have long been recognized as hallmarks in epithelial cancer^[17-22], including pancreatic ductal adenocarcinoma (PDAC), which accounts for about 90% of pancreatic cancer. Glycosylation abnormalities can be characterized by one or both of the following changes: (1) composition and structural alterations of glycan; and (2) change in the density of glycosylation at protein sites (hyper, hypo or neoglycosylation). Ultimately, malignant transformation is usually associated with one or both of these types of glycosylation alterations, leading to the expressional and functional changes of tumor-specific glycoproteins. Malignancy associated glycosylation abnormalities can influence cancer cell proliferation, invasion and viability, as well as interactions with tumor micro environment. Disruption or inhibition of glycosylation and carbohydrate-dependent cellular

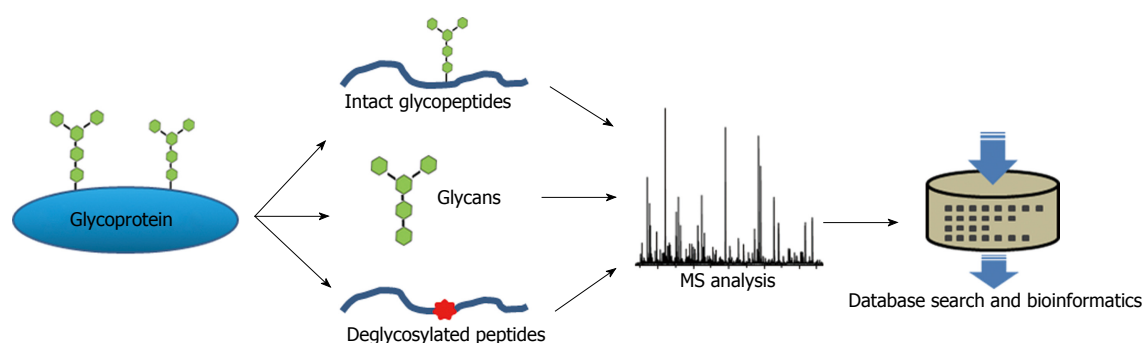


Figure 2 Mass spectrometry based glycoproteomics analysis.

pathways may represent potential modalities for cancer therapies^[23,24]. Receptor tyrosine kinases (RTKs), which are transmembrane glycoproteins that play important roles in malignancy and drug resistance, have been targets of anti-cancer drug development for various malignancies, including pancreatic adenocarcinoma^[25,26]. In addition, many of the current blood-based tumor markers are glycoproteins, including CA 19-9 for pancreatic cancer, CA 125 for ovarian cancer, CA 15-3 for breast cancer, and CA 242 for gastrointestinal cancer. CA 19-9, which detects the epitope of sialyl Lewis (a) on mucins and other adhesive molecules such as carcinoembryonic antigen^[27-29], is widely used for monitoring the clinical course of pancreatic cancer patients^[30]. To date, while implication of aberrant protein glycosylation in malignancy has been well recognized^[21,31], limited information is available describing the site specific glycoproteome changes associated with pancreatic cancer.

GLYCOPROTEOMICS METHODS

A number of proteomics studies in pancreatic cancer have been reported^[32-39]. As a subfield of proteomics, glycoproteomics uniquely focuses on analyzing glycosylated proteins to reveal glycoproteome alterations associated with pancreatic cancer. The major challenge for a comprehensive glycoproteomics analysis in a clinical sample arises from the biological intricacy within the molecule of a glycoprotein, including the variety in glycan composition and structure, as well as the complex linkage to the corresponding protein. Mass spectrometry has been the most effective and versatile instrument platform for both glycan and protein analysis. Although various sample preparation strategies may be applied to collect glycoproteins or glycans from different biological specimens, a glycoproteomics pipeline typically consists of glyco-enrichment, MS analysis and bioinformatics interpretation. The technical details of global analysis of glycoprotein can be found in a number of reviews on the subject of glycomics (analysis of glycans)^[40-43] and glycoproteomics (analysis of glycoproteins and

glycosites)^[15,44-52]. Figure 2 illustrates the overall approaches for MS analysis of glycoproteins. Prior to MS acquisition, glyco-enrichment strategies, including lectin affinity^[53-56], hydrazide chemistry^[57-60], boronic acid^[61], size-exclusion chromatography^[62], and hydrophilic interaction^[63], may be applied to enrich glycoproteins from complex biological samples, and thus, enhance analytical sensitivity. The direct analysis of intact glycopeptides with carbohydrate attachments is analytically challenging, but allows complementary identification of the peptide backbone and the glycan structure in a single measurement, providing site-specific glycosylation characterization directly. However, this approach is complicated by the mixed information obtained from the MS signals from the peptide backbone, the carbohydrate group and the combinations of both, and therefore, is largely limited for analyzing purified glycoproteins or simple systems. Alternatively, glycans, especially N-linked glycans, can be enzymatically or chemically cleaved from proteins or peptides and analyzed separately by MS. Using glycan databases and bioinformatics tools, MS analysis enables global identification of glycan species in a complex biological sample. On the other hand, de-glycosylated glycopeptides can also be profiled in a global fashion using shotgun proteomics approach to identify the amino acid sequence of the backbone peptides. The N-glycosylation sites can be precisely mapped using the consensus sequence of Asn-X-Ser/Thr, in which asparagine is converted to aspartic acid after PNGase F enzymatic cleavage, which introduces a mass difference of 0.9840 Dalton for MS identification. By defining the glycan structures and profiling the glycoproteins in complex clinical samples, disease associated aberrant glycan forms and site-specific occupancy on proteins can be revealed. For quantitative analysis, additional steps, such as differential stable isotope labeling of the sample and controls, may be required. Ultimately, to comprehensively address disease associated aberrant glycosylation, all the data obtained from different aspects of the workflow need to be integrated, so that the full extent of glycosylation changes with site-specific information can be better

revealed.

DISCOVERY OF ABERRANT GLYCOSYLATION IN BODILY FLUIDS

Identification and detection of abnormal protein glycosylation associated with pancreatic cancer in bodily fluids may present meaningful targets for cancer detection. A variety of carbohydrates and glycoproteins have been investigated for pancreatic cancer detection. Currently, CA19-9 is the only clinical biomarker test for management of pancreatic cancer^[64]. While CA19-9 is widely used for monitoring the clinical course of cancer patients, it does not provide adequate accuracy for pancreatic cancer diagnosis and early detection, underscoring the importance of obtaining molecular details on specific glycosylation events involved in neoplastic progression. Mucin (MUC) proteins, including MUC1, MUC5AC, and MUC16 are major protein carriers of CA 19-9, and play important roles in pancreatic cancer tumorigenesis, invasiveness and metastasis, in part through their characteristic glycoforms^[28,65-67]. Changes of MUC1 and MUC5AC in pancreatic cancer serum involved distinct glycan alterations, including Thomsen-Friedenreich antigen and fucose and Lewis antigens^[28]. The measurement of CA 19-9 antigen on MUC1, MUC5AC and MUC16 individually did not improve the performance of cancer detection, owing to the biological heterogeneity of the patients in their CA 19-9 protein carriers^[68]. However, the combined measurement of standard CA 19-9 assay and the detection of the CA 19-9 antigen on MUC5AC and MUC16 did improve the performance of pancreatic cancer detection^[68].

In addition to CA19-9, other aberrant protein glycosylations associated with pancreatic cancer have also been investigated in bodily fluids. The glycosylation of serum ribonuclease 1 (RNASE1) - another well-studied pancreas associated protein, showed a 40% increase in core fucosylation in pancreatic cancer^[69]. Using *Concanavalin A* lectin affinity chromatography for N-glycopeptide enrichment and LC MS/MS, one study identified 92 individual glycosylation sites and 105 unique carbohydrate structures in serum, and observed increased branching of N-linked oligosaccharides, as well as increased protein fucosylation and sialylation in the sera from pancreatic cancer patients^[70]. Increased level of sialyl Lewis X of major serum acute-phase proteins, including alpha-1-acid glycoprotein (AGP1 or ORM1), haptoglobin (HP), fetuin (AHSG), alpha-1-antitrypsin (SERPINA1) and transferrin (TF) were observed in the sera from patients with advanced pancreatic cancer and chronic pancreatitis - an alteration possibly associated with inflammatory response^[71]. In addition, the observation of an increase in core fucosylation on AGP1 and HP in the serum of advanced pancreatic cancer may represent a potential

cancer associated signal^[71]. Although the detection of increase level of fucosylated HP alone does not provide sufficient accuracy for pancreatic cancer diagnosis, it is possible that fucosylated HP might be used as an indication of liver metastasis if the biomarker undergoes further validation^[72,73]. The changes in protein fucosylation and sialylation in pancreatic cancer were also investigated by analyzing intact glycopeptides. Using immunoprecipitation, partial deglycosylation and LC MS/MS, one study suggested that the core-fucosylation levels at site N396 and N1424 in alpha-2-macroglobulin (A2M) were decreased in serum of both pancreatic cancer and chronic pancreatitis compared to non-diseased controls^[74]. The investigation of sialylated N-glycopeptide levels in sera from pancreatic cancer patients in comparison to non-diseased controls and acute pancreatitis patients identified 13 glycoforms, mainly from high-abundant serum proteins, with changes associated with pancreatic cancer group^[75]. Mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) are pancreatic cysts that are subject to high risk of malignant transformation. Proteomic and glycomic investigation of cyst fluids collected from patients with MCN and IPMN led to the identification of 80 N-linked glycans, and several hyper-fucosylated glycoproteins, including triacylglycerol lipase and pancreatic α -amylase^[76].

GLYCOPROTEOMICS OF PANCREATIC CANCER CELLS AND TISSUES

Known tumor-specific glycoproteins, such as mucins and carcinoembryonic antigen-related cell adhesion molecules, have been extensively studied for their roles in neoplastic progression and metastasis of pancreatic cancer^[77-81]. The emerging technology of glycoproteomics has been recently applied to interrogate broader changes of glycoproteome in pancreatic cancer cells and tissue. A large number of cell surface proteins are transmembrane glycoproteins, including many of cell-surface receptors such as RTKs, which play pivotal roles in signaling, trafficking and cell-cell interactions. These cell-surface receptors, such as epithelial growth factor receptor (EGFR), integrins, and TGF β receptor (TGF β R) have been important targets for anti-cancer therapy, and their glycosylation forms impact their functionality^[82-85]. Using a biocytin hydrazide cell surface capturing technique^[86], or azido sugar based bioorthogonal chemical reporter for metabolic glycan labeling^[87] for glycopeptide enrichment, studies were carried out to profile N-linked glycopeptides derived from surface glycoproteins of pancreatic cancer cells using LC MS/MS^[88,89]. The studies indicated the overexpression of CD109^[88] and ecto-50-nucleotidase^[89] in pancreatic cancer cells and tissues. Using multi-lectin affinity chromatography

and LC-MS/MS, another study investigated the differential glycoproteins associated with pancreatic cancer CD24⁺CD44⁺ stem-like cells in comparison with CD24⁻CD44⁺ cells^[90]. The study indicated that the high expression and high positive rate of CD24 was significantly associated with late-stage pancreatic adenocarcinomas, while CD13 expression and positive rate were negatively associated with tumor progression. By manipulating exogenous substrate supply, a study reported that increases in metabolic flux through the sialic acid pathway could dramatically enhance the sialylation of certain N-linked glycoproteins to influence cancer cell adhesive and mobility properties of SW1990 pancreatic cancer cells^[91].

Glycoproteomic techniques were also applied to investigate the glycoproteome of pancreatic cancer tissues. In our study, we observed an overall increase in N-glycosylation level on many glycoproteins in PDAC tissue in comparison with normal pancreas^[92]. Supplemental Table 1 summarizes some of the glycoproteins with at least one N-glycopeptide overexpressed (≥ 2 fold) in pancreatic cancer, including many pancreatic cancer associated proteins, such as MUC5AC, carcinoembryonic antigen-related cell adhesion molecule 5, insulin-like growth factor binding protein (IGFBP3), cathepsin D (CTSD), as well as a number of CD antigens (including CD44 - a marker of pancreatic cancer stem-like cells) and integrins. Pathway analysis suggested that increased N-glycosylation activities of these proteins were implicated in several pancreatic cancer pathways, including TGF- β , TNF, and NF-kappa-B^[92]. Other glycoproteins, such as Thy-1 membrane glycoprotein (THY1), which was recently developed into an ultrasound molecular imaging marker for pancreatic cancer detection^[93], was found heavily N-glycosylated in pancreatic cancer tissues. Further mapping of N-glycosylation sites revealed that the change of N-glycosylation level in pancreatic cancer was not only protein specific, but also glycosylation site specific. Specific N-glycosylation sites within certain individual proteins can have significantly altered glycosylation occupancy (e.g., a change in glycan density) in pancreatic cancer, reflecting the complex nature of glycosylation events underlying pancreatic tumorigenesis. Notably, the increase of N-glycosylation of many of these proteins was also found in chronic pancreatitis tissue, supporting the notion that pancreatic cancer and chronic pancreatitis share many common clinical and molecular features^[94-98]. It is also noteworthy to mention that in contrast to many glycoproteins with increased N-glycosylation, pancreatic secretory granule membrane major glycoprotein (GP2) - a pancreas specific glycoprotein, showed a reduced N-glycosylated level in both cancer and chronic pancreatitis tissues. While the global data have revealed the aberrant N-glycosylation changes of many relevant proteins in pancreatic cancer tissues,

the orchestrated glycosylation mechanism underlying pancreatic tumorigenesis, immune response and pancreatic functional changes, remains poorly understood and warrant further investigation.

MUCIN GLYCOSYLATION

Mucins are high molecular weight glycoproteins produced by various epithelial cells, and have 21 family members. The mucins are heavily glycosylated in O- and N-linked glycosylation and implicated in PDAC through their characteristic glycoforms influencing tumorigenicity, invasiveness, metastasis and drug resistance. Mucins have been extensively studied in PDAC, and showed various expressional and glycosylation changes not only in pancreatic carcinoma, but also in pancreatic intraepithelial neoplasia (PanIN), IPMN and MCN^[66,67,99]. Several mucins, including MUC1, MUC4, MUC5AC and MUC16, are frequently upregulated in PDAC. Mucin core protein expression and the differential localization in PDAC and its precursor lesions have been well documented in the literature^[66,67,100-103]. In addition, mucin glycoforms also play an important role in modulating their functionality in tumorigenesis as well as cancer cell interaction with the tumor microenvironment. In fact, the glycan component can make up more than 50% of the molecular weight of a mucin glycoprotein.

The glycosylation of cancer associated mucins is largely associated with Tn antigen, sialyl Tn and fucosylated core 1 structures, forming the so-called tumor-associated antigens^[104]. Altered glycoforms of MUC1, MUC4 and MUC5AC were observed early in pancreatic cancer progression (PanINs) to late stage metastatic disease^[105]. The elevation of fucosylated core structures, fucose and Lewis antigen have frequently been detected on MUC1 and MUC5AC in the blood from patients with pancreatic cancer^[28]. Additionally, MUC16 and its sialofucosylated structures were reported overexpressed in pancreatic cancer cell and acted as a functional ligand for E- and L-selectin to enhance cancer cell metastatic spread^[106]. By stimulating pancreatic cancer cells with pro-inflammatory conditions, such as oxidative stress and cytokines, mucin glycosylation can be significantly altered in specific pancreatic cancer cell lines, suggesting a possible molecular link between inflammation, glycosylation alteration and adaptive responses of those pancreatic cancer cells^[107]. Efforts have also been made to use proteomic approaches to profile mucins in cyst fluids to enhance the discrimination of malignant pancreatic cyst lesions from those that are benign^[108].

ECM GLYCOPROTEINS, PROTEOGLYCANS AND HYALURONAN

In our proteomic study, we observed a large group of

ECM associated proteins overexpressed in pancreatic cancer and chronic pancreatitis tissues^[97]. Many of these proteins are glycoproteins and are involved in stellate cell activation and ECM organizational and structural changes, which regulate pancreatic fibrosis - one of the fundamental histological abnormalities observed in pancreatic adenocarcinoma and chronic pancreatitis. ECM components, including matrix proteins, proteoglycan proteins, galectins and hyaluronan, which interact with each other and form supramolecular complexes, are subjected to alterations during cancer progression, leading to cancer associated ECM^[109-115]. Abnormal protein glycosylation can significantly affect the mechanical properties of ECM, enhancing tumor cell migration^[110,116]. Studies have shown that ECM components associated with integrin-ECM axis are highly up-regulated in pancreatic cancer^[117]. The glycoforms of integrins, such as the presence of N-linked oligosaccharides, can regulate integrin function, affecting the cell-ECM interactions. In pancreatic cancer tissues, we observed increased levels of N-glycosylation, not only on several integrins (both α and β subunits), but also on ECM adhesion proteins, including collagens, fibronectin, vitronectin, and laminin (Supplemental Table 1)^[92]. These observations warrant mechanistic study to better understand how aberrant glycosylation of integrins and ECM adhesion ligands influence pancreatic cancer migration and malignant phenotypes. Galectins and fibulins play a role in organization of ECM supramolecular structure, such as basement membranes, by forming intramolecular bridges, binding to complex carbohydrates and ECM adhesive proteins^[118-120]. The core protein expression and N-glycosylation level of fibulin 1 were both found up-regulated in pancreatic cancer tissues^[92,97]. Galectin 1 (LGALS1) is a human extracellular lectin that specifically binds to β -galactoside sugars, including N- and O-linked glycans. Galectin 1 was overexpressed in the stroma of both pancreatic cancer and PanINs lesions^[121], and its expression was related to pancreatic cancer survival^[122,123]. Concurrently, the N-glycosylation level of endogenous ligands of galectin-1 in the ECM, including fibronectin (FN1), laminins and galectin-3-binding protein (LGALS3BP), were all up-regulated in pancreatic cancer tissue (Supplemental Table 1), implying an intensified interaction of galectins and their major binding partners in pancreatic cancer^[92]. Periostin (POSTN), an ECM protein involved in cell mobility and neovascularization^[124], has both up-regulated core protein expression and N-glycosylation levels in pancreatic cancer tissue^[92,97]. Cathepsins are proteases that are implicated in cancer invasion by degrading ECM, including proteoglycans and collagens. We observed up-regulation of both core protein expression and N-glycosylation level of cathepsins (CTSD, CTSL) (Supplemental Table 1) in pancreatic cancer tissue, suggesting its possible functional role in

pancreatic tumorigenesis^[92,94].

Proteoglycans are heavily glycosylated proteins with serine attached glycosaminoglycans (GAGs), such as heparan sulphate and chondroitin sulphate (Figure 1). Proteoglycans are an important component of ECM and affect multiple biological processes, including cell differentiation and proliferation, binding to cytokines, growth factors and morphogens. During tumorigenesis, the expression and glycosylation patterns of proteoglycans change in the stroma surrounding cancer, influencing tumor growth and neoplastic progression^[125]. In proteomics and other studies, the increased expression of proteoglycan proteins, including lumican, decorin, versican, and biglycan, has been observed in pancreatic cancer tissues or cells^[97,121,126-130]. Since GAGs are large, linear polysaccharides, to a certain extent, the biological function of proteoglycans can be governed by the interaction of the attached GAGs with other proteins. Most of proteoglycans also contain N- and O-linked glycans. In a quantitative glycoproteomics study, N-glycosylation levels of several major ECM proteoglycans, including decorin (DCN), biglycan (BGN), lumican (LUM), versican (VCAN), and aggrecan (ACAN), were found elevated in pancreatic cancer tissues (Supplemental Table 1)^[92].

Hyaluronan is a non sulfated glycosaminoglycan that is not covalently attached to proteoglycans and can have a very high molecule weight^[131]. CD44 and receptor for HA-mediated motility are the two main receptors for the anchorage of hyaluronan-rich ECM to the cell surface^[132,133]. Although it has relatively simple chemical composition, as one of the major components of ECM, hyaluronan is involved in promoting pancreatic cancer progression and chemoresistance^[132-134]. Aberrant production and deposition of hyaluronan provide a favorable microenvironment to enhance cancer cell proliferation, migration, invasion, angiogenesis, and limit the delivery of anti-cancer agents^[134-137]. Studies have also shown that the interaction between hyaluronan and its CD44 receptor is involved in the stemness and survival of cancer stem cells^[138], and may be relevant to pancreatic cancer CD24⁺CD44⁺ stem-like cells.

IMPLICATIONS IN ANTI-CANCER DRUG DEVELOPMENT

Protein glycosylation has become a prominent target for drug development. One strategy involves disruption of the protein glycosylation process, such as inhibition of glycosylation enzymes and hexosamine biosynthetic pathway, to reduce pancreatic cancer progression and tumor growth^[23,24]. Silencing O-GlcNAc transferase has shown to inhibit pancreatic cancer growth^[139]. Inhibition of N-glycosylation can influence the maturation and surface expression of RTKs (e.g., EGFR, IGF1R), and enhance chemosensitivity

of drug-resistant pancreatic cancer cells^[82]. Lewis-Y carbohydrate antigen is expressed by many epithelial cancers, including pancreatic cancer^[24,140], and has been a target for cancer vaccines and immunoconjugated chemotherapy^[141,142].

Mucins (especially MUC1, MUC4, MUC5AC, MUC16) are an important group of glycoproteins in pancreatic cancer and have been targeted for therapeutic treatment. Multiple efforts have been made to develop MUC peptide based vaccination for pancreatic cancer, unfortunately with no significant clinical effects^[99]. New data suggests that it may be important to incorporate cancer associated glycoforms in the vaccine design so that the specific immunogenic epitopes expressed in tumors can be better mimicked^[143-145]. Other mucin based targeted therapeutic approaches include radioimmunoconjugate of MUC1 antibodies and gene therapy designed to suppress MUC1 or mucin gene promoters^[99].

The ECM is consisted of various biopolymers, and plays a pivotal role in cancer invasion and metastasis. Several anti-cancer therapeutic strategies targeting different ECM components have been considered, including inhibition of heparanase and proteases, anti-integrin therapy, and inhibition of GAGs of proteoglycans^[146]. In addition, anti-cancer therapies targeting hyaluronan metabolic enzymes and hyaluronan-CD44 interactions have also been investigated^[147,148]. In pancreatic cancer, recent studies demonstrated that depletion of stromal hyaluronan surrounding tumor improved drug delivery and significantly enhanced the efficacy of gemcitabine treatment^[134,137,149].

CONCLUSION

Protein glycosylation is deeply involved in pancreatic tumorigenesis. Cancer associated changes in protein glycosylation and polysaccharides can profoundly affect cellular function and ECM organization, supporting tumor growth and metastasis, as well as influencing immuno-response and chemoresistance. The emerging technologies of glycoproteomics, glycomics and other chemical biology approaches provide powerful tools to interrogate the complex nature of protein glycosylation involved in pancreatic cancer. While significant efforts have been made, ranging from mechanistic investigation, to biomarker discovery and therapeutic development, many aspects of how glycosylation events orchestrate changes in cancer signaling pathways at the genomic, proteomic and metabolomic level to facilitate cancer progression remain to be elucidated. To analyze complex clinical samples and obtain an in-depth, comprehensive understanding of site specific glycosylation changes requires a concerted approach drawing from a variety of techniques. With the development of molecular techniques and bioinformatics, many of the current

technical obstacles may be transient. Nonetheless, many strategies have been demonstrated to target protein glycosylation and polysaccharides for diagnostic and therapeutic gains in pancreatic cancer. These studies have laid foundation and will provide experimental guidance for future investigations.

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Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases

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Abstract

Anti-tumor necrosis factor (TNF) antibodies are successfully used in the therapy of inflammatory bowel diseases (IBD). However, the molecular mechanism of action of these agents is still a matter of debate. Apart from neutralization of TNF, influence on the intestinal barrier function, induction of apoptosis in mucosal immune cells, formation of regulatory macrophages as well as other immune modulating properties have been discussed as central features. Nevertheless, clinically effective anti-TNF antibodies were shown to differ in their mode-of-action *in vivo* and *in vitro*. Furthermore, the anti-TNF agent etanercept is effective in the treatment of rheumatoid arthritis but failed to induce clinical response in Crohn's disease patients, suggesting different contributions of TNF in the pathogenesis of these inflammatory diseases. In the following, we will review different aspects regarding the mechanism of action of anti-TNF agents in general and analyze comparatively different effects of each anti-TNF agent such as TNF neutralization, modulation of the immune system, reverse signaling and induction of apoptosis. We discuss the relevance of the membrane-bound form of TNF compared to the soluble form for the immunopathogenesis of IBD. Furthermore, we review reports that could lead to personalized medicine approaches regarding treatment with anti-TNF antibodies in chronic intestinal inflammation, by predicting response to therapy.

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Key words: Mucosal immunology; Lamina propria mononuclear cells; Crohn's disease; Ulcerative colitis; Transmembrane tumor necrosis factor; Apoptosis

Core tip: To improve the response rates of patients with inflammatory bowel diseases to anti-tumor necrosis

factor (TNF) therapy, it is mandatory to understand the pleiotropic effects of these agents. Herein, we overview and discuss several immunological targets of anti-TNF antibody application and highlight the most important findings.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders mainly affecting the gut. The two main forms are Crohn's disease (CD) and ulcerative colitis (UC) that differ in several aspects, *e.g.* regarding the location and distribution of inflammation and in the mucosal cell populations involved in the immune reaction. CD is a segmental, transmural disorder that can affect the whole gastrointestinal tract and type 1 T helper cells have been associated to be involved in the pathogenesis^[1]. In contrast, UC is characterized by continuous inflammation of the colon, and a modified Th2 cytokine profile has been described^[2]. Additionally, the recently described Th17 cells have been reported to be present in both CD and UC^[3]. However, the precise etiology of IBD is still a matter of debate but there is general consensus that genetic predisposition, environmental factors and immunological dysfunction of tolerance against the intestinal microflora are involved in the immunopathogenesis^[4]. In this regard, mucosal CD4+ T cells as key mediators in driving immune responses are critically involved in the pathogenesis of IBD and in accordance, a high number of infiltrating T lymphocytes can be found in both CD and UC in the gut^[5]. Furthermore, recent studies strongly suggest that the proinflammatory cytokine tumor necrosis factor (TNF) is one of the major pathogenic cytokines involved in the pathogenesis of IBD as elevated levels of TNF are present in the serum of both UC and CD patients^[6]. In addition, an elevated number of TNF-secreting cells in the inflamed mucosa of IBD patients has been repeatedly reported^[7-9]. Herein, lamina propria mononuclear cells (LPMCs) isolated from colonic biopsies from IBD patients spontaneously produced increased amounts of TNF which correlated with the degree of tissue involvement and mucosal inflammation^[10], strengthening the importance of TNF in the inflamed gut.

In accordance, application of antibodies targeting TNF in IBD patients have been shown to induce clinical response in up to 60% of CD patients, and inducing long-term maintenance of remission in a large amount of patients^[11-13]. Comparable results have also

been described for the therapy of UC patients^[14,15]. Nevertheless, the molecular mechanism of action of TNF agents is still discussed and not all commercially available anti-TNF antagonists are effective in the therapy of IBD patients. Regarding the mechanism of action, TNF neutralization, diverse effects on the immune system, outside-to-inside signaling and importantly, induction of direct or indirect apoptosis have been suggested. Furthermore, first studies focusing on successful prediction of clinical response have been conducted, either *via* expression of various biomarkers^[16,17] or by endoscopic *in vivo* molecular imaging^[18]. In this review, TNF biology and different anti-TNF antibodies will be assessed, and the results of different aspects of the biological function of anti-TNF agents for the treatment of IBD will be discussed.

TNF BIOLOGY AND ITS SIGNALING PATHWAY

TNF is a crucial mediator in driving inflammatory processes in the gut. It is produced by a variety of mucosal cells, mainly macrophages and T cells, as a preform on the plasma membrane^[19]. In addition, Paneth cells in CD affected segments of the terminal ileum were shown to strongly express *TNF* RNA in contrast to Paneth cells in normal tissue, indicating an induction under pathogenic conditions^[20]. The transmembrane precursor form (mTNF), a homotrimer of 26 kDa subunits, is cleaved by the matrix metallo-proteinase TNF alpha converting enzyme (TACE/Adam17) into a soluble form (sTNF, a homotrimer of 17 kDa monomers)^[21-23]. The expression of mTNF by CD14+ macrophages has been reported to be relevant in IBD^[24] (Figure 1).

Both forms of TNF are biologically active and signal through two distinct receptors that differ in molecule mass: the 55-kDa TNFR1 (TNFRSF1A/CD120a) and the 75-kDa TNFR2 (TNFRSF1B/CD120b) glycoproteins^[25]. TNFR2 is mainly expressed on lymphocytes and endothelial cells, whereas TNFR1 is ubiquitously expressed and possesses an intracellular death domain^[26]. TNF and its receptors are crucially involved in the pathogenesis of IBD. For example, elevated levels of the soluble form of TNFR1 and TNFR2 have been detected in both CD and UC patients and their expression correlated with disease activity^[27].

Signal transduction of the membrane-bound form of TNF can be transmitted through both TNFR1 and TNFR2, whereas sTNF mainly signals through TNFR1. Binding affinity studies revealed that sTNF preferentially binds to TNFR1 with higher affinity^[28]. In contrast, TNFR2 is mainly activated by mTNF^[29]. Activation of TNFR1 by TNF induces an intracellular signaling cascade with pleiotropic effects involving apoptosis, cell proliferation or cytokine secretion. Activation of the nuclear factor kappa B (NFκB) following stimulation of TNFR1 results in translocation

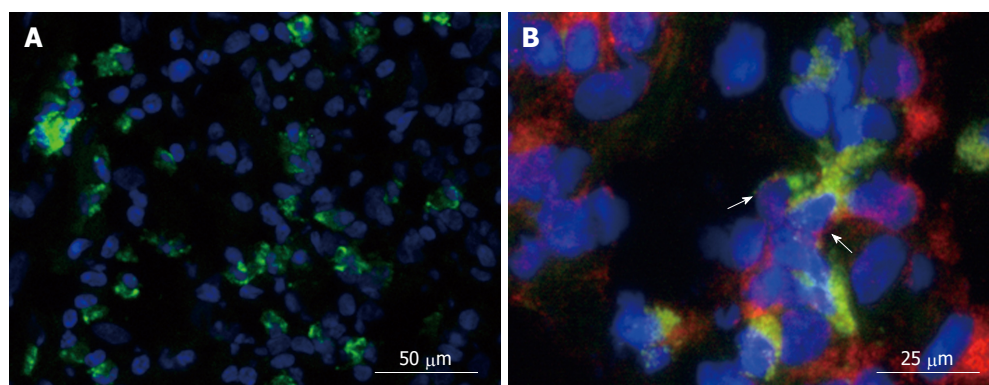


Figure 1 Expression of membrane-bound tumor necrosis factor in the gut. Gut tissue of patients with Crohn's disease (CD) was cryo-frozen and stained for cell markers by immunofluorescence. Nuclei were counterstained with DAPI. A: Staining for mTNF (green). B: Staining for mTNF (green) and CD14 (red). Co-expressing cells are labeled with an arrow.

to the nucleus and transcriptional upregulation of several genes such as IL-8, IL-1, IL-6, COX2 and TNF^[21]. Alternatively, TNFR1 can activate a Caspase-8 dependent signaling pathway *via* FADD resulting in apoptosis. The TNFR2 pathway does not contain a death domain and its stimulation can result in proliferation, migration and cytokine production such as IL-1 and IL-6. Furthermore, binding of mTNF to TNFR2 not only activates an intracellular signaling pathway, but can also result in reverse signaling within the TNF-expressing cell^[30] which will be later discussed in detail.

The role of the receptors of TNF in the pathogenesis of IBD remains only partly understood. A study using a colitis mouse model suggests that both TNFR1 and TNFR2 have protective functions in intestinal inflammation^[31]. Herein, intestinal inflammation was provoked by oral application of dextran sulfate in mice deficient for TNFR1 or TNFR2 as well as wildtype controls. TNFR1 or TNFR2 ablation resulted in exacerbation of colitis, possibly due to increased apoptosis of colonic epithelial cells^[31]. However, in other studies a central role of TNFR2 in mucosal inflammation has been proposed. For example, mutations in the gene of TNFR2 have been linked with IBD^[32,33], suggesting that this polymorphism could increase the disease risk. In accordance, its overexpression drives inflammation in a transgenic mouse strain overexpressing human TNFR2, causing a severe multiorgan inflammatory syndrome mainly affecting liver, pancreas, kidney and lung^[34]. Regarding IBD, TNFR2 expression was found to be upregulated on lamina propria and peripheral blood T cells in patients with CD^[35]. In accordance, TNFR2 was found to promote experimental colitis. Herein, T cells overexpressing TNFR2 were transferred into SCID mice that do not express T and B cells. In comparison to SCID mice that received wildtype T cells, transfer of T cells overexpressing TNFR2 resulted in more severe colitis and enhanced expression of T helper cells type 1^[35], underlining the importance of TNFR2 in intestinal inflammation. It could moreover be shown

that activation of mucosal TNFR2 expressing CD4+ T cells by mTNF expressing CD14+ led to heightened resistance of the intestinal lymphocytes to apoptosis, resulting in perpetuation of chronic intestinal inflammation in IBD^[24].

STRUCTURE AND APPLICATION OF ANTI-TNF ANTIBODIES

Infliximab

In 1998, infliximab (Remicade®) was the first antibody targeting TNF to be approved in the United States for the treatment of CD and later for the treatment of UC. It is a chimeric monoclonal antibody with 25% murine and 75% human sequences. Infliximab therapy is intravenously initiated at weeks 0, 2 and 6 and then applied every 8 wk for maintenance of remission. The starting concentration for both CD and UC is 5 mg/kg^[6].

CT-P13

The infliximab biosimilar CT-P13 (Remsima®, Inflectra®) was approved in Europe for the treatment of both CD and UC based on a comprehensive non-clinical comparability exercise and extrapolation of clinical data from two studies with rheumatoid arthritis patients. The biosimilar product is highly similar to its originator biological drug infliximab and is therefore clinically used in the same way.

Adalimumab

In contrast to infliximab, adalimumab (Humira®) is a monoclonal human antibody produced by CHO cells. It is approved for the treatment of both CD and UC. Adalimumab is administered subcutaneously and can be injected by the patient himself. At therapy initiation, adalimumab is administered at 160 mg and then 80 mg two weeks later. For maintenance of remission adalimumab is administered every 2 wk at 40 mg.

Golimumab

In 2013 golimumab (Simponi®), a fully human mono-

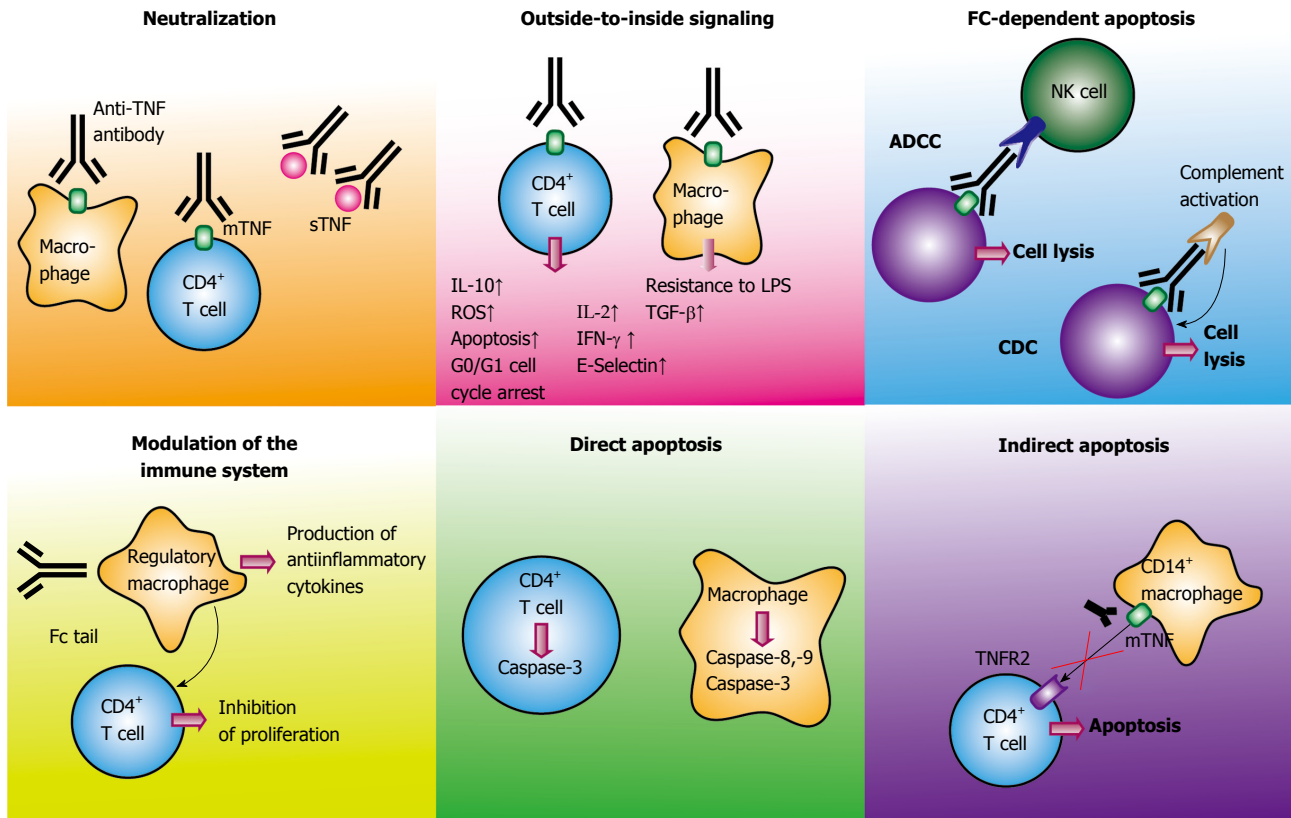


Figure 2 Mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel disease. Schematic illustration of different modes of action of anti-TNF antibodies in inflammatory bowel diseases. TNF: Tumor necrosis factor; mTNF: Membrane-bound TNF; sTNF: Soluble TNF; TNFR: TNF receptor; NK cell: Natural killer cell; ADCC: Antibody-dependent cellular cytotoxicity; CD: Crohn's disease; CDC: Complement-dependent cytotoxicity; LPS: Lipopolysaccharide.

clonal antibody was approved for the treatment of UC^[36]. Like adalimumab, it is administered subcutaneously. The induction dose is 200 mg and then 100 mg two weeks later. Maintenance of remission is achieved by 50 mg (< 80 kg body weight) and 100 mg (\geq 80 kg bodyweight) every 4 wk.

Certolizumab pegol

Certolizumab pegol (Cimzia®), a PEGylated humanized Fab' fragment is approved for the treatment of CD in the United States and Switzerland^[37] but not by the European Medicines Agency. In contrast to the other anti-TNF antibodies, this agent does not possess a Fc fragment and correspondingly, is not recognized by Fc receptors. The subcutaneous administration is performed at week 0, 2 and 4 with 400 mg. For maintenance of remission, certolizumab pegol is given every 4 wk in a dose of 400 mg.

Etanercept

Etanercept (Enbrel®) is a chimeric fusion protein that consists of the p75 part of TNFR2 and a human IgG1 Fc domain. Importantly, in combination with methotrexate, etanercept has similar efficacy in the therapy of rheumatoid arthritis as infliximab and adalimumab^[38]. It nevertheless failed to be effective in a clinical study with CD patients, when 25 mg were

subcutaneously applied twice per week^[39]. Etanercept is therefore not approved for treatment of CD.

MECHANISM OF ACTION OF ANTI-TNF ANTIBODIES

Although anti-TNF antibodies are approved for the therapy of IBD and other inflammatory diseases for many years, the mechanism of action is still a matter of debate. Initially, it was suggested that anti-TNF agents inactivate the proinflammatory cytokine TNF by direct neutralization, thus resulting in suppression of inflammation. Given the complexity of TNF signaling, a lot of studies indicate that anti-TNF antibodies exert more complex functions beyond simple blockade. Furthermore, functional characterization of etanercept and other anti-TNF agents can elucidate the signaling pathways that are important in IBD in contrast to other autoimmune diseases such as rheumatoid arthritis. In the following part we will list several described effects of anti-TNF antibody therapy that are suggested to be relevant in IBD (see also Figure 2).

TNF NEUTRALIZATION

Although all anti-TNF agents have the same target, the affinity of the antibodies to TNF has been reported not

Table 1 Comparison of affinity to tumor necrosis factor by anti-tumor necrosis factor agents

Target	Method /source	Affinity	Ref.
sTNF	SPR	GOL = ETA > IFX > ADA	[40]
sTNF	SPR	ETA > ADA = IFX	[41]
mTNF	mTNF-expressing cells (Sp2/0-11A5-1 myeloma cells)	IFX = ADA = ETA	[41]
mTNF	mTNF-expressing cells (K2 murine myeloma cells)	IFX = GOL > ADA > ETA	[40]
mTNF	PBMCs (T cells)	IFX = ADA > Certo >>	[42]
	Flow cytometry	No binding of ETA	
mTNF	PBL	IFX >> No binding of	[43]
	Flow cytometry	ETA	
sTNF	Immunoassay	IFX > ETA (rapid dissociation)	[44]

ADA: Adalimumab; ETA: Etanercept; GOL: Golimumab; IFX: Infliximab; Certo: Certolizumab pegol; PBL: peripheral blood lymphocytes; TNF: Tumor necrosis factor; mTNF: Membrane-bound TNF; sTNF: Soluble TNF; SPR: Surface plasmon resonance.

Table 2 Comparison of avidity to tumor necrosis factor by anti-tumor necrosis factor agents

Target	Method/source	Avidity	Ref.
sTNF	KinExA® (automated flow immunoassay system)	ETA > ADA IFX	[41]
mTNF	[44]	IFX > ETA	[44]

ADA: Adalimumab; ETA: Etanercept; IFX: Infliximab; TNF: Tumor necrosis factor; mTNF: Membrane-bound TNF; sTNF: Soluble TNF.

to be equal. An overview of respective affinities and avidities of anti-TNF agents is given in Tables 1 and 2. In TNF bioassays, infliximab, etanercept, adalimumab, certolizumab pegol and golimumab all neutralize sTNF^[23]. However, using the method of surface plasmon resonance, golimumab was shown to bind to soluble TNF with a similar affinity as etanercept, but with greater affinity than infliximab and adalimumab, the later showing the weakest affinity in the used setting^[40]. However, affinity of adalimumab and infliximab were reported to be similar although less compared to etanercept in another study^[41]. Regarding avidity, etanercept was shown to bind to sTNF with a 10- to 20-fold greater avidity as compared to adalimumab or infliximab^[41].

For mTNF, affinity of anti-TNF antibodies has been reported to be lower than to sTNF^[40,41]. Using mTNF-transfected cells, binding affinities of infliximab, adalimumab and etanercept were shown to be similar^[41]. In contrast, affinities for golimumab and infliximab to another mTNF-expressing cell line were comparable and greater than that of adalimumab, whereas all three anti-TNF agents had significantly greater affinity than etanercept^[40]. Furthermore, in other studies binding of etanercept to mTNF could not be detected, perhaps due to the fact that peripheral blood mononuclear cells (PBMCs) and not cell lines stably expressing mTNF were examined^[42,43], arising

the question to what extent the binding of etanercept to mTNF expressing cell lines represents the situation *in vivo*. Furthermore and in discrepancy to the above mentioned study, etanercept was shown to form relatively unstable complexes with sTNF, resulting in fast release of dissociated TNF in contrast to infliximab that bound to both sTNF and mTNF by forming stable complexes with high stability^[44]. However, interpretations of all presented data have to take into account the different cells, methods and techniques used for respective assessment of affinity, which could have a profound effect on the data obtained.

Importantly, another aspect is the variation of the different anti-TNF agents in regard to cross-linking towards mTNF. Herein, it has been reported that up to three infliximab molecules can bind to each TNF homotrimer. In contrast, etanercept was shown to bind to TNF trimer in a 1:1 ratio, suggesting that not all binding sides are blocked^[44]. This aspect together with the fact that infliximab forms more stable complexes with mTNF^[44] is a possible explanation why infliximab is effective in the therapy of IBD, whereas etanercept failed to show any clinical efficacy in a trial with Crohn's disease patients and is therefore not approved for IBD treatment. Recent findings indicate that the neutralization of mTNF rather than sTNF is crucial in the therapy of IBD. For example, in a mouse model of IBD the neutralization of sTNF alone was ineffective, in contrast to anti-TNF antibodies that block both sTNF and mTNF, indicating that neutralization of mTNF is crucial in the therapy of IBD^[45]. The pivotal role of mTNF was further demonstrated in an experimental colitis model by transferring CD4+ T cells into RAG2 deficient mice^[46]. Herein, by transferring TNF mutant T cells, disease activity was compared in absence of TNF, in absence of sTNF and in presence of both sTNF and mTNF. Whereas the expression of TNF was essential for the induction of colitis, the absence of sTNF did not protect the mice from intestinal inflammation^[46], clearly underlining the pathogenic role of mTNF in the pathogenesis of mucosal inflammation in the intestine.

MODULATION OF THE IMMUNE SYSTEM

Cytokines

Cytokines as mediators of inflammation are in imbalance in IBD. On the one hand proinflammatory cytokine levels such as TNF, IL-13, IL-17 or IFN- γ ^[1-3,6] are elevated and drive the massive tissue damage in the gut. On the other hand, anti-inflammatory cytokines that usually regulate overwhelming immune reactions are supposed to be also involved in the pathogenesis of IBD. For example, mice deficient of IL-10 spontaneously develop a chronic intestinal inflammation, which is dependent on the influence of the microflora^[47]. Interestingly, the enterocolitis exacerbates in mice deficient for both IL-10 and TNF, indicating that TNF has some protective effects in this context^[48]. Crucial mediators of inflammation in the gut

are neutrophils and granulocytes that accumulate in inflamed areas and secrete proinflammatory cytokines, chemokines and recruit effector cells to the site of inflammation. In this setting, Agnholt *et al*^[49] reported that GM-CSF, a growth factor for granulocytes and neutrophils, was decreased in CD patients after application of infliximab. In accordance, also reduced peripheral blood neutrophils and diminished infiltration of polymorphonuclear cells in the lamina propria were observed after anti-TNF application. Furthermore, *in vitro* experiments using an intestinal T cell culture system showed that GM-CSF secretion by T cells was inhibited by infliximab, suggesting direct effects of infliximab on T cells. Apart from GM-CSF, the release of the proinflammatory cytokine IL-1 beta after stimulation of human monocytes with lipopolysaccharide (LPS) was inhibited by anti-TNF agents. Herein, certolizumab pegol was the most potent inhibitor. Infliximab and adalimumab were also able to inhibit IL-1 beta release at higher concentrations whereas etanercept only partially prevented IL-1 beta release^[50]. Furthermore, *in vitro* analysis using PBMCs from patients with UC showed that incubation of the cells with infliximab inhibited secretion of the proinflammatory cytokines IFN- γ , IL-13, IL-17A and TNF^[51], cytokines reported to be critically involved in the pathogenesis of IBD. In addition, infliximab reduced the expression of TNF, IL-1, IL-6 and IL-8 by LPS-stimulated monocytes *in vitro*^[21,52]. In addition, the key Th1 cytokine IL-12 and the anti-inflammatory cytokine IL-10 were described to be affected by anti-TNF antibodies. Monocytes that were isolated from blood of healthy controls or CD patients expressed less IL-10 or IL-12 when they were incubated in the presence of adalimumab or infliximab, whereas etanercept had no effect in this setting^[53].

Interestingly, a recent study analysed the regulation of 14169 different genes by anti-TNF antibodies and compared the changes in anti-TNF responders versus non-responders. Intestinal biopsies from CD patients were used and compared to controls^[54]. They identified some genes that were regulated by anti-TNF treatment, such as IL-6, CD69, GPR183 and MMP9. Interestingly, the proinflammatory cytokine IL-6 was downregulated by anti-TNF agents but independently of clinical response to therapy. Of note, IL-1 beta and IL-17A were found to be significantly upregulated in patients with active CD and patients refractory to anti-TNF, in accordance with an enhanced Th17 cell population in IBD^[3], thereby representing possible targets in anti-TNF refractory patients. In contrast to this study, Katz *et al*^[55] found IL-17 levels not to be affected by anti-TNF therapy in CD patients but showed IL-2 to be significantly decreased during anti-TNF therapy. These contradictory findings may reflect biological differences between gene expression and protein expression.

T helper cell subsets

T cells are critically involved in the pathogenesis of IBD and therefore, modulation of immune responses by influencing the presence of T helper cell subsets is suggested to be an important effect of anti-TNF agents. Response and clinical remission could be the result of either a reduction in proinflammatory T cell subsets such as Th1, Th2 or Th17 cells or an enhanced presence of anti-inflammatory T helper cells, or both. In this regard, several studies investigated the number of pro- and anti-inflammatory T helper cell subsets before and after anti-TNF therapy. The influence of infliximab *in vitro* on isolated T cells from the peripheral blood of UC patients was examined by Dahlén *et al*^[51]. The T cell activation marker CD25 was reduced on both CD4+ and CD8+ blood T cells and their proliferation was inhibited after incubation with infliximab. In accordance, the levels of proinflammatory cytokines such as IFN- γ , IL-13, IL-17A, TNF and granzyme A were inhibited. The authors also investigated effects of infliximab on regulatory T cell populations; however, the results regarding CD4+ Foxp3+ T cells were ambiguous^[51]. Importantly, effects of infliximab on blood or mucosal T cells were shown not to be identical. Herein, Li *et al*^[56] described that infliximab therapy resulted in an increase in CD4+CD25+Foxp3+ and CD4+CD25-Foxp3+ regulatory T cell populations in the blood of both UC and CD patients; in contrast, *Foxp3* mRNA and protein were downregulated in the mucosa, possibly reflecting increased apoptosis rates or down-regulated T cell activation in the intestine during therapy. Other studies described general reduction of T cells in the mucosa after infliximab therapy for both CD^[57] and UC patients^[58]. A direct effect of adalimumab on T cell populations could be elegantly demonstrated by Maggi *et al*^[59]. Local injection of adalimumab along perianal fistulas in CD patients resulted in decrease of infiltrating CD4+ CD161+ T cells whereas no systemic effects on circulating T cells could be detected^[59].

In addition, infliximab has been shown not only to suppress CD4 T cell activation, but also to down-regulate IL-21 expression by mucosal CD4+ T cells and consequently, inhibit Th17 differentiation. Using intestinal biopsies of CD patients before and after infliximab therapy, the authors could clearly show that intestinal mucosal healing during therapy was due to down-regulation of mucosal infiltration of the highly proinflammatory Th17 cells through diminished IL-21 expression, a cytokine that is pivotal to Th17 cell differentiation^[60]. In summary, these studies clearly indicate that effects of anti-TNF antibodies on T cells are complex and differ between peripheral and mucosal T cells, as well as *in vivo* and *in vitro*.

Epithelial cells, angiogenesis and immunosuppressive macrophages

In addition to shifts in cell populations, effects of anti-

TNF antibodies on the epithelial barrier function have been also examined. For example, adalimumab has been shown to prevent barrier dysfunction induced by TNF *in vitro* using the intestinal epithelial cell lines Caco-2 and T-84^[61]. Furthermore, a role of infliximab in restoring epithelial barrier dysfunction in CD has been described. Herein, downregulation of epithelial apoptosis was noticed in 10 of 11 patients undergoing therapy with infliximab^[62]. Additionally, effects of anti-TNF antibodies on the mucosal microcirculation have been reported. In this context, infliximab was shown to downregulate CD40 expression by intestinal microvessels *in vivo* in the mucosa of CD patients on the one hand and to reduce the levels of sCD40L in the circulation on the other hand, indicating an inhibition of vascular inflammation in the gut^[63]. In addition, infliximab downregulated mucosal angiogenesis in CD patients^[64]. A direct effect of infliximab on myofibroblasts has been described by Di Sabatino *et al.*^[65]. Myofibroblasts isolated from CD patients expressed more mTNF than control myofibroblasts and infliximab significantly augmented the migration ability from CD myofibroblasts in a tissue inhibitor of metalloproteinases (TIMP)-1-dependent manner, suggesting another mode-of-action of anti-TNF antibodies that results in enhanced mucosal healing. However, the TIMP-1 production was also increased by adalimumab and etanercept, so it is still unclear if this effect is relevant for induction of clinical remission in CD. Interestingly, the induction of immunosuppressive macrophages has been suggested as mechanism of action of anti-TNF antibodies^[42]. Therefore, the influence of infliximab, adalimumab, certolizumab pegol and etanercept was investigated in PBMCs from healthy controls. The T cell proliferation was diminished by infliximab and adalimumab in a mixed lymphocyte reaction, whereas certolizumab pegol and etanercept had no effect. The inhibition of proliferation was driven by immunosuppressive CD14+ macrophages that secreted large amounts of the anti-inflammatory cytokine IL-10 and were activated by the Fc region of the antibodies^[42]. The same group later showed that regulatory macrophages were induced in IBD patients with mucosal healing upon infliximab treatment and functionally, these macrophages had the ability to induce wound healing *in vitro*^[66].

In summary, several effects of anti-TNF antibodies on the immune system have been described. The reduction of proinflammatory T cell subsets on the one hand and the induction of regulatory T cells on the other hand clearly contributes to resolution of inflammation. In addition, the induction of regulatory macrophages^[42] and effects on mucosal healing^[61,62,65] have been suggested.

OUTSIDE-TO-INSIDE SIGNALLING

As mentioned before, activation of the membrane-bound form of TNF can result in bidirectional signalling.

The function of mTNF not only as a ligand but also as a receptor is called "outside-to-inside signal" or reverse signalling. Reverse signalling by mTNF on monocytes/macrophages confers resistance to bacterial LPS^[67], suggesting that this pathway represents a silencing signal. A very recent paper demonstrated that the immune-suppressive effect of mTNF following LPS stimulation in macrophages was mediated by TGF- β *via* activation of the MAPK kinase, interestingly this effect was mediated by infliximab and golimumab whereas etanercept failed to induce TGF- β in human macrophages^[68]. Functional studies showed that LPS resistance downstream of mTNF is mediated through protein kinase-C dependent and independent pathways in monocytic cells^[67]. In addition, enhanced secretion of IL-2 and IFN- γ ^[69] and an upregulation of E-selectin in T cells have been reported^[70]. Furthermore, apoptosis induction as consequence of reverse signalling has also been proposed in CD patients^[43,71]. A study by Mitoma *et al.*^[72] shed light on the reverse signalling pathway induced by infliximab and etanercept. Herein, human Jurkat T cells were stably transfected with a wild-type and a mutant form of mTNF. E-selectin expression was induced by both agents, but only infliximab induced the expression of the anti-inflammatory cytokine IL-10, activation of ROS accumulation and apoptosis by upregulation of Bax and Bak^[72]. Interestingly, their finding that infliximab further induced G0/G1 cell cycle arrest elucidated another mechanism of action of anti-TNF antibodies. Later the induction of cell cycle arrest was shown to be mediated by adalimumab as well^[73]. In the context of reverse signalling, a downregulation of the growth and differentiation factor-1 (GDF-1) mediated by infliximab and certolizumab pegol has been described^[74]. Using gene expression assays, GDF-1 was found to be upregulated in inflamed tissue from CD patients, and downregulated after infliximab treatment in clinical responders. Furthermore, GDF-1 was shown to act as proinflammatory mediator by IL-6 and STAT-3 induction^[74], indicating that a down-regulation of proinflammatory effectors can also be a consequence of reverse signalling by anti-TNF agents in contrast to immune-suppressive effects.

APOPTOSIS

The programmed cell death of immune cells is a fundamental mechanism of resolution of inflammation. Apoptosis induction by anti-TNF antibodies has therefore been addressed by several groups. The induction of apoptosis can either be direct or indirect as side effect of TNF signaling. Furthermore, apoptosis can be induced by the Fc region of anti-TNF antibodies involving the complement or natural killer cells or can be a consequence of the activation of TNF itself by antibody binding. An overview of the apoptosis inducing capabilities of the different anti-TNF antibodies is given in Table 3.

Table 3 Mechanism of action of anti-tumor necrosis factor agents

Agent	Cell type	Mechanism of action	Ref.
ADA	CHO cell line stably expressing mTNF cocultured with human PBMCs	Induction of ADCC	[75]
ADA	CHO cell line stably expressing mTNF with human normal serum	Induction of CDC	[75]
IFX, ADA, ETA, Certo	TNF6.5 cells (murine NSO cell line stably expressing human TNF) with baby rabbit complement	CDC: IFX, ADA, ETA ++; Certo -	[50]
IFX, ADA, ETA, Certo	TNF6.5 cells in coculture with CD14-depleted PBMCs	ADCC: IFX, ADA ++, ETA+, Certo -	[50]
IFX, ADA, ETA, Certo	Peripheral blood lymphocytes	Apoptosis: IFX, ADA ++; ETA+, Certo -	[50]
IFX, ADA, ETA, Certo	Peripheral blood monocytes	Apoptosis: IFX, ADA ++; ETA+, Certo -	[50]
IFX, ADA	mTNF transfected cell line (SP2/0-11A5-1, murine)	Induction of CDC	[41]
IFX, ADA	Activated human PBMCs	No induction of CDC	[41]
GOL, ADA, IFX, Certo	mTNF expressing Jurkat cells with PBMCs/human serum	ADCC, CDC: GOL, ADA, IFX ++; ETA+, Certo-	[77]
ADA, IFX, GOL, Certo	mTNF expressing Jurkat cells	Apoptosis: ADA, IFX ++, GOL+, Certo+	[77]
ADA, IFX, ETA	cultured monocytes	Caspase-dependent apoptosis: ADA, IFX ++; ETA -	[53]
IFX, ETA	Peripheral blood lymphocytes and lamina propria T cells	Caspase-3 activation and apoptosis: IFX +; ETA -	[43]
ADA, IFX, ETA, Certo	Intestinal CD4/CD14 cells from IBD patients	Apoptosis by targeting mTNF/TNFR2: ADA, IFX, Certo +; ETA -	[24]

Apoptosis induction of anti-TNF agents. ++: Strong effect; +: Effect visible; -: No effect; ADA: Adalimumab; ETA: Etanercept; IFX: Infliximab; Certo: Certolizumab pegol; GOL: Golimumab; TNF: Tumor necrosis factor; mTNF: Membrane-bound TNF; sTNF: Soluble TNF; PBMCs: Peripheral blood mononuclear cells; ADCC: Antibody-dependent cellular cytotoxicity.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY AND COMPLEMENT-DEPENDENT CYTOTOXICITY

Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism of the adapted immune system in order to kill antibody-tagged target cells, for example infected cells. Therefore, after binding of an antibody to its target cell, the Fc domain is recognized by the Fc receptor of effector immune cells, typically natural killer cells. The natural killer cell then releases cytotoxic proteins such as perforins and granzymes that subsequently results in lysis of the target cell. ADCC is a mechanism of action of anti-TNF antibodies that possess a Fc domain. A recent study indicated that ADCC measured as cell lysis of co-incubation of mTNF-expressing CHO cells and PBMCs could be induced by adalimumab in up to 76% of cells in contrast to a control isotype that caused ADCC in less than 5% of the cells^[75]. In a comparative study, infliximab and adalimumab were shown to induce ADCC in a similar way and more potent as etanercept whereas certolizumab pegol did not show any effect due the fact that the later agent does not possess the Fc domain^[50].

Apart from ADCC, binding of antibodies to a target cells can result in complement activation. The complement consists of several different proteins that act as a cascade which finally leads to the formation of a membrane attacking complex, inducing a pore within the cell and subsequent cell death. In a CDC assay, 10 µg/mL adalimumab were shown to induce 90.6% cell death in the presence of heat-inactivated human serum^[75]. Comparing different agents, infliximab and adalimumab had the same capacity in inducing CDC-dependent cell death and were more effective than

etanercept and as expected, certolizumab pegol did not show any effect^[50], which was in accordance to other studies^[41,73]. Of note, CDC was induced in mTNF-transfected cells by adalimumab and infliximab, but not in activated human PBMCs^[41], raising the question if cell lines over-expressing mTNF reflect the situation *in vivo*. In a functional study, affinity of infliximab and adalimumab to FcγII and FcγIII receptors was found to be low but significantly increased in the presence of exogenous TNF^[76]. Similarly, both antibodies bound to the complement protein C1q in the presence of TNF. In both settings, etanercept binding was shown to be low. The more recently approved anti-TNF antibody golimumab induced both CDC and ADCC to a similar extent as infliximab and adalimumab^[77].

Fc-mediated apoptosis has been shown to be most potently induced by infliximab and adalimumab. However, the fact that certolizumab pegol is effective in CD despite its incapacity to induce ADCC or CDC strongly suggests that Fc-mediated apoptosis by anti-TNF antibodies is not a central mechanism of action.

APOPTOSIS OF INFLAMMATORY IMMUNE CELLS

Apoptosis of inflammatory immune cells as consequence of anti-TNF treatment has been discussed as a major mechanism of action explaining the fast onset of therapeutic effects upon successful therapy. Thus, the induced cell death of monocytes/macrophages or T cells has been analyzed. Regarding monocytes, in a comparative study both adalimumab and infliximab induced apoptosis in cultured monocytes rapidly in a caspase-dependent way whereas etanercept did not^[53]. In addition, apoptosis induction *via* transmembrane

Table 4 Prediction of response to anti-tumor necrosis factor therapy - possible biomarkers

Material/method	Predictor for response	Disease	Ref.
Intestinal biopsies	IL1B (upregulated in non-responders)	CD	[54]
Intestinal biopsies	IL17A (upregulated in non-responders)	CD and UC	[16]
	IL13RA2		
	PTGS2		
	WNT5A		
Blood	high CAI	UC	[17]
	negative ANCA		
	IL23R		
Blood	High API	CD	[83]
Blood	C-reactive protein	CD and UC	[84]
Intestinal biopsies	TNFRSF11B (osteoprotegerin)	UC	[85]
	STC1 (stanniocalcin-1)		
	PTGS2 (prostaglandin-endoperoxide synthase 2)		
	IL13Ralpha2 (IL13R alpha 2)		
	IL11 (IL-11)		
SPECT	High apoptosis rate	CD	[80]
Endoscopic molecular imaging	High mucosal mTNF expression in vivo	CD	[18]

CD: Crohn's disease; UC: Ulcerative colitis; SPECT: Single-photon emission computer tomography.

TNF in a human monocytic cell line by adalimumab and infliximab could be detected in a chimeric mouse model and furthermore, apoptosis could be abrogated by *in vivo* treatment with a pan-caspase inhibitor, indicating a caspase-dependent mechanism of action^[78]. Infliximab-induced apoptosis of monocytes required the activation of members of the caspase-family, such as caspase-8, -9 and -3 in peripheral blood monocytes derived from CD patients^[79]. Activation of caspase was induced independently from the CD95/CD95L signaling pathway by release of mitochondrial cytochrome C, apparently triggered by Bax and Bak^[79].

The effect of anti-TNF agents on lymphocytes was studied by Van den Brande *et al.*^[43]. They could show that etanercept, which is ineffective in the treatment of CD, in contrast to infliximab was incapable of inducing apoptosis in peripheral blood lymphocytes from healthy controls and lamina propria T cells from CD patients. Here, infliximab rapidly activated caspase 3 which possibly can also be a consequence of reverse signaling. Later the same authors demonstrated that this was also relevant *in vivo*. A rapid increase of apoptosis was detected in experimental colitis and importantly, also in CD patients where infliximab mediated apoptosis occurred within 24 h after starting the therapy^[80]. In this *in vivo* molecular imaging study, patients underwent single-photon emission computer tomography (SPECT) 24 h after infliximab infusion, and apoptosis was measured by radiolabeled annexinV uptake using scintigraphic imaging. A strong correlation of detected apoptosis signals and diseased regions was observed and furthermore, annexinV uptake was significantly higher in patients subsequently responding to infliximab therapy. In addition, in some patients LPMCs from biopsies were isolated and a marked increase in apoptosis of CD4 T cells after infliximab treatment was observed^[80].

Another study analyzing apoptotic markers such as Bcl-2, Bax, Caspase3 and Fas in biopsies from CD patients before and after infliximab/adalimumab therapy showed that in LPMCs, a significant increase of active Caspase-3 and an increase in the pro-apoptotic Bax/Bcl-2 ratio could be detected upon anti-TNF therapy, but no change of TNFR1 or Fas expression could be measured^[81].

In recent studies of our group, another mechanism of action apart from direct induction of apoptosis by clinically effective anti-TNF antibodies was identified. We showed that T cell apoptosis was only induced if mucosal T cells expressing TNFR2 were co-cultivated with mTNF-expressing CD14+ macrophages^[24]. Binding of mTNF to TNFR2 on T cells resulted in activation of TNFR-2 dependent signaling pathways leading to TRAF2 and NF- κ B induction followed by heightened IL-6 production, and subsequently resulting in T cell resistance to apoptosis, which leads to perpetuation of intestinal inflammation. This concept proposes an indirect apoptosis induction by neutralizing the mTNF binding site for TNFR2 by anti-TNF antibody treatment, resulting in apoptosis of TNFR2 expressing T cells^[24].

PREDICTION OF CLINICAL RESPONSE

Given that approximately 30% of the patients fail to respond to anti-TNF therapy (primary non-responders), and up to 50% lose the response during therapy (secondary non-responders)^[82], there is a clinical need for biomarkers allowing prediction of clinical response. Expression of biomarkers in blood or intestinal biopsies has been investigated in this regard. Table 4 presents a selection of so far collected data regarding possible predictors of response to anti-TNF therapy in IBD. A study using intestinal biopsies of patients with CD responding and non-responding to anti-TNF therapy

revealed that modulation of TNF-dependent genes has also been observed in non-responsive patients, indicating biological activity of anti-TNF independently of clinical response^[54]. On the other hand, in this study IL1B and IL17A remained altered in non-responders, suggesting these cytokines as relevant targets in this subgroup of patients^[54]. A database search validated possible biomarkers in gene expression in patients with IBD under infliximab therapy. Of interest, datasets of PBMCs and biopsies were not comparable. Regarding intestinal biopsies, the genes *IL13RA2*, *PTGS2* and *WNT5A* were shown to possibly predict the responsiveness to infliximab in IBD^[16].

Another study investigating UC patients before and after therapy with infliximab identified a high CAI, a negative antineutrophil cytoplasmic auto-antibody status and the IL23R genotype as predictors of response to therapy^[17]. As apoptosis is a central mechanism of action of anti-TNF antibodies, a predictive model based on an apoptotic pharmacological index (API) has been proposed in another study. Herein, response rates of infliximab in CD patients have been investigated in regard to polymorphisms of apoptosis genes (FAS ligand -843C/T; Fas -670G/A; Caspase-9 93 C/T). Patients with an low API had the lowest response whereas patients with an high API displayed high response and remission rates^[83]. In addition, high C-reactive protein levels have been described as predictors for response to anti-TNF therapy^[84]. Gene array analyses of biopsies from UC patients taken before anti-TNF treatment identified mainly five differentially expressed genes involved in adaptive immunity (osteoprotegerin, stanniocalcin-1, prostaglandin-endoperoxide synthase 2, IL13R alpha 2 and IL-11) in patients later responding to infliximab as compared to patients that did not^[85]. However, these promising biomarkers will have to be validated in larger cohorts of patients to verify if they serve as reliable markers for prediction of therapy in the clinic.

Another approach for prediction of response is based on biomarkers derived from the molecular mechanism of action of anti-TNF antibodies and transition into corresponding *in vivo* molecular imaging modalities. As described earlier, apoptosis induction by infliximab was determined by SPECT imaging and detected a significantly higher apoptosis rate in patients later responding to infliximab^[80]. The visualization of mTNF expressing cells by molecular imaging was the fundament of another recently published clinical trial. Based on the previous finding that the interaction of mTNF expressing cells and TNFR2 expressing T cells is targeted by anti-TNF antibodies^[24], it was possible to predict clinical response to subsequent anti-TNF therapy using GMP-conform fluorescent anti-TNF antibodies during endoscopy. Here, prior to initiation of anti-TNF therapy, fluorescent anti-TNF antibodies were topically applied *in vivo* to the inflamed mucosa

in 25 CD patients and the number of mTNF positive mucosal cells were localized with molecular imaging using a confocal laser endomicroscope. It could be demonstrated that CD patients with high amounts of mTNF+ cells showed significantly higher response rates at week 12 (92%) upon subsequent anti-TNF therapy as compared to patients with low amounts of mTNF+ cells (15%). Clinical response was defined as the reduction of the Crohn's Disease Activity Index > 100 points at week 12 after the initiation of the anti-TNF therapy. A high number of mTNF positive cells also predicted sustained clinical response at week 52, decreased steroid use and high mucosal healing rates^[18]. These findings represent a first step towards individualized medicine, making it possible to determine the most suitable biological therapy based on molecular level analysis thereby circumventing ineffective antibody therapy in IBD patients.

DISCUSSION AND FUTURE RESEARCH DIRECTIONS

Given the complexity of TNF signaling, the identification of the mechanism of action of anti-TNF therapy remains challenging. In this review, several aspects of potential targets for anti-TNF antibodies have been discussed. However, as reviewed here, contradictory results concerning the influence of anti-TNF antibodies on cells *in vivo* and *in vitro* have been reported, which possibly reflect the different experimental conditions used in each study. Furthermore, although infliximab, adalimumab, golimumab, certolizumab pegol and etanercept target the same epitope, fundamental differences in their mode-of-action *in vivo* have been demonstrated, beginning with the fact that etanercept is effective in the treatment of RA, but did not show therapeutic efficacy in the treatment of CD. However, in summary some conclusions can be drawn: First, as many IBD patients benefit from long-term remission induced by anti-TNF therapy, additionally to TNF neutralization fundamental biological changes must be induced, especially in those patients that can stop the medication due to stable remission. Second, it is unclear if Fc-dependent apoptosis such as ADCC and CDC by anti-TNF agents is relevant *in vivo* in IBD. Certolizumab pegol is incapable of inducing ADCC and CDC due to its structure but is successfully used for the treatment of CD. Third, direct and indirect apoptosis induction of anti-TNF agents and the modulation of the immune system possibly might be connected. For example, apoptosis induction could possibly directly influence the mucosal cytokine production^[53]. Fourth, the effects of anti-TNF agents apparently are dependent on the cell type; in some studies no detection of apoptosis induction in peripheral blood T cells could be observed, while induction of apoptosis in mucosal T cells were described *in vitro* and *in vivo*. Lastly, several findings suggest binding

of anti-TNF antibodies to mTNF rather than binding to sTNF is critical in IBD. In order to improve the response rates of anti-TNF antibody treatment, beside pharmacokinetic reasons, it is mandatory to better understand the mechanism of action of these agents and unravel the key signaling pathways involved. First clinical studies using *in vivo* imaging are opening the field for individualized therapeutic approaches in the treatment of IBD.

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Acute kidney injury and post-reperfusion syndrome in liver transplantation

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Abstract

In the past decades liver transplantation (LT) has become the treatment of choice for patients with end stage liver disease (ESLD). The chronic shortage of cadaveric organs for transplantation led to the utilization of a greater number of marginal donors such as older donors or donors after circulatory death (DCD). The improved survival of transplanted patients has increased the frequency of long-term complications, in particular chronic kidney disease (CKD). Acute kidney injury (AKI) post-LT has been recently recognized as an important risk factor for the occurrence of de

novo CKD in the long-term outcome. The onset of AKI post-LT is multifactorial, with pre-LT risk factors involved, including higher Model for End-stage Liver Disease score, more severe ESLD and pre-existing renal dysfunction, either with intra-operative conditions, in particular ischaemia reperfusion injury responsible for post-reperfusion syndrome (PRS) that can influence recipient's morbidity and mortality. Post-reperfusion syndrome-induced AKI is an important complication post-LT that characterizes kidney involvement caused by PRS with mechanisms not clearly understood and implication on graft and patient survival. Since pre-LT risk factors may influence intra-operative events responsible for PRS-induced AKI, we aim to consider all the relevant aspects involved in PRS-induced AKI in the setting of LT and to identify all studies that better clarified the specific mechanisms linking PRS and AKI. A PubMed search was conducted using the terms liver transplantation AND acute kidney injury; liver transplantation AND post-reperfusion syndrome; acute kidney injury AND post-reperfusion syndrome; acute kidney injury AND DCD AND liver transplantation. Five hundred seventy four articles were retrieved on PubMed search. Results were limited to title/abstract of English-language articles published between 2000 and 2015. Twenty-three studies were identified that specifically evaluated incidence, risk factors and outcome for patients developing PRS-induced AKI in liver transplantation. In order to identify intra-operative risk factors/mechanisms specifically involved in PRS-induced AKI, avoiding confounding factors, we have limited our study to "acute kidney injury AND DCD AND liver transplantation". Accordingly, three out of five studies were selected for our purpose.

Key words: Liver transplantation; Acute kidney injury; Post-reperfusion syndrome; Donation after circulatory death; Chronic kidney disease

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Core tip: Post-reperfusion syndrome (PRS)-induced acute kidney injury (AKI) has been recognized as an important complication occurring after liver transplantation (LT) that characterizes kidney involvement caused by PRS with mechanisms not clearly understood and implication on graft and patient survival. Since pre-LT risk factors may influence intra-operative events responsible for post-reperfusion syndrome-induced AKI (PRS-induced AKI), we aim to consider all the relevant aspects involved in PRS-induced AKI in the setting of LT and to identify all studies that better clarified the specific mechanisms linking PRS and AKI, in particular in LT recipients from donation after circulatory death.

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INTRODUCTION

Liver transplantation (LT) is the treatment of choice for patients with end stage liver disease (ESLD). Ongoing progress in organ preservation, surgical and anaesthetic techniques and immunosuppression has improved outcomes with 1-year survival > 85% and 5-year survival > 75%. The chronic shortage of cadaveric organs for transplantation led to the utilization of a greater number of marginal donors such as older donors or donors deceased after circulatory death (DCD). The improved survival of transplanted patients has increased the frequency of long-term complications, in particular chronic kidney disease (CKD). The incidence of CKD at 10 years after LT can be as high as 28%^[1] leading to 4-fold increase in mortality^[1,2].

Acute kidney injury (AKI) post-LT has been recently recognized as an important risk factor for the occurrence of *de novo* CKD in the long-term follow up after LT^[3].

Risk factors for AKI may be related to pre-LT conditions, such as severe ESLD, higher model for end-stage liver disease (MELD) score and pre-existing renal dysfunction, either to intra-operative conditions, in particular ischaemia reperfusion injury (IRI)^[4-6]. The latter is responsible for the post-reperfusion syndrome (PRS), which is an intra-operative complication, whose definition is still under debate, that may influence morbidity and mortality of recipients. PRS involves intra-operative events such as reduction of mean arterial pressure (MAP), presence of haemodynamic arrhythmias, need for inotropic drugs during transplantation^[7]. This condition promotes multi-organ damage, with particular kidney involvement through systemic inflammatory and haemodynamic mechanisms in addition to a direct damage with tubular cell death. Post-reperfusion syndrome-induced AKI (PRS-induced AKI) is an important complication post-LT that characterizes kidney involvement caused by PRS with mechanisms not clearly understood and implication on graft and patient survival.

Furthermore, after LT period, graft dysfunction, reoperation, bacterial infection, sepsis and acute CNIs toxicity may be associated to renal dysfunction^[8-12].

Since pre-LT risk factors may influence intra-operative events responsible for PRS-induced AKI, we aim to consider all the relevant aspects involved in PRS-induced AKI in the setting of LT and to identify all studies that find out the specific mechanisms linking PRS and AKI.

LITERATURE SEARCH

A PubMed search was conducted using the terms

Table 1 Summary of studies evaluating acute kidney injury in donors after circulatory death liver transplant recipients

Study	Journal and Year	Outcome	Diagnosis
Doyle <i>et al</i> ^[96]	J Am Coll Surg 2015	AKI	RRT
Ruebner <i>et al</i> ^[95]	Transpl Int 2014	End stage renal disease	
Leithead <i>et al</i> ^[112]	J Hepatol 2014	AKI	KDIGO criteria
Elaffandi <i>et al</i> ^[113]	Liver Transpl 2014	Prehospital cardiac arrest	
Leithead <i>et al</i> ^[99]	Am J Transplant 2012	AKI	RIFLE classification

AKI: Acute kidney injury; RRT: Renal replacement therapy; KDIGO: Kidney disease improving global outcomes; RIFLE: Risk, injury, failure, loss of kidney function, and end-stage kidney disease.

liver transplantation AND acute kidney injury; liver transplantation AND post-reperfusion syndrome; acute kidney injury AND post-reperfusion syndrome; acute kidney injury AND DCD AND liver transplantation. Five hundred seventy four articles were retrieved on PubMed search. Results were limited to title/abstract articles published in English between 2000 and 2015; studies on children, case reports, editorials and review articles were not considered. We have identified twenty-three papers that specifically evaluated incidence, risk factors and outcome for patients developing PRS-induced AKI in LT.

In order to identify intra-operative risk factors/mechanisms specifically involved in PRS-induced AKI, avoiding confounding factors, we have limited our study to "acute kidney injury AND DCD AND liver transplantation". Accordingly, three out of five studies were selected for our purpose (Table 1).

ACUTE KIDNEY INJURY POST-LT

Acute kidney injury is a significant complication after LT. It is reported to be associated to an increased mortality^[13,14] and to the development of CKD after LT^[15]. Recently, reversible AKI has been recognised as an important risk factor for *de novo* CKD in the non-transplant setting, suggesting important implications also for the surveillance in patients without pre-existing and clinically evident kidney disease^[16].

The incidence of post-operative AKI in the setting of LT ranges between 12% and 80%, depending on the definition adopted^[17,18], with a 30-d mortality of up to 50% in case of renal dysfunction at transplantation and as high as 90% if renal replacement therapy (RRT) is required^[4,19].

Different criteria have been used to diagnose and classify AKI. A recent new definition of AKI has been proposed on the basis of the universal consensus of KDIGO criteria, which are an evolution of RIFLE and AKIN criteria^[20,21].

Moreover, since all risk factors for AKI in the setting of LT are intertwined between the pre-LT and the intra-

operative period, we should consider all risk factors in order to evaluate the PRS-induced AKI.

PRE-TRANSPLANT RISK FACTORS FOR POST-LT PRS-INDUCED AKI

MELD

MELD score was created to predict poor survival in patients with liver cirrhosis and portal hypertension complications, undergoing elective transjugular intrahepatic porto-systemic shunts placement^[22]. The formula for calculation of MELD includes only 3 objective parameters [serum creatinine (sCr), serum bilirubin and INR]; it was validated by the Organ Procurement and Transplantation Network in 2002 as an accurate predictor of poor survival in patients with various stages of liver disease, as well as in patients of different geographic origin^[23]. The main use of MELD score is regarded to liver graft allocation, identifying those patients in greater immediate need for LT^[24], and in predicting outcome and survival post-LT^[25,26]. Pre-operative sCr was identified as an independent predictor of post-transplant mortality before the introduction of MELD score^[27].

Other factors affect sCr, such as gender. It is reported that women are disadvantaged in MELD era possibly due to the inclusion of sCr. Women are less likely than men to receive a LT and have greater 3-mo mortality, probably due to low sCr and MELD compared to men. However within most MELD strata, women had more deranged serum bilirubin and INR^[28].

End stage liver disease

In advanced cirrhosis, the systemic vascular resistance is particularly decreased, and any additional increase in cardiac output can no longer compensate, leading to under filling of arterial circulation^[29]. In this scenario, systemic arterial pressure and effective arterial blood volume are maintained through the activation of vasoconstrictor systems, including renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system, and, later, non-osmotic hyper-secretion of antidiuretic hormone. This leads to sodium and solute-free water retention and renal failure due to intra-renal vasoconstriction and hypoperfusion^[29].

Recently, a new specific form, named acute-on-chronic liver failure has been recognised to be associated to high risk for short-term mortality in advanced ESLD. This syndrome is defined as an acute decompensation of cirrhosis (development of ascites, bacterial infections, gastrointestinal hemorrhage and/or encephalopathy) associated to organ failure (liver, kidney, coagulation, circulation, respiration, brain) and high short-term mortality, $\geq 15\%$ within a period of 28 d^[30]. The type of organ failure is obviously a risk factor for mortality and it is $> 15\%$ in patients with kidney failure compared to $< 15\%$ for single non-kidney organ failures^[31].

The aetiology of ESLD in some cases seems to be involved in the occurrence of AKI. Non-alcoholic steatohepatitis (NASH), now regarded as the hepatic manifestation of the metabolic syndrome^[32-34], was found to be more frequently associated with post-LT AKI compared to other liver diseases leading to ESLD. This association is not surprising, since the increasing prevalence of NASH and non-alcoholic fatty liver disease (NAFLD) contributes to hyperlipidaemia and diabetes.

Clear evidences suggest the existence of a link between NAFLD/NASH and renal impairment. Different mechanisms have been hypothesized, involving multiple pro-inflammatory substances that originate in the steatotic and inflamed liver or through the contribution of systemic insulin resistance and atherogenic dyslipidemia^[35].

Patients with NAFLD have significantly low levels of adiponectin, higher levels of hemostatic and inflammatory factors, oxidative stress and endothelial dysfunction biomarkers^[34,36-39]. Similarly, patients with renal disease showed reduced adiponectin levels, as well as increased levels of oxidative stress and systemic inflammation biomarkers, hypofibrinolysis and hypercoagulation^[40-43]. The supposed mechanisms that link NAFLD and renal disease may begin from the inflamed and expanded visceral adipose tissue that releases multiple molecules, such as free fatty acids, hormones, tumor necrosis factor- α , interleukin-6, and other pro-inflammatory cytokines, that seem to be implicated in the insulin resistance and kidney damage^[44,45]. In this context, the liver is both source of several mediators and target of these systemic abnormalities, amplifying kidney damage. Even though the mechanisms by which oxidative stress and chronic inflammation can damage kidney are not completely described, some studies on animal models showed that cytokine imbalance may have a role in the pathogenesis of kidney involvement through the activation of different pro-inflammatory pathways, up-regulation of adhesion molecules, induction of oxidative stress and endothelial dysfunction, as well as reduction of adiponectin expression^[43,46-49]. As a result, patients with NAFLD should be considered at increased risk for the occurrence of pre-transplant renal disease^[35]. Furthermore, macrovesicular steatosis was described as a risk factor for PRS by Chung *et al*^[50].

Evaluation of renal function

The evaluation of renal function in patients with ESLD waiting for LT remains a challenge. Serum creatinine may be influenced by several factors including age, race, gender, body weight, medications, diet and laboratory techniques. In the context of cirrhosis, sCr can be affected by reduced hepatic production of creatine (> 40%), protein malnutrition, decreased muscle mass, oedema and increased tubular secretion of creatinine^[51]. As a result, low levels of sCr are the

result of an underestimation of glomerular filtration rate (GFR). Therefore, the evaluation of sCr modifications from baseline is the best way to assess renal function in patients with ESLD^[8].

Since the multiple limitations of sCr as a measure of renal function in these patients, alternative methods were proposed such as GFR estimation by inulin clearance^[52]. This is the gold standard for renal function assessment but its routinely use is limited due to high cost and technical difficulties^[52]. Other direct methods are characterized by the use of exogenous radiolabelled substances or non-radioactive agents^[53]. Nevertheless they have not been widely confirmed in patients with ESLD. Furthermore, creatinine clearance is not better than sCr in evaluating renal function in patients with cirrhosis due to several variables including potential errors in collection and measurement of urine, variations in re-absorption or excretion of creatinine and sCr dilution caused by fluid retention^[54].

Serum cystatin C is freely filtered by glomeruli and then re-absorbed and catabolized by proximal tubules^[55]. For this reason, it is recognised as a sensitive indicator of renal function in liver cirrhosis^[56,57], significantly different from inulin or exogenous radio-labelled substances^[58,59]. However, further studies are needed to develop specific cystatin C-based GFR estimation formulas in patients with ESLD.

Nevertheless, sCr remains the clinical marker of kidney function for routinely practice, and changes in sCr are currently used specifically to define AKI.

Therefore, in order to standardize its definition, the classification of AKI was recently revised by KDIGO group including all causes of acute renal dysfunction as indicated by an increase in sCr to 1.5-1.9 times from baseline within 7 d or an increase in sCr by ≥ 26.5 mmol/L (≥ 0.3 mg/dL) in < 48 h, allowing patients with less severe degrees of renal dysfunction to receive treatment^[60].

Pre-existing renal disease

Kidney failure among patients with ESLD is closely related to liver disease severity. This is due to circulatory disturbances characterized by reduced systemic vascular resistance secondary to splanchnic arterial vasodilatation due to portal hypertension^[29,61].

Hepatic and renal disorders are frequently associated as a result of a number of systemic conditions that concurrently affect liver and kidney. Furthermore, renal dysfunction is also a common complication of primary liver disorders with renal histological changes mainly of glomerular type, named hepatic glomerulosclerosis. It was initially described predominantly in patients with advanced liver cirrhosis and recently in HCV-related cirrhosis^[62,63]. This is characterised by mesangial expansion, thickening of capillary walls, a mild increase in size and number of endothelial and epithelial cells, and deposits of immunoglobulins and electron-dense material in the mesangium and capillary walls^[63,64],

as demonstrated in renal biopsies performed at the time of LT^[63,65]. Recently, trans-jugular renal biopsies performed in cirrhotic patients with clotting problems showed an incidence > 70% of glomerular lesions with a prevalence of IgA deposition^[66].

In advanced cirrhosis, renal dysfunction results mainly from renal hypoperfusion due to intravascular volume depletion secondary to diuretic use, lactulose-induced diarrhoea, gastrointestinal bleeding, infections, use of nephrotoxic agents such as nonsteroidal anti-inflammatory drugs, contrast agents, and aminoglycosides. Intense renal vasoconstriction, as seen during hepatorenal syndrome, can in turn lead to renal tubular necrosis. The histological changes are very similar to those reported for chronic nephrotoxicity from calcineurin inhibitors and differential diagnosis may be difficult^[61,67-69].

Hepatitis B virus-related cirrhosis is associated with membranous glomerulonephritis (GN) and membranoproliferative GN, but also with vasculitis, such as polyarteritis nodosa^[70]. Hepatitis C virus-related cirrhosis is associated primarily with membranoproliferative GN and cryoglobulinaemia, but also with fibrillary GN^[71,72]. In addition, the occurrence of type 2 diabetes mellitus in HCV-infected patients is associated with earlier loss of renal function compared with uninfected patients^[73]. Alcoholic cirrhosis is linked mainly with IgA nephropathy^[74]. Primary sclerosing cholangitis and primary biliary cirrhosis are associated with anti-neutrophil cytoplasmic autoantibody-positive vasculitis and membranous nephropathy^[74,75].

A few primary diseases can affect both liver and kidney, including polycystic disease, primary oxaluria, alpha1-antitrypsin deficiency and Wilson's disease. A secondary involvement of the kidney may result such as in diabetic nephropathy.

In literature it is increasingly reported a close association between renal failure and severe liver dysfunction in patients with ESLD^[76-79]. In the setting of LT the interval of pre-transplant renal dysfunction is predictive of 6- and 12-mo sCr post-transplant^[80].

MELD score, ESLD, pre-existing Renal Disease and PRS-induced AKI

Recipient characteristics including older recipient age^[81], higher MELD score, sCr levels^[82], bilirubin and higher pre-operative heart rate^[50] seem to have a role in the development of PRS. This may reflect the occurrence of haemodynamic and cardiovascular complications that characterise patients with cirrhosis^[83]. These patients develop splanchnic arteriolar vasodilatation, leading to reductions of systemic vascular resistance, central volume, and arterial filling. The activation of RAAS and sympathetic nervous system, and the release of vasopressin all contribute to maintain the arterial blood pressure, thus inducing a hyperdynamic circulatory state^[83]. The progression of cirrhosis leads to more pronounced splanchnic vasodilatation with failure of the hyperdynamic compensation, lower

systemic vascular resistance index, higher cardiac output, and decreased arterial blood pressure^[83,84]. The state of advanced cirrhosis is in line with the higher MELD score and the more pronounced haemodynamic changes with the occurrence of renal dysfunction.

The loss of integrity of the vasoconstrictive response during graft reperfusion is also believed to be, at least partially, responsible for the development PRS^[85,86].

In an experimental model of rats with portal hypertension the pressor response generated by the sympathetic system was found to be impaired^[87]. Similarly, the peripheral vascular response to angiotensin II and noradrenaline is attenuated in patients with liver cirrhosis^[88,89]. Garutti Martinez *et al.*^[85] reported a lower increase in vascular muscle tone reflected by an increased systemic vascular resistance index in patients experiencing PRS, suggesting a reduced vascular adaptability in these patients. A significant reduction in sympathetic tone has also been reported in patients who developed PRS^[86]. Although altered cardiovascular autonomic control may not clarify the reason for the occurrence of graft reperfusion-related hypotension during LT, the assessment of autonomic indices may be helpful in predicting PRS occurrence and may help anaesthesiologist management in preventive treatment with vasoconstrictors before any substantial decrease in MAP in the first minutes after reperfusion.

Left ventricular diastolic dysfunction was found to be a risk factor for the development of PRS in a Chinese population of liver transplanted patients by Xu *et al.*^[90]. Diastolic dysfunction has been entitled as cause of perioperative haemodynamic instability and an adverse outcome following cardiac surgery^[91] and may contribute to the development of PRS by inducing haemodynamic instability during LT.

SPECIFIC INTRA-OPERATIVE RISK FACTORS FOR POST-LT PRS-INDUCED AKI

PRS

PRS is an intra-operative complication that characterizes specific surgical phases related to instability at reperfusion involving cardiovascular and metabolic alterations, together with a PRS-induced AKI, that can influence recipient's morbidity and mortality. Pathophysiology of PRS has not been clarified yet; several mechanisms have been hypothesized, considering factors related to recipient or donor characteristics, graft quality and intra-operative factors.

Donors deceased after circulatory death characterized by prolonged ischaemic phase of the graft compared to traditional brain death donors. Although LT from DCD donors demonstrates satisfactory long-term outcomes, these are burdened by a higher degree of delayed graft function compared to donation after brain death (DBD) and a higher frequency of AKI as well as CKD^[92-95], despite similar pre-LT renal

function^[96]. In this setting, the damage sustained by the graft during cold preservation following recovery from the donor and during subsequent warm at implantation into the recipient^[97] is intensified by the period of warm ischaemia in the donor. The functional donor warm ischemic time (dWIT) is defined as the interval between hypotension (Systolic Blood Pressure < 50 mmHg) or hypoxia (O₂ saturations < 70%) and cold perfusion of the aorta^[98]. Functional dWIT adds complementary injury to cold ischaemia. Elevation of serum aminotransferase enzymes is the first clinical evidence of IRI of the graft, which can lead up to clinical liver dysfunction and progressive graft failure. In a study by Leithead *et al.*^[99] on AKI in DCD LT, the only consistent predictor of renal outcomes was peak peri-operative aspartate aminotransferase, which represents a surrogate marker of hepatic IRI. The hepatic IRI is known to evoke a systemic inflammatory response, which can cause distant organ dysfunction and AKI through haemodynamic mechanisms and direct tubular cell death, associated to PRS^[23,100-103]. Therefore, graft injury, by driving a systemic inflammatory response, may be a contributing factor of PRS-induced AKI.

Furthermore, in the same single-centre case-controlled study it has been shown for the first time that LT from DCD donors was associated with higher incidence of AKI and RRT, evaluated by RIFLE criteria, compared to propensity score-matched DBD recipients, despite a significant (53.4% vs 31.8%, $P = 0.004$) better renal function in DCD vs DBD^[99].

Donor and graft characteristics

Cold ischaemia time (CIT) and graft steatosis have been reported to be associated with PRS in several studies^[6,13,17,21,22]. The increased CIT is likely to increase the IRI which is associated with release of increased amounts of pro-inflammatory mediators, generation of free radicals, neutrophil sequestration and activation^[50]. Steatotic livers tolerate less the IRI and it is conceivable that the higher percentage of steatosis is linked to a higher degree of damage. The release of vasoactive substances, including reactive oxygen species^[32], pro-inflammatory chemokines and cytokines^[33], from the transplanted liver is also considered as a possible mechanism of PRS.

An important link between IRI and PRS is similarly accounted by Pan *et al.*^[104] in their retrospective study, where they report a double incidence of PRS in DCD compared to DBD (25.7% vs 12.2%, respectively). These authors also suggest that the severe IRI experienced by DCD grafts during the additional warm ischaemia occurring before organ procurement may play a role in the development of PRS.

Donor age and donor risk index (DRI) are also reported being associated with the development of PRS^[105] and with greater haemodynamic instability and delayed intra-operative haemodynamic recovery.

A role for poor tolerance of ischemia-reperfusion phenomenon or age-related steatotic parenchymal changes (senescence) has been advocated^[105].

Intra-operative factors

Intra-operative events additionally play a role in transfusion requirements^[50,81,106]. These are associated to the severity of liver disease, characterized by anaemia and coagulation abnormalities. Intra-operative blood loss thereby increases, requiring transfusion of blood products, which itself is linked to fibrinolysis associated with severe PRS^[81,106].

The use of piggy-back technique and porto-caval shunt seems to reduce the occurrence of PRS^[107,108]. The piggy-back technique is proven to provide better haemodynamic stability and the severity of PRS has been associated to the surgical technique utilised. Currently, piggy-back technique is widely used making difficult to clearly ascertain the role of operation technique as a risk factor^[108].

Otherwise the preservation of caval flow does not prevent hypotension during graft reperfusion^[109]. The creation of a temporary portocaval shunt in patients with cirrhosis during the anhepatic phase, reducing splanchnic congestion, in theory limits splanchnic ischaemia and the subsequent release of toxic mediators at reperfusion^[110]. This hypothesis may in part account for the protective effect of a portocaval shunt toward the incidence of intra-operative PRS^[107].

The need for emergent and unplanned intra-operative RRT (IORRT) during LT is associated with a greater incidence of PRS compared to both patients not receiving IORRT and patients receiving planned IORRT^[111]. IORRT become part of a complex multifactorial cause of reduced patients short-term survival. A role has been advocated for planned IORRT to correct metabolic derangements due to electrolyte and acid/base disturbances occurring in patients with pre-transplant renal failure^[111].

CONCLUSION

Although advances in medical and surgical techniques have been done to improve survival among LT recipients, mortality rates related to post-LT renal complications remain a concern. The onset of AKI post-LT is multifactorial, with pre-LT risk factors involved, including higher MELD score, ESLD and pre-existing renal dysfunction, either with intra-operative conditions such as IRI and PRS. Recently, PRS-induced AKI is considered an important complication post-LT that characterizes kidney involvement caused by PRS with mechanisms not clearly understood and implication on graft and patient survival. Pre-LT risk factors for AKI and PRS are closely intertwined and the impact of PRS-induced AKI on the onset of long-term CKD is very important. Furthermore, a strong influence of donor quality has also been described on the development of CKD post-LT^[95]. Receiving a liver from DCD or with

higher DRI increase the risk of end-stage renal disease by 40%, despite favourable recipient characteristics, supporting the current knowledge that donor quality is associated with short and long-term outcomes, renal injury in particular^[95]. The better understanding of predisposing factors for post-LT AKI may give the possibility to improve methods to prevent or ameliorate injury. Therefore, this new understanding may possibly help researchers to develop further studies in order to better clarify PRS and PRS-induced AKI, in particular in DCD recipients.

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Clinical relevance of endoscopic assessment of inflammation in ulcerative colitis: Can endoscopic evaluation predict outcomes?

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel condition characterised by a relapsing and remitting course. Symptom control has been the traditional mainstay of medical treatment. It is well known that histological inflammatory activity persists despite adequate symptom control and absence of endoscopic inflammation. Current evidence suggests that presence of histological inflammation poses a greater risk of disease relapse and subsequent colorectal cancer risk. New endoscopic technologies hold promise for developing endoscopic markers of mucosal inflammation. Achieving endoscopic and histological remission appears to be the future aim of medical treatments for UC. This review article aims to evaluate the use of endoscopy as a tool in assessment of mucosal inflammation in UC and its correlation with disease outcomes.

Key words: Ulcerative colitis; Inflammation; Endoscopy; Disease activity indices; Mucosal healing

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Core tip: Endoscopy is the mainstay of assessing disease activity in ulcerative colitis. Mucosal healing (MH) is an accepted end point in clinical trials. Recent data suggest that complete MH is associated with lower relapse rates and better long term outcomes. Advanced imaging techniques like high definition endoscopy, narrow band imaging, magnification endoscopy, chromoendoscopy and endomicroscopy help in detailed assessment of mucosa and the submucosal vasculature. In this review article we aim to look at the correlation

between these endoscopic assessment modalities and clinical outcomes.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel condition characterised by mucosal inflammation of the rectum and colon. It is associated with a relapsing and remitting disease course. The exact aetiology of the disease remains elusive although genetic linkage, auto immune causes and environmental influences have been postulated. Approximately 25% of patients with UC experience acute exacerbation of their disease activity during the course of their disease^[1]. Colectomy rate increases with more than one hospital admissions with acute severe UC, reaching up to 40% after two admission^[1]. Truelove and Witts criteria established over 60 years ago, estimates the severity of the disease and predicts the need for colectomy using clinical and biochemical scores^[2]. Current treatment goals in UC focus on keeping the disease in remission and a colectomy free survival.

There have been significant scientific advances in both diagnosis and management of UC in the last two decades. The use of Immunomodulators like Azathioprine, Cyclosporine, and biologic agents like Anti-Tumour necrosis factors alpha has changed the way patients with UC are managed in modern day practice. Advances in medical management of UC have seen a fall in colectomy rates^[3].

A flare up in disease activity in UC is difficult to predict but a reliable biomarker would be important in guiding appropriate therapy. Commercially available serum and faecal biomarkers have been ineffective in positively predicting disease relapse in UC. Serological markers available for clinical and research use includes C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cells, Platelets, 1-acid glycoprotein, serum amyloid A-protein, 2-globulin, lactoferrin, orosomucoid and thrombopoietin. Faecal biomarkers are thought to be non-invasive and are relatively inexpensive. Available faecal biomarkers include a1-antitrypsin excretion, lysozyme excretion, calprotectin, lactoferrin, Myeloperoxidase. Faecal calprotectin has generated the most interest among researchers and clinicians. However, in a meta-analysis consisting of 6 prospective studies looking at the use of faecal calprotectin in predicting clinical flares in inflammatory bowel disease (IBD), Mao *et al*^[4] report a pooled sensitivity of only 78% and specificity of

73%. Endoscopy is the main tool used by physicians in assessing severity and extent of the disease in UC in clinical practice. It is a reliable tool in assessment of disease activity during flare up of symptoms. But in inactive disease persistent microscopic inflammation is often seen despite the normal appearance of colonic mucosa on standard white light endoscopy (WLE)^[5]. Histologically active disease is associated with greater risk of subsequent relapse^[6-8]. Studies using standard WLE fail to predict relapse in quiescent UC^[5,7,8], whereas studies using advanced endoscopic imaging modalities seem to hold promise^[9-11].

In this review article we aim to discuss the use of endoscopic modalities in assessment of disease activity in UC, its correlation with clinical outcomes, and endoscopic predictors of relapse.

ENDOSCOPY IN UC

Endoscopy is essential in diagnosing UC, obtain biopsies and distinguishing from Crohn's disease. Direct mucosal visualisation allows physicians to assess extent and severity of the disease during flare ups and observe effectiveness of treatment during follow up. In addition to this it is the only available test to identify and resect dysplastic lesions during surveillance for colorectal cancer in patients with long standing colitis.

Endoscopic assessment of the mucosa for pathological diagnosis is largely operator dependant. Although agreement among beginners was good at the extremes of the disease, concordance for certain endoscopic features like granularity, erosions and friability was still poor and identified the need for training to improve endoscopic diagnosis^[12,13]. Training has shown to improve diagnostic yield in endoscopy in trainee endoscopists. Studies suggest that among experienced endoscopists there is a good inter-observer agreement in UC related endoscopic findings^[13].

There are at least ten scoring systems designed to assess the disease activity in UC since the development of first such score by Baron *et al*^[14] in 1964. Table 1 provides details of scores using endoscopic activity alone and Table 2 details of scores with mainly clinical and biochemical parameters with or without endoscopic features. Many of these scoring systems use clinical, biochemical and endoscopic components in an attempt to grade the disease activity^[15]. Endoscopic parameters of assessment include mucosal vascular pattern (MVP), friability and mucosal damage. Mayo endoscopic sub score is an endoscopic component of full Mayo score^[16]. Both Baron score and Mayo endoscopic sub score have been used in clinical trials; however these scores have not been validated rigorously^[15]. Recently Travis *et al*^[13,17] have designed and validated a new scoring system using endoscopic "descriptors" called ulcerative colitis endoscopic index of severity (UCEIS). Ten IBD experts evaluated sigmoidoscopic videos of varying degree of endoscopic inflammation seen in UC. Inter and intra-investigator reliability was tested using

Table 1 Disease activity indices with endoscopic component alone

Disease activity index	Endoscopic variables
Baron score ^[14] 1964	Bleeding and MVP
Rachmilewitz endoscopic index ^[18] 1989	Granulation, MVP, Mucosal vulnerability, Mucosal damage
UC colonoscopic index of severity (UCCIS) ^[19] 2013	MVP, Granularity, Ulceration, Bleeding, Segmental assessment of endoscopic severity, Global assessment of endoscopic severity
UC endoscopic index of severity (UCEIS) ^[13] 2013	MVP, Bleeding, Erosions and Ulcers

MVP: Mucosal vascular pattern; UC: Ulcerative colitis.

Table 2 Disease activity indices with endoscopic and non-endoscopic components

Disease activity index	Endoscopic variables	Non-endoscopic variable
Powell-Tuck score ^[20] 1982	Bleeding	Wellbeing, Abdominal pain, stool frequency and consistency, Bleeding, Anorexia, nausea and vomiting, EIM, Temperature
Sutherland index ^[21] 1987	Friability, Bleeding	Stool frequency, Bleeding, Physician's rating of disease activity
Mayo score ^[16] 1987	Erythema, MVP, Friability, erosions, ulcers, spontaneous bleeding	Stool frequency, Bleeding, Physician's global assessment
Improvement based on individual symptom scores ^[22] 2002	Mucosal oedema, MVP, Granularity, Friability, Petechiae, Ulceration, Spontaneous bleeding	Rectal bleeding, Stool frequency, Abdominal pain, PFA, PGA

EIM: Extra-Intestinal Manifestations; MVP: Mucosal vascular pattern; PGA: Physician global assessment; PFA: Patient functional assessment.

Kappa statistics. In the validation phase they report a satisfactory intra and inter-investigator reliability using this score. No significant difference was observed when investigators were tested with or without the knowledge of clinical details of the subjects.

ENDOSCOPY IN ACUTE SEVERE COLITIS

Endoscopy plays a vital role in disease assessment in acute flares of UC. Limited examination of the colon by flexible sigmoidoscopy is enough to establish the diagnosis and obtain biopsies. Radiological examinations like abdominal X-rays and sometimes computed tomography (CT) scans are carried out prior to endoscopic examinations. Minimal air insufflation is used during endoscopic procedure to avoid misinterpretation of subsequent X-ray images as toxic megacolon.

Sigmoidoscopy is commonly performed during UC flare ups and it is thought to be sufficient for assessing disease severity. Colonoscopy is avoided until the disease is settled, mainly due to fear of complications such as perforation during severe flare. However there are few prospective studies to validate this widely used practice. In the only published study to date, Carbonnel *et al.*^[23] demonstrated that colonoscopic examination is safe in acute flare up of UC, and helps in identifying patients at high risk of colectomy. In their cohort of 85 consecutive patients with acute severe colitis, extensive deep ulcerations were found in 46 patients. Forty-three/forty-six patients with deep ulceration underwent colectomy and histology in 42/43 patients showed

deep ulcerations extending up to muscular layer. Thirty of thirty-nine patients with moderate colitis responded to medical therapy. They did not report any major complications apart from one dilated colon in their cohort. The authors conclude that a full colonoscopy was safe in acute severe flare of colitis and also helped in predicting course of the disease and short term outcome. It is important to know that all endoscopic procedures in this study group were performed by an experienced colonoscopist; hence care must be taken in generalising these findings to all endoscopists. Secondly this study was conducted in the pre-biological treatment era which could account for the high rates of colectomy.

ENDOSCOPY IN DISEASE REMISSION

The aims of endoscopy performed during clinical disease remission are to assess if there is reduction of endoscopic activity after a flare, ascertain if mucosal healing (MH) is achieved, to obtain biopsies and screen for dysplastic lesions.

MH is increasingly recognised as a therapeutic endpoint in clinical trials. Although there is no consensus definition of MH, the International organisation of IBD proposed the following criteria to define MH: absence of friability, blood, erosions and ulcers in all visualised segments of the colonic mucosa^[15]. Essentially disappearance of endoscopic lesions such as erosions and ulcers is called as MH. Drugs such as 5-aminosalicylates, immunomodulators like azathioprine, methotrexate and biological agents

Table 3 Correlation of endoscopic activity with clinical symptoms

Ref.	Study characteristics	Results
Karoui <i>et al</i> ^[35] 2011	Prospective observational study. 101 patients with UC in remission.	CRP correlated well with DAI and Rachmilewitz score Correlation between DAI and Rachmilewitz was not statistically significant
Tunisia	CRP, Disease activity index and Rachmilewitz scores used	
Osada <i>et al</i> ^[36] 2008	Prospective observational study. 54 patients with UC.	Clinical symptoms correlated with left sided disease activity. CRP and ESR correlated well with right sided inflammation.
Japan	CRP, ESR, Mayo endoscopic subscore, Lichtiger's clinical activity scores used.	
Turner <i>et al</i> ^[37] 2009	Prospective observational study. 86 patients with UC. Disease activity was measured using 9 different activity indices	Disease activity was best assessed by Walmsley and PUCAI followed by Partial Mayo score and Rachmilewitz
Canada		

CRP: C-reactive protein; DAI: Disease activity index; ESR: Erythrocyte sedimentation rate; PUCAI: Paediatric ulcerative colitis activity index; UC: Ulcerative colitis.

(infliximab, adalimumab, golimumab, vedolizumab, etc.) are used in the induction of remission and maintenance of MH in UC^[24-30]. MH is associated with favourable short and long term clinical outcomes like reduced hospitalisation due to flares of disease, decreased colectomy rates and lower incidence of subsequent colorectal cancers^[6,31-34].

DOES ENDOSCOPY CORRELATE WITH CLINICAL SYMPTOMS?

Generally it is considered that clinical symptoms, biochemical markers of inflammation, endoscopic findings and histological grading help in assessing the severity of the disease in UC. It is not uncommon to find that clinical symptoms and endoscopic findings do not correlate. Table 3 contains the studies comparing endoscopic activity with clinical symptoms. Karoui *et al*^[35] compared the endoscopic findings of patients in remission whereas Osada *et al*^[36] examined patients with varying grades of severity. In both the studies serological markers (CRP and ESR) correlated well with the disease activity. However conflicting results were noted when comparing clinical symptoms with endoscopic findings (Table 3). Osada *et al*^[36] reported that clinical symptoms correlated well with the disease of left colon whereas CRP and ESR reflected well with right sided disease. No significant association was noted by Karoui *et al*^[35]. Different clinical activity indices used in the above two studies (Rachmilewitz score and Lichtiger index respectively) may have contributed to the differences. However, in a prospective study Turner *et al*^[37] compared different clinical activity indices and their respective abilities to assess disease activity. They noted that the Rachmilewitz score and Lichtiger index had comparable "discriminative average" which is the ability to differentiate patients in clinical remission with those patients with active disease [Rachmilewitz score- 0.92 (95%CI: 0.87-0.98) and Lichtiger index- 0.90 (95%CI: 0.84-0.97)].

DOES STANDARD WLE CORRELATE WITH HISTOLOGICAL ACTIVITY?

The presence of deep ulcerations, extensive disease, higher median inflammation seen on WLE corresponds to more severe disease and are associated with higher colectomy rates^[23,38]. MH is associated with better outcomes such as decreased relapse rates and the need for surgical interventions^[6,30,31,33,39]. Use of conventional colonoscopy is restricted to assessment of disease activity and extent of the disease during disease flare; however colonoscopic findings in remission does not correlate well with the histological activity nor are they predictive of relapses. Table 4 provides details of studies comparing white light endoscopic activity and histological activity and their potential in predicting disease outcomes in UC.

Histological inflammation has been shown to persist despite normal endoscopic findings in both prospective and retrospective studies^[5-8,40,41]. Histological markers of inflammation such as basal plasmacytosis, basal lymphocytosis and chronic inflammatory infiltrates were found in biopsies from endoscopically normal looking mucosa. These histological markers were associated with increased risk of subsequent relapse. The rate of relapse was reported to be between 20%-57.7% among UC patients with quiescent disease (Table 4).

DOES ENDOSCOPY PREDICT DISEASE RELAPSE?

In a recent prospective study involving 41 patients with UC who had undergone colonoscopy before and after receiving Tacrolimus, Ikeya *et al*^[43] studied the outcomes of patients assessed by two of the disease activity score; the Mayo endoscopic subscore and the UCEIS. They reported better correlation of endoscopic assessment of disease activity using UCEIS and also in predicting relapse free survival. In another prospective

Table 4 Correlation between white light endoscopy and histology in ulcerative colitis

Ref.	Study characteristics and aims	Results
Bitton <i>et al</i> ^[8] 2001	Prospective observational study 74 patients in clinical and endoscopic remission were included	36.4% patients relapsed Younger age, multiple previous relapses (women), and basal plasmacytosis on histology predicted relapse.
United States Azad <i>et al</i> ^[41] 2011	Followed up for a year or until the patients relapsed. Prospective observational study 26 patients with clinical and endoscopic remission were included	CRP, ESR, IL-1b, -6, 15, ANCA was non-predictive of relapse. 57.7% patients relapsed Increased Eosinophils and Neutrophils were predictors of relapse.
India Bessissow <i>et al</i> ^[7] 2012	Monthly follow up for a year or until the patients relapsed. Retrospective study 75 patients with endoscopically inactive disease (Mayo score 0)	Hb, CRP, ESR, IL-6 were not predictive of relapse. Microscopic inflammation was found in 40% of patients. Basal plasmacytosis and histological activity (Geboes score \geq 3.1) predicted relapse.
Belgium Lemmens <i>et al</i> ^[40] 2013	Time to relapse was noted Retrospective study 131 patients with known UC	Significant correlation with Mayo endoscopic subscore and histology noted in extremes of disease (inactive and acute severe disease)
Belgium Rosenberg <i>et al</i> ^[5] 2013	Correlation of endoscopy and histology Prospective observational study 103 UC patients in clinical remission	54% of patients with quiescent disease had signs of histological inflammation.
United States Feagins <i>et al</i> ^[6] 2013	Correlation of endoscopy and histology Retrospective study of 51 patients. colonoscopy for surveillance	20% of patients had flare up within 12 mo. Basal lymphocytosis, disruption of crypt architecture, erosions and ulcers predicted relapse.
United States Zenlea <i>et al</i> ^[42] 2016	Correlation of endoscopic and histological activity Prospective study 179 patients included	23% of patients relapsed Histological activity with Geboes score \geq 3.1 was strongest predictor of relapse.
United States	Baseline Mayo endoscopic score and Geboes score for histology noted Follow up period was 12 mo	

UC: Ulcerative colitis; Hb: Haemoglobin; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; IL-6: Interleukin-6; ANCA: Anti-nutrophil cytoplasmic antibody.

study of 82 patients with UC, a score of 0-1 on UCEIS after treatment with Infliximab had favourable long term outcomes^[44].

Does advanced imaging modalities predict relapse

Advanced imaging modalities such as magnification colonoscopy (MC), narrow band imaging (NBI), iScan, Fujinon intelligent colour enhancement (FICE), autofluorescence imaging (AFI), chromoendoscopy, confocal laser endomicroscopy (CLE) and endocytology, etc. enable real time mucosal assessment in greater detail. Imaging modalities such as NBI, MC and magnification chromoendoscopy have been evaluated, mainly by Japanese investigators, for their ability to predict relapse in UC (Table 5). Figure 1A-D shows the appearances of inflamed colonic mucosa in patients with colitis using standard definition, high definition, NBI and chromoendoscopy respectively. Image enhanced endoscopic techniques appear to improve the visualization of inflammation in colonic mucosa but large scale clinical studies are needed to ascertain the relevance of these findings to clinical outcomes.

MC: Optical enhancement of image from six to 150 fold occurs due to a moving camera at the tip of the endoscope^[45]. In MC the image undergoes optical enhancement and hence the pixels are not distorted and the image quality is not compromised. Hence

the image appears sharp and allows assessment of surface pattern in detail. Regular pit pattern seen under MC is associated with a significantly reduced risk of relapse^[9,10]. Patients with distorted MVP, abnormalities in epithelium or pit pattern have a higher grade of inflammation on histology and relapse subsequently^[9-11,46,47]. In one recent study MC with NBI-lead target biopsies seems to predict long term outcomes^[48].

Chromoendoscopy and NBI: Chromoendoscopy is examination of the colonic mucosa after spraying dye which contrast enhances and highlights mucosal abnormalities allowing precision biopsies. NBI, also called as "virtual chromoendoscopy" or "dye-less chromoendoscopy", utilises optical filters and uses shorter wavelengths of light (between 415-540 nm) which intensely absorbed by haemoglobin. This allows examination of the vasculature and surface pattern in detail. Use of NBI in predicting relapse is controversial. Kudo *et al*^[46] in their prospective study evaluated the MVP observed under WLE and NBI. NBI findings of obscure MVP correlated well with the histological markers of inflammation. Although this study did not report any outcome data on relapse, we know that the histological inflammation leads to subsequent relapse. More recently a prospective study from Spain of 67 patients with UC in sustained clinical

Table 5 Relapse prediction using advanced imaging techniques

Ref.	Imaging modality	Study characteristics	Results
Watanabe <i>et al</i> ^[9] 2009	Magnification colonoscopy with chromoendoscopy	Prospective study 57 patients with clinical and endoscopic remission were enrolled for MC examination and followed up for 12 mo	70% of patients with mucosal defects identified by MC had a flare up within 12 mo
Japan Nishio <i>et al</i> ^[10] 2006	Magnification colonoscopy with chromoendoscopy	Prospective study 113 patients with UC in remission were enrolled. Pit pattern in rectal mucosa assessed using MC. Followed up for 12 mo	29% of patients relapsed. Significant correlation seen between pit pattern abnormalities and relapse rate.
Japan Fujiya <i>et al</i> ^[11] 2002	Magnification colonoscopy	18 patients with UC in remission underwent MC and follow up	7 out of 9 (77.7%) with minute epithelial defect had a flare.
Japan Kudo <i>et al</i> ^[46] 2009	NBI	Prospective study 157 colonic segments among 30 patients were examined under WLE and NBI	Obscured MVP had good correlation with the histological activity.
Japan Jauregui-Amezaga <i>et al</i> ^[49] 2014	Chromoendoscopy and NBI	Prospective study 64 patients with clinical and endoscopic remission for at least 3 mo were included. 1 year follow up.	27% relapsed during follow up Neither NBI nor chromoendoscopy predicted relapse
Spain Osada <i>et al</i> ^[55] 2011	AFI	Retrospective study 572 images from 42 patients were correlated with histological activity	The green component of AFI correlated closely with the inflammatory activity
Japan			

MC: Magnification colonoscopy; MVP: Mucosal vascular pattern; AFI: Autofluorescence imaging; NBI: Narrow band imaging.

remission investigated chromoendoscopy, NBI and faecal calprotectin in predicting clinical flares. In this study advanced endoscopy using NBI failed to predict relapse within one year^[49].

CLE: CLE allows visualisation of cellular structures and assessment of their function in real time. Contrast agent such as fluorescein is administered systemically and laser light is emitted *via* CLE. The reflected endoscopic image is reprocessed for microscopic examination in such a way that the resultant image is enhanced to a 1000 fold magnification. CLE detects barrier dysfunction in the epithelium in patients with IBD^[50]. Mucosal inflammation in IBD results in barrier dysfunction which is seen as increased fluorescence leak and widening of crypt diameter along with intercept distance on CLE. A composite score developed by Buda *et al*^[51] using fluorescence leak, and crypt diameter have shown predictive capabilities for disease outcomes in quiescent UC patients during 12 mo follow up^[50-52]. A recent study from Karstensen *et al*^[53] reported parameters for distinguishing active and inactive UC with CLE. In this prospective study the authors examined colonic mucosa from twenty two patients with clinical symptoms of relapse and 7 patients with inactive disease referred for surveillance purposes served as controls. This study demonstrated that fluorescein leak, microerosions, tortuosity of crypts, distortion of crypt opening, decreased crypt density and presence of inflammatory infiltrates

were significantly higher in active compared to inactive colitis. They also noticed improvement in the crypt architecture was associated with histological improvement following treatment of active colitis.

Endocytoscopy: Data on use of endocytoscopy (EC) in prediction of relapse in UC is limited. Maeda *et al*^[54], in a retrospective study of patients who underwent endocytoscopic-NBI (EC-NBI) compared the images with histological inflammation. EC-NBI was found to be highly useful in assessing histological activity with a sensitivity, specificity, positive predictive value, negative predictive value and accuracy of EC-NBI for diagnosis of acute inflammation to be 84%, 100%, 87.1%, 100% and 92.3%. There was not data on relapse rate provided in this study.

Endoscopic assessment with AFI and iScan have been found to correlate well with histological activity, they have not been used to assess relapse prediction in UC^[55,56]. The FICE was however found not helpful in improving and further characterisation of endoscopic findings in IBD^[57].

Spectroscopy: More recently we studied the use of Raman spectroscopy to identify MH and inflammation in UC. We observed that three carotenoid peaks were twice as intense in the inflamed mucosa and two phospholipid peaks were significantly lower in the normal mucosa. These five peaks seen on the spectroscopy could be used reliably to distinguish

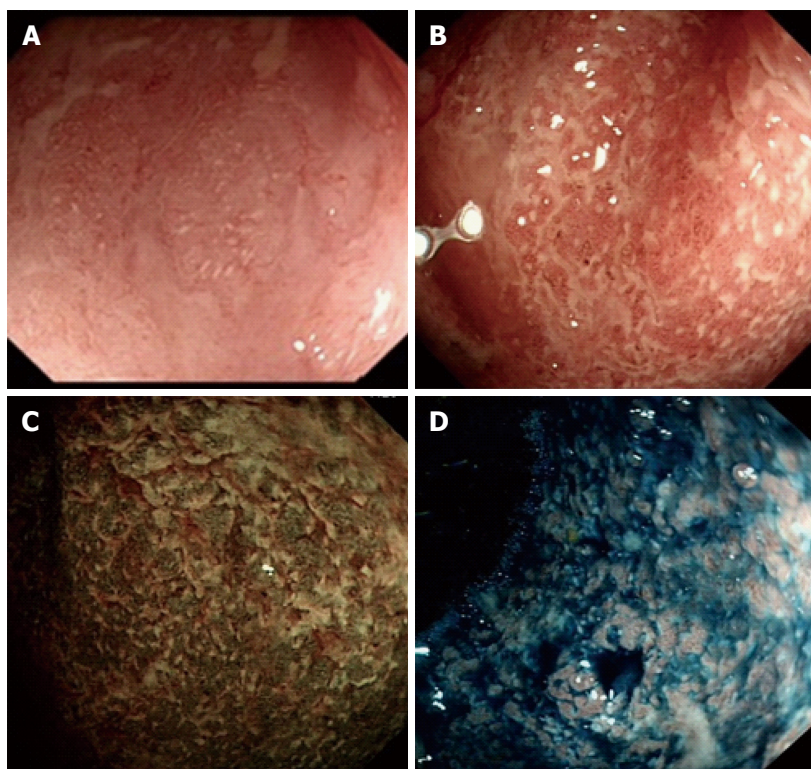


Figure 1 Assessment of inflamed colon with white light endoscopy, narrow band imaging and chromoendoscopy. A: White light assessment with standard definition endoscope reveals areas with superficial ulceration interspersed with areas of patchy obliteration of mucosal vascular pattern; B: High resolution endoscope allows more detailed assessment including crypt openings and disrupted vascular architecture; C: NBI assessment of moderately active UC shows obscured vascular pattern; white mucosal spots which represent mucous exudates giving the characteristic appearance of “Coral reef” like mucosa; D: Chromoendoscopy shows the mucosal damage with disruption of pit pattern and complete destruction of vascular pattern. Ulcer margins are seen more prominent with contrast enhancement.

active from quiescent UC^[58].

CONCLUSION

Endoscopy is a useful tool in the clinical management of UC. Although standard WLE is the commonly used in day to day practice, it has its limitations in assessing disease activity and predicting disease course. Advanced imaging modalities show promising results but they are expensive, involve a steep learning curve and are time consuming. Endoscopic modalities such as CLE and EC are still restricted to research use and cannot be advocated for routine assessment of IBD. Advanced endoscopy improves visualisation of mucosal surface structure and vascularity and hold promise for predicting disease outcomes. Development of endoscopic markers using these advanced technologies in well-designed prospective clinical studies is essential to develop robust markers for predicting disease course in patients with UC.

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Basic Study

Carbon monoxide contributes to the constipating effects of granisetron in rat colon

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Abstract

AIM

To investigate the mechanisms underlying the potential contribution of the heme oxygenase/carbon monoxide (HO/CO) pathway in the constipating effects of granisetron.

METHODS

For *in vivo* studies, gastrointestinal motility was evaluated in male rats acutely treated with granisetron [25, 50, 75 µg/kg/subcutaneous (sc)], zinc protoporphyrin IX [ZnPPiX, 50 µg/kg/intraperitoneal (ip)] and hemin (50 µmol/L/kg/ip), alone or in combination. For *in vitro* studies, the contractile neurogenic response to electrical field stimulation (EFS, 3, 5, 10 Hz, 14 V, 1 ms, pulse trains lasting 10 s), as well as the contractile myogenic response to acetylcholine (ACh, 0.1-100 µmol/L) were evaluated on colon specimens incubated with granisetron (3 µmol/L, 15 min), ZnPPiX (10 µmol/L, 60 min) or CO-releasing molecule-3 (CORM-3, 100, 200, 400 µmol/L) alone or in combination. These experiments were performed under co-treatment with

or without atropine (3 $\mu\text{mol/L}$, a muscarinic receptor antagonist) or N^G-nitro-L-Arginine (L-NNA, 100 $\mu\text{mol/L}$, a nitric oxide synthase inhibitor).

RESULTS

Administration of granisetron (50, 75 $\mu\text{g/kg}$) *in vivo* significantly increased the time to first defecation ($P = 0.045$ *vs* vehicle-treated rats), clearly suggesting a constipating effect of this drug. Although administration of ZnPPiX or hemin alone had no effect on this gastrointestinal motility parameter, ZnPPiX co-administered with granisetron abolished the granisetron-induced constipation. On the other hand, co-administration of hemin and granisetron did not modify the increased constipation observed under granisetron alone. When administered *in vitro*, granisetron alone (3 $\mu\text{mol/L}$) did not significantly modify the colon's contractile response to either EFS or ACh. Incubation with ZnPPiX alone (10 $\mu\text{mol/L}$) significantly reduced the colon's contractile response to EFS ($P = 0.016$) but had no effect on contractile response to ACh. Co-administration of ZnPPiX and atropine (3 $\mu\text{mol/L}$) abolished the ZnPPiX-mediated decrease in contractile response to EFS. Conversely, incubation with CORM-3 (400 $\mu\text{mol/L}$) alone increased both the contractile response to EFS at 10 Hz (10 Hz: 71.02 ± 19.16 *vs* 116.25 ± 53.70 , $P = 0.01$) and the contractile response to ACh (100 $\mu\text{mol/L}$) ($P = 0.012$). Co-administration of atropine abolished the CORM-3-mediated effects on the EFS-mediated response. When granisetron was co-incubated *in vitro* with ZnPPiX, the ZnPPiX-mediated decrease in colon contractile response to EFS was lost. On the other hand, co-incubation of granisetron and CORM-3 (400 $\mu\text{mol/L}$) further increased the colon's contractile response to EFS (at 5 Hz: $P = 0.007$; at 10 Hz: $P = 0.001$) and to ACh (ACh 10 $\mu\text{mol/L}$: $P = 0.001$; ACh 100 $\mu\text{mol/L}$: $P = 0.001$) elicited by CORM-3 alone. L-NNA co-administered with granisetron and CORM-3 abolished the potentiating effect of CORM-3 on granisetron on both the EFS-induced and ACh-induced contractile response.

CONCLUSION

Taken together, findings from *in vivo* and *in vitro* studies suggest that the HO/CO pathway is involved in the constipating effects of granisetron.

Key words: Granisetron; Carbon monoxide; Heme oxygenase; Colon; Contraction; Neurogenic response; Myogenic response

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Core tip: We studied whether *in vivo* and *in vitro* effects of granisetron might be influenced, at least in part, by the heme oxygenase/carbon monoxide (HO/CO) pathway. Our findings demonstrate for the first time that the HO/CO pathway takes part in the contractile colon activity in rats. Interestingly, the constipating effects of granisetron are positively correlated with

levels of carbon monoxide, thus suggesting that treatments able to modulate carbon monoxide levels may potentially reduce the constipation mediated by granisetron.

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INTRODUCTION

In recent decades, the role played by carbon monoxide (CO) in several biochemical processes has been increasingly recognized^[1-3]. Once considered only for its lethal effects, the therapeutic use of CO has been proposed after the discovery of its potential "positive" functions (<http://clinicaltrials.gov/ct2/search>, "carbon monoxide").

CO is a gas that is produced, together with iron and biliverdin, from the catalysis of heme by the microsomal heme oxygenase (HO) enzyme. Of the two HO isoforms, HO-2 is the constitutive one, whereas HO-1 is a highly inducible isoform whose activity is intended to provide protection against oxidative stress, injury and inflammation^[1,2].

The first physiological role suggested for CO was in non-adrenergic non-cholinergic (NANC) neurotransmission at the gastrointestinal level^[4]. The hypothesis of CO as a neurotransmitter is strongly supported by the wide expression of HO-2 throughout the gastrointestinal tract in the enteric nerves, as well as in the non-neuronal cells of the mucosal epithelium, smooth muscle cells, endothelium of blood vessels and interstitial cells of Cajal^[3-5]. Moreover, HO-1 is upregulated in several gastrointestinal pathologies such as colitis, inflammatory bowel disease and gastric ulcers (see^[3] for references). Because endogenously produced CO diffuses to blood where it binds to hemoglobin, increased HO-1 expression may result in augmented blood levels of carboxyhemoglobin (normal levels 0.8%). However, high levels of carboxyhemoglobin are more typically the consequence of smoking habits or environmental pollution^[2]. Either from endogenous or exogenous sources, altered CO levels may affect physiological processes or modulate pathological conditions *via* several distinct mechanisms^[6]. Ion channels have been shown to be, among others, the target of CO; thus, it is possible that CO may modulate the effects of other signals by acting directly on the same target or indirectly on the shared pool of second messengers^[6-8]. A similar modulating activity of CO might also be plausible toward specific drugs; indeed, in a previous report, we observed the involvement of the

HO/CO pathway in granisetron-mediated effects on rat duodenal motility^[9].

Granisetron is a highly selective competitive antagonist of the 5-HT₃ receptor, the only serotonin-gated ion channel that, if activated, allows an influx of cations^[10]. Granisetron is currently used for the chemotherapy-induced nausea and vomiting^[11], and constipation is reported among its side effects^[12]. On the other hand, constipation is the desired effect for 5-HT₃ receptor antagonists such as alosetron and cilansetron in the treatment of irritable bowel syndrome with diarrhea^[13] in which the delayed transit in the large bowel may reduce pain and discomfort in those patients^[14]. Unfortunately, despite their clinical efficacy, the potential use of these drugs has been restricted due to reports of severe ischemic colitis (see^[15] for review). Nevertheless, these observations support the ability of 5-HT₃ receptor antagonists to induce constipation.

To explore potential mechanisms linking the activity of the HO/CO pathway to granisetron-induced constipation, we investigated whether the constipating effects of granisetron administered *in vivo* may be modulated by agents that induce (such as hemin) or inhibit (such as zinc protoporphyrin, ZnPPiX) the endogenous HO activity. A 3 µmol/L concentration of granisetron was chosen for the present investigation based on dose-response curves previously obtained^[9]. Moreover, because constipation has been ascribed to abnormalities of various contractile activities of the colon^[16-19], parallel *in vitro* studies on isolated colon preparations were performed to evaluate (1) the neurogenic contractile responses to electrical field stimulation indicative of cholinergic and non-cholinergic transmitter release from enteric neurons^[20,21] in the absence and in the presence of the muscarinic antagonist atropine as well as the nitric oxide synthase inhibitor L-NNA; and (2) the myogenic contractile response to ACh, one of the major contractile neurotransmitters at the gastrointestinal level in the absence and in the presence of L-NNA.

MATERIALS AND METHODS

Experimental animal model

All experimental procedures were performed in accordance with the Guidelines and Authorization for the Use of Laboratory Animals (Italian Government, Ministry of Health) and according to the European Community Guidelines for Animal Care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 - 86/609/EEC).

Ten-week-old male Sprague-Dawley rats weighing 220-250 g at arrival (Envigo, San Pietro al Natisone, Udine, Italy) were used. The animal protocol was designed to minimize pain or discomfort to the animals.

Rats were housed in an animal facility with monitored temperature and light (12-h cycle and 21 ± 2 °C). All cages were floored with sawdust, and bedding was replaced on a regular basis. The animals were

allowed to acclimate to the environment for at least 7 d. Rats undergoing *in vivo* treatments were randomly chosen and allocated into individual cages before initiating the study, with the remaining rats caged together (4 rats/cage) in close proximity to allow experimental animals to see and smell their companions. Rats had free access to water and food when they were not under testing. All animals were handled and trained for at least 1 wk to minimize the possible stress of the drug administration procedure.

Gastrointestinal motility test

A repeated measures protocol was designed for *in vivo* study, so that each rat, at one-week intervals, received the following treatments either subcutaneously (sc) or intraperitoneally (ip): vehicle (1 mL/kg), granisetron (25, 50, 75 µg/kg/sc soon before testing), ZnPPiX (50 µg/kg/ip, 60 min before testing), hemin (50 µmol/L/kg/ip 24 h before testing), ZnPPiX (50 µg/kg/ip, 60 min before granisetron) with granisetron (25, 50, 75 µg/kg/sc), or hemin (50 µmol/L/kg/ip 24 h before granisetron) with granisetron (25, 50, 75 µg/kg/sc). The timing and dosing for ZnPPiX and hemin were carefully chosen to obtain the greatest level of HO inhibition or induction, respectively^[9,22,23]. In a pilot study, we observed that the average time to first defecation in vehicle-treated rats was between 80-110 min (median 105 min; interquartile range 90-110; full range 80-180). Based on these preliminary findings, the observation cut-off time was set at 180 min. In the late afternoon preceding the test day, rats were fasted with free access to water. On the test day, animals were weighed and then allowed to free feed for 20 min. The amount of food eaten and the weight of the fed rats were calculated.

Following drug administration, each rat was monitored every 10 min for 180 min, and the time to first defecation was assumed as an index of whole-gut transit^[24,25].

Tensiometric studies

After induction of general anesthesia (pentobarbital 80 mg/kg ip), rats were killed by cervical dislocation. A 3-cm section of proximal colon (1 cm from the ileocecal sphincter), obtained through a midline incision of the abdomen, was immediately placed in a cooled modified Krebs' solution (pH = 7.4) of the following composition (mmol/L): NaCl 113, KCl 4.8, MgSO₄ 1.2, CaCl₂ (H₂O) 2.2, NaH₂PO₄ 1.2, NaHCO₃ 25, glucose 5.5, and ascorbic acid 5.5. The specimen was then cleaned and rinsed, and a circular ring (0.5-cm length) was mounted in an organ bath (20 mL) filled with modified Krebs' solution, maintained at 37 °C and gassed with a mixture of 95% O₂ and 5% CO₂. One end of the circular ring was connected to a metal rod, while the other end was attached to a strain gauge transducer (FORT 25, WPI, Sarasota, FL, United States). Isometric tension was measured by the PowerLab data acquisition system and recorded using Chart 5.5.5 (ADIn-

struments, Castle Hill, Australia). The colon ring was allowed to equilibrate for at least 30 min prior to the experiment. An initial load of 0.5 g tension was applied to the preparation.

The neurogenic contractile response was measured by applying a transmural stimulation (Electrical Field Stimulation, EFS) at frequencies of 3, 5, and 10 Hz (14 V, 1 ms pulse, trains lasting 10 s) through two parallel platinum electrodes connected to a stimulator (Digital Stimulator, LE 12106, Letica, Ugo Basile, Italy). The EFS results in an immediate relaxation, followed at the end of EFS by a so-called off-contraction. This contractile response is indicative of a nervous reflex that is abolished by tetrodotoxin and reduced by atropine and tachykinin antagonists^[26]. Activation of enteric nerves by EFS mimics the *in vivo* conditions in which neurotransmitters are released by motor neurons to the neuroeffector apparatus; the interaction between the interstitial cells of Cajal, neurons, glial cells and smooth muscle cells generates contraction^[27,28].

The myogenic contractile response was explored by calculating the extent of contraction induced by acetylcholine (ACh, 0.1–100 $\mu\text{mol/L}$).

Both neurogenic and myogenic contractile responses were measured after incubation with the following agents alone or in combination: granisetron hydrochloride (3 $\mu\text{mol/L}$, 15 min), ZnPPiX (10 $\mu\text{mol/L}$, 60 min), L-NNA (100 $\mu\text{mol/L}$, 20 min), and CORM-3 (100, 200, 400 $\mu\text{mol/L}$). For the last compound, CORM-3, a water-soluble Ru-containing compound releasing one mole of CO per mole^[29], the effect was evaluated within 10 min from administration to avoid its spontaneous breakdown.

The neurogenic contractile responses were expressed as a percentage of three consecutive contractile responses to EFS (10 Hz, 14 V, 1 ms pulse, trains lasting 10 s) recorded and averaged before drug administration.

The myogenic contractile responses to ACh (0.1–100 $\mu\text{mol/L}$) were expressed as a percentage of tension values elicited by the highest ACh concentration (100 $\mu\text{mol/L}$) before drug administration.

The activity of ZnPPiX and CORM-3 (indicative of a specific CO-dependent effect) on neurogenic contractile response was measured in the absence and in the presence of atropine (3 $\mu\text{mol/L}$).

Drugs and chemicals

The following drugs were used: atropine sulphate and granisetron hydrochloride dissolved in saline (Sigma Chemical Co., St. Louis, Missouri, United States). Zinc protoporphyrin IX and hemin were dissolved in 0.1 N NaOH and equilibrated to a pH of 7.4 with HCl (Sigma Chemical Co., St. Louis, Missouri, United States). Tricarbonyl Chloro(glycinato)ruthenium (II) (CORM-3) and N^G-nitro-L-Arginine (L-NNA) were dissolved in distilled water (Sigma Chemical Co., St. Louis, Missouri, United States). In *in vivo* studies, vehicle-treated rats

received the same amount of vehicle as did drug-treated animals. In *in vitro* experiments, vehicle-treated preparations were exposed to the same amount of vehicle as drug-treated preparations.

Statistical analysis

For *in vivo* study, Friedman's ANOVA for repeated measures followed by a *post hoc* test was performed. For *in vitro* study, two-way ANOVA for repeated measures (treatment effect, frequencies or concentrations effect and interaction effect, with frequency or concentrations as repeated measure) was performed. When the interaction effect was significant, a one-way ANOVA at each frequency or concentration was performed with pre-planned multiple comparison tests for each treatment vs vehicle.

The results are presented as individual observations ($n = 8$) for each *in vivo* treatment; results are expressed as the mean \pm SD of 6–8 preparations for each *in vitro* treatment. Statistical analysis was performed by the biomedical statistician Dr. Margherita Fanelli (coauthor) using SPSS software (version 20.0). A P value < 0.05 was considered to indicate statistical significance.

RESULTS

In vivo study

Effect of granisetron, ZnPPiX and hemin on the time to first defecation: The average amount of food eaten before drug administration was 5 g. After 20 min of free access to food, the body weight increased by approximately 8 g in all animals.

Consistent with results obtained in our previous study^[9], acute administration of granisetron increased the time to first defecation. Interestingly, the delay to first defecation was dose-dependent, with no significant effect measured for the lowest dose of granisetron used (25 $\mu\text{g/kg}$) and with a substantial increase in the time to first defecation observed in animals administered higher doses of granisetron; in this respect, both 50 and 75 $\mu\text{g/kg}$ of granisetron were equally effective (Friedman's test = 13, $P = 0.005$, *post hoc*: granisetron 25 $\mu\text{g/kg}$ vs vehicle, $P = 0.132$; granisetron 50 $\mu\text{g/kg}$ vs vehicle, $P = 0.045$; granisetron 75 $\mu\text{g/kg}$ vs vehicle: $P = 0.045$) (Figure 1). A preliminary comparison of the amount of food eaten before vehicle or drug administration showed no statistically significant differences among treatments (Friedman's test = 0.958, $P = 0.811$).

Although ZnPPiX (50 $\mu\text{g/kg}$) alone did not modify the time to first defecation, co-administration of ZnPPiX (50 $\mu\text{g/kg}$) with granisetron (25, 50, 75 $\mu\text{g/kg}$) was able to counteract the constipating effect of granisetron: Friedman's test = 10.486, $P = 0.033$; *post hoc* comparisons: ZnPPiX vs vehicle: $P = 1$; granisetron 25 $\mu\text{g/kg}$ with ZnPPiX vs vehicle: $P = 1$; granisetron 50 $\mu\text{g/kg}$ with ZnPPiX vs vehicle: $P = 1$;

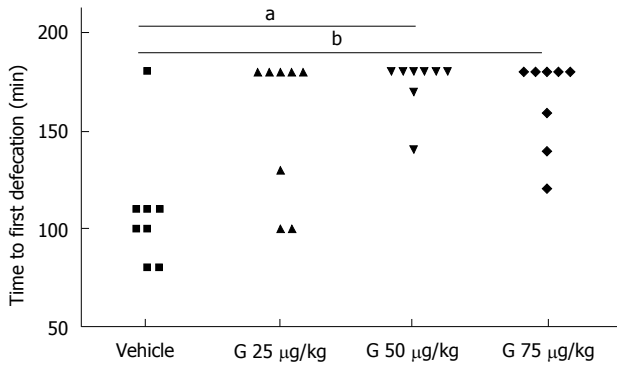


Figure 1 Effect of *in vivo* administration of granisetron on the time to first defecation. *In vivo* treatment with granisetron (G) significantly increased the time to first defecation at doses of 50 and 75 µg/kg. Friedman's test = 13 $P = 0.005$, *post hoc*: G 25 µg/kg vs vehicle, $P = 0.132$; G 50 µg/kg vs vehicle, $^aP = 0.045$; G 75 µg/kg vs vehicle, $^bP = 0.045$. Each point represents an individual observation.

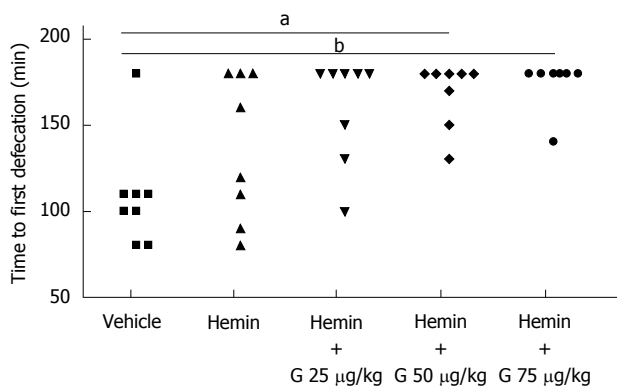


Figure 3 Effect of *in vivo* administration of hemin alone and with granisetron on the time to first defecation. Hemin (50 µmol/L/kg) did not affect the time to first defecation. Co-administration of hemin (50 µmol/L/kg) with granisetron (G) (50, 75 µg/kg) resulted in an increased time to first defecation. Friedman's test = 20.364 $P = 0.000$; *post-hoc* comparisons: hemin vs vehicle: $P = 1$; G 25 µg/kg + hemin vs vehicle: $P = 0.108$; G 50 µg/kg + hemin vs vehicle: $^aP = 0.028$; G 75 µg/kg + hemin vs vehicle: $^bP = 0.004$. Each point represents an individual observation.

granisetron 75 µg/kg with ZnPPiX vs vehicle: $P = 0.132$ (Figure 2). Similar to the previous case, a preliminary comparison of the amount of food eaten before vehicle or drug administration showed no statistically significant differences among treatments (Friedman's test = 1.077, $P = 0.898$).

On the other hand, hemin (50 µmol/L/kg) alone or co-administered with granisetron (25, 50, 75 µg/kg) showed the following results: Friedman's test = 20.364, $P = 0.000$; *post hoc* comparisons: hemin vs vehicle: $P = 1.000$; granisetron 25 µg/kg with hemin vs vehicle: $P = 0.108$; granisetron 50 µg/kg with hemin vs vehicle: $P = 0.028$; granisetron 75 µg/kg with hemin vs vehicle: $P = 0.004$, thus suggesting that hemin does not alter the time to first defecation when administered alone and does not modify the constipating effect of granisetron when administered in combination (Figure 3). Similar to the previous

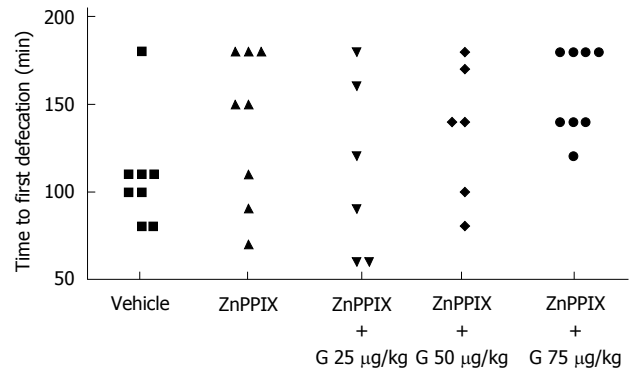


Figure 2 Effect of *in vivo* administration of zinc protoporphyrin alone and with granisetron on the time to first defecation. Zinc protoporphyrin (ZnPPiX) (50 µg/kg) did not affect the time to first defecation. Co-administration of ZnPPiX (50 µg/kg) with granisetron (G) (25, 50, 75 µg/kg) abolished the effect of G on its own. Friedman's test = 10.486, $P = 0.033$; *post-hoc* comparisons: ZnPPiX vs vehicle: $P = 1$; G 25 µg/kg + ZnPPiX vs vehicle: $P = 1$; G 50 µg/kg + ZnPPiX vs vehicle: $P = 1$; G 75 µg/kg + ZnPPiX vs vehicle: $P = 0.132$. Each point represents an individual observation.

case, a preliminary comparison of the amount of food eaten before vehicle or drug administration showed no statistically significant differences among treatments (Friedman's test = 2.205, $P = 0.698$).

In vitro studies

Effects of granisetron on EFS-induced and ACh-induced contractile response of colon preparations: Incubation of colon specimens with granisetron did not significantly modify the contractile response to EFS obtained in vehicle-treated samples ($F_{\text{treatments}} = 1.26$, $df = 1/9$, $P = 0.29$; $F_{\text{frequencies}} = 22.50$, $df = 2/18$, $P = 0.001$; $F_{\text{treatments} \times \text{frequencies}} = 1.79$, $df = 2/18$, $P = 0.21$) (Figure 4A). Interestingly, a trend to increase the contractile effect induced by ACh (0.1–100 µmol/L) was measured in samples incubated with granisetron, although no statistical significance was measured with respect to vehicle-treated samples ($F_{\text{treatments}} = 3.48$, $df = 1/9$, $P = 0.09$; $F_{\text{concentrations}} = 21.35$, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 0.08$, $df = 3/27$, $P = 0.85$) (Figure 4B).

Effects of ZnPPiX on EFS-induced and ACh-induced contractile response of colon preparations: When compared to vehicle-treated preparations, a significant decrease in the contractile response to EFS was observed in specimens incubated with ZnPPiX (10 µmol/L, 60 min) ($F_{\text{treatments}} = 8.78$, $df = 1/9$, $P = 0.016$; $F_{\text{frequencies}} = 50.33$, $df = 2/18$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.79$, $df = 2/18$, $P = 0.21$) (Figure 5A). Interestingly, the ZnPPiX-mediated effect on EFS was abolished by concomitant incubation with atropine (3 µmol/L, 20 min) ($F_{\text{treatments}} = 1.44$, $df = 1/11$, $P = 0.25$; $F_{\text{frequencies}} = 37.66$, $df = 2/22$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 2.74$, $df = 2/22$, $P = 0.09$), therefore suggesting that ZnPPiX may exert its effects by inhibiting the EFS-mediated release of endogenous ACh (Figure 5B). However, ZnPPiX did not affect the contractile

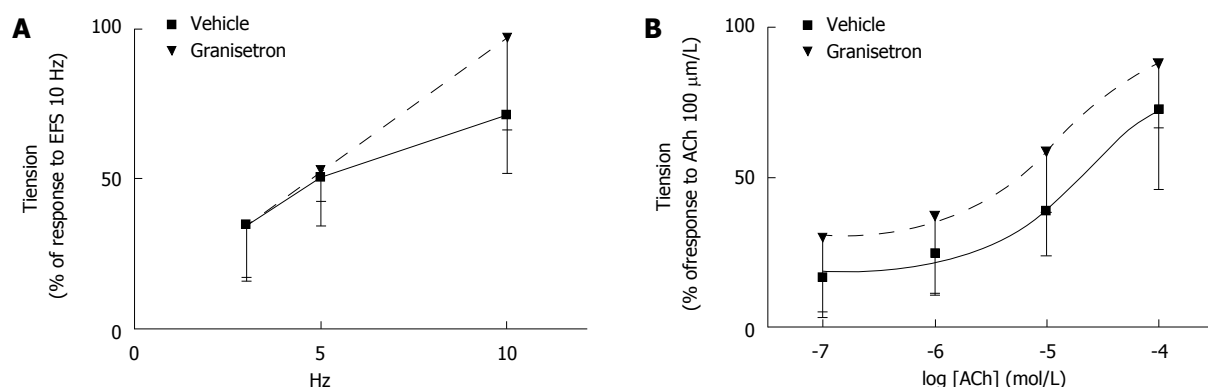


Figure 4 Effects of *in vitro* treatment with granisetron on rat colon contractile response to electrical field stimulation and to acetylcholine. A: Incubation with granisetron (G) (3 $\mu\text{mol/L}$, 15 min) did not significantly modify the electrical field stimulation (EFS)-induced contractile response compared to vehicle. ANOVA results: $F_{\text{treatments}} = 1.26$, $df = 1/9$, $P = 0.29$; $F_{\text{frequencies}} = 22.50$, $df = 2/18$, $P = 0.001$; $F_{\text{treatments} \times \text{frequencies}} = 1.79$, $df = 2/18$, $P = 0.21$; B: Incubation with G (3 $\mu\text{mol/L}$, 15 min) did not affect the contractile response to acetylcholine (ACh) (0.1–100 $\mu\text{mol/L}$) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 3.48$, $df = 1/9$, $P = 0.09$; $F_{\text{concentrations}} = 21.35$, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 0.08$, $df = 3/27$, $P = 0.85$. Values are expressed as the mean \pm SD of 6–8 experiments.

response to exogenous ACh (0.1–100 $\mu\text{mol/L}$) compared to vehicle ($F_{\text{treatments}} = 0.006$, $df = 1/9$, $P = 0.94$; $F_{\text{concentrations}} = 36.89$, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 0.84$, $df = 3/27$, $P = 0.45$) (Figure 5C).

Effects of CORM-3 on EFS-induced and ACh-induced contractile response of colon preparations: Assessment of the EFS-induced contractile response after CORM-3 (100–400 $\mu\text{mol/L}$) administration shows that CORM-3 (400 $\mu\text{mol/L}$) significantly increased the EFS-induced contractile response compared to vehicle at 10 Hz [$F_{\text{treatments}} = 2.75$, $df = 3/20$, $P = 0.07$; $F_{\text{frequencies}} = 55.38$, $df = 2/40$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 4.36$, $df = 6/40$, $P = 0.002$; at 10 Hz: CORM-3 (400 $\mu\text{mol/L}$) vs vehicle $^aP = 0.01$] (Figure 6A).

When repeated after 20-min incubation with atropine (3 $\mu\text{mol/L}$, 20 min), the increased EFS-induced contractile response by CORM-3 (400 $\mu\text{mol/L}$) administration was abolished: $F_{\text{treatments}} = 3.06$, $df = 3/20$, $P = 0.052$; $F_{\text{frequencies}} = 50.05$, $df = 2/40$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.14$, $df = 6/40$, $P = 0.36$. Consistent with the results obtained with ZnPPiX, these observations suggest that CORM-3 may enhance the EFS-induced release of endogenous ACh (Figure 6B).

Analysis performed to determine the effect of CORM-3 administration (100–400 $\mu\text{mol/L}$) on the contractile response to exogenous ACh (0.1–100 $\mu\text{mol/L}$) showed that incubation with CORM-3 (400 $\mu\text{mol/L}$) increases the contractile response to the highest ACh concentration (100 $\mu\text{mol/L}$) compared to vehicle-treated samples [$F_{\text{treatments}} = 2.28$, $df = 3/22$, $P = 0.11$; $F_{\text{concentrations}} = 86.22$, $df = 3/66$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.49$, $df = 9/66$, $P = 0.02$; for ACh 100 $\mu\text{mol/L}$: CORM-3 (400 $\mu\text{mol/L}$) vs vehicle: $P = 0.012$] (Figure 6C).

Effects of co-administration of granisetron with ZnPPiX or CORM-3 on EFS-induced and ACh-induced contractile response of colon preparations: When co-administered with granisetron (3

$\mu\text{mol/L}$, 15 min), incubation with ZnPPiX (10 $\mu\text{mol/L}$, 60 min) did not significantly modify the EFS-induced contraction compared to vehicle-treated samples ($F_{\text{treatments}} = 0.43$, $df = 1/8$, $P = 0.53$; $F_{\text{frequencies}} = 55.35$, $df = 2/16$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.66$, $df = 2/16$, $P = 0.22$) (Figure 7A). Because incubation with ZnPPiX alone decreased the contractile response to EFS (Figure 5A), it is plausible to infer that co-administration of granisetron was responsible for the abolished effects of ZnPPiX on EFS-induced colon contraction.

Co-administration of ZnPPiX (10 $\mu\text{mol/L}$, 60 min) and granisetron (3 $\mu\text{mol/L}$, 15 min) did not modify the myogenic contractile response to ACh ($F_{\text{treatments}} = 0.22$, $df = 1/8$, $P = 0.65$; $F_{\text{concentrations}} = 39.19$, $df = 3/24$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 4.06$, $df = 3/24$, $P = 0.02$). (Figure 7B).

When the effects of CORM-3 (100–400 $\mu\text{mol/L}$) on the EFS-induced contractile response were analyzed in combination with granisetron (3 $\mu\text{mol/L}$, 15 min), the results showed that co-incubation of CORM-3 (400 $\mu\text{mol/L}$) and granisetron significantly increased the EFS-induced contractile response when compared to vehicle-treated samples at 5 and 10 Hz [$F_{\text{treatments}} = 5.47$, $df = 3/19$, $P < 0.01$; $F_{\text{frequencies}} = 55.40$, $df = 2/38$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 3.05$, $df = 6/38$, $P = 0.04$; granisetron (3 $\mu\text{mol/L}$, 15 min) and CORM-3 (400 $\mu\text{mol/L}$) vs vehicle at 5 Hz: $P = 0.007$ and at 10 Hz: $P = 0.001$ (Figure 7C).

Interestingly, when compared to vehicle-treated samples, the concomitant incubation of CORM-3 (400 $\mu\text{mol/L}$) with granisetron significantly increased the myogenic response to ACh at 10 and 100 $\mu\text{mol/L}$ ($F_{\text{treatments}} = 7.40$, $df = 3/19$, $P = 0.002$; $F_{\text{concentrations}} = 61.69$, $df = 3/57$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.55$, $df = 9/57$, $P = 0.027$; at ACh 10 $\mu\text{mol/L}$: $P = 0.001$ and at ACh 100 $\mu\text{mol/L}$: $P = 0.001$) (Figure 7D).

Effects of co-administration of granisetron, ZnPPiX, L-NNA and granisetron, CORM-3, L-NNA on EFS-induced and ACh-induced contractile response of colon preparations: When co-adminis-

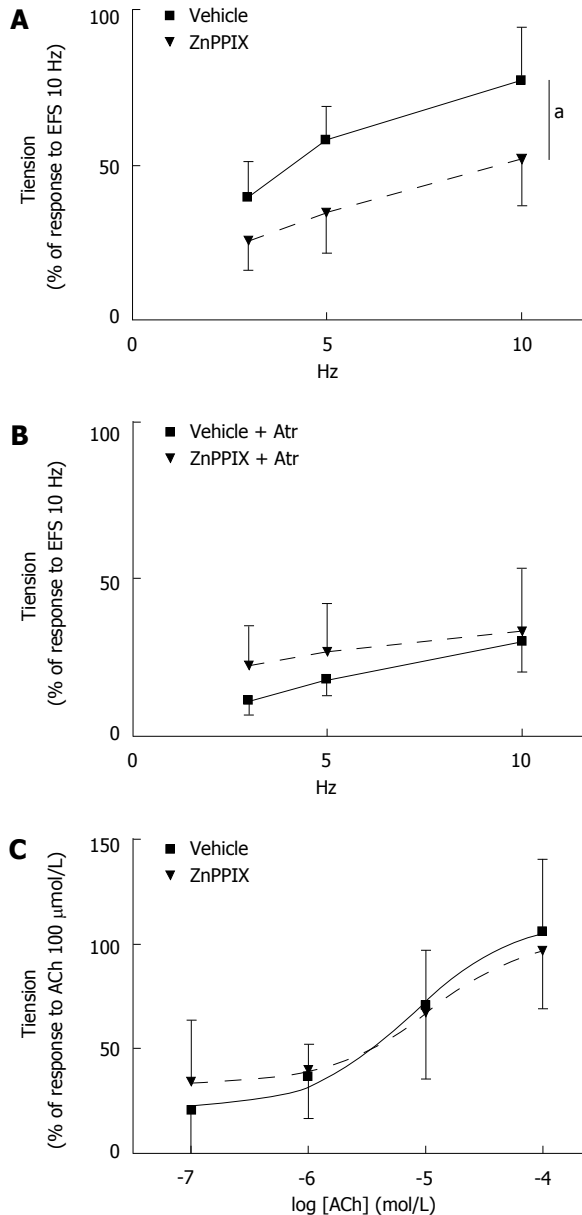


Figure 5 Effects of *in vitro* treatment with zinc protoporphyrin on rat colon contractile response to electrical field stimulation, without and with atropine, and to acetylcholine. A: Incubation with zinc protoporphyrin (ZnPPiX) (10 $\mu\text{mol/L}$, 60 min) significantly reduced the electrical field stimulation (EFS)-induced contractile response compared to vehicle. ANOVA results: $F_{\text{treatments}} = 8.78$, $df = 1/9$, $^aP = 0.016$; $F_{\text{frequencies}} = 50.33$, $df = 2/18$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.79$, $df = 2/18$, $P = 0.21$; B: Co-incubation with ZnPPiX (10 $\mu\text{mol/L}$, 60 min) with atropine (3 $\mu\text{mol/L}$, 20 min) abolished the effect of ZnPPiX alone. ANOVA results: $F_{\text{treatments}} = 1.44$, $df = 1/11$, $P = 0.25$; $F_{\text{frequencies}} = 37.66$, $df = 2/22$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 2.74$, $df = 2/22$, $P = 0.09$; C: Incubation with ZnPPiX (10 $\mu\text{mol/L}$, 60 min) had no effect on contractile response to atropine (Atr) (0.1–100 $\mu\text{mol/L}$) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.006$, $df = 1/9$, $P = 0.94$; $F_{\text{concentrations}} = 36.89$, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 0.84$, $df = 3/27$, $P = 0.45$. Values are expressed as the mean \pm SD of 6–8 experiments.

tration of granisetron (3 $\mu\text{mol/L}$, 15 min) and ZnPPiX (10 $\mu\text{mol/L}$, 60 min) was combined with L-NNA (100 $\mu\text{mol/L}$, 20 min), no difference in EFS-induced contractile effects was observed compared to vehicle-treated samples ($F_{\text{treatments}} = 0.08$, $df = 1/9$, $P = 0.79$, $F_{\text{frequencies}} = 24.89$, $df = 2/18$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}}$

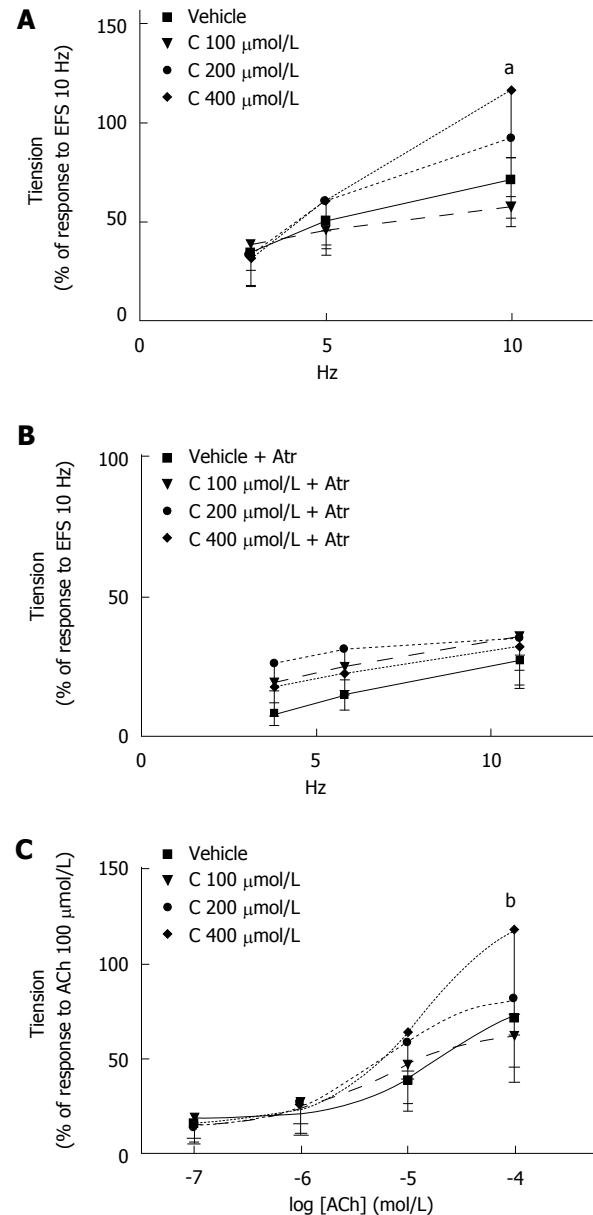


Figure 6 Effects of *in vitro* treatment with CORM-3 on rat colon contractile response to electrical field stimulation, without and with atropine, and to acetylcholine. A: Incubation with CORM-3 (C) (400 $\mu\text{mol/L}$) significantly increased the electrical field stimulation (EFS)-induced contractile response compared to vehicle at 10 Hz. ANOVA results: $F_{\text{treatments}} = 2.75$, $df = 3/20$, $P = 0.07$; $F_{\text{frequencies}} = 55.38$, $df = 2/40$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 4.36$, $df = 6/40$, $P = 0.002$. At 10 Hz: C (400 $\mu\text{mol/L}$) vs vehicle $^aP = 0.01$; B: Co-incubation of C (100–400 $\mu\text{mol/L}$) with atropine (Atr) (3 $\mu\text{mol/L}$, 20 min) abolished the effect of C when administered alone. ANOVA results: $F_{\text{treatments}} = 3.06$, $df = 3/20$, $P = 0.052$; $F_{\text{frequencies}} = 50.05$, $df = 2/40$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.14$, $df = 6/40$, $P = 0.36$; C: Incubation with C (400 $\mu\text{mol/L}$) increased the contractile response to acetylcholine (ACh) (100 $\mu\text{mol/L}$) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 2.28$, $df = 3/22$, $P = 0.11$; $F_{\text{concentrations}} = 86.22$, $df = 3/66$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.49$, $df = 9/66$, $P = 0.02$. For ACh 100 $\mu\text{mol/L}$: C (400 $\mu\text{mol/L}$) vs vehicle: $^bP = 0.012$. Values are expressed as the mean \pm SD of 6–8 experiments.

$= 0.03$, $df = 2/18$, $P = 0.91$) (Figure 8A). Similarly, contractile responses to exogenous ACh administration were not modified by concomitant administration of granisetron, ZnPPiX and L-NNA (vs vehicle-treated samples) ($F_{\text{treatments}} = 0.03$, $df = 1/9$, $P = 0.87$; $F_{\text{concentra-}}$

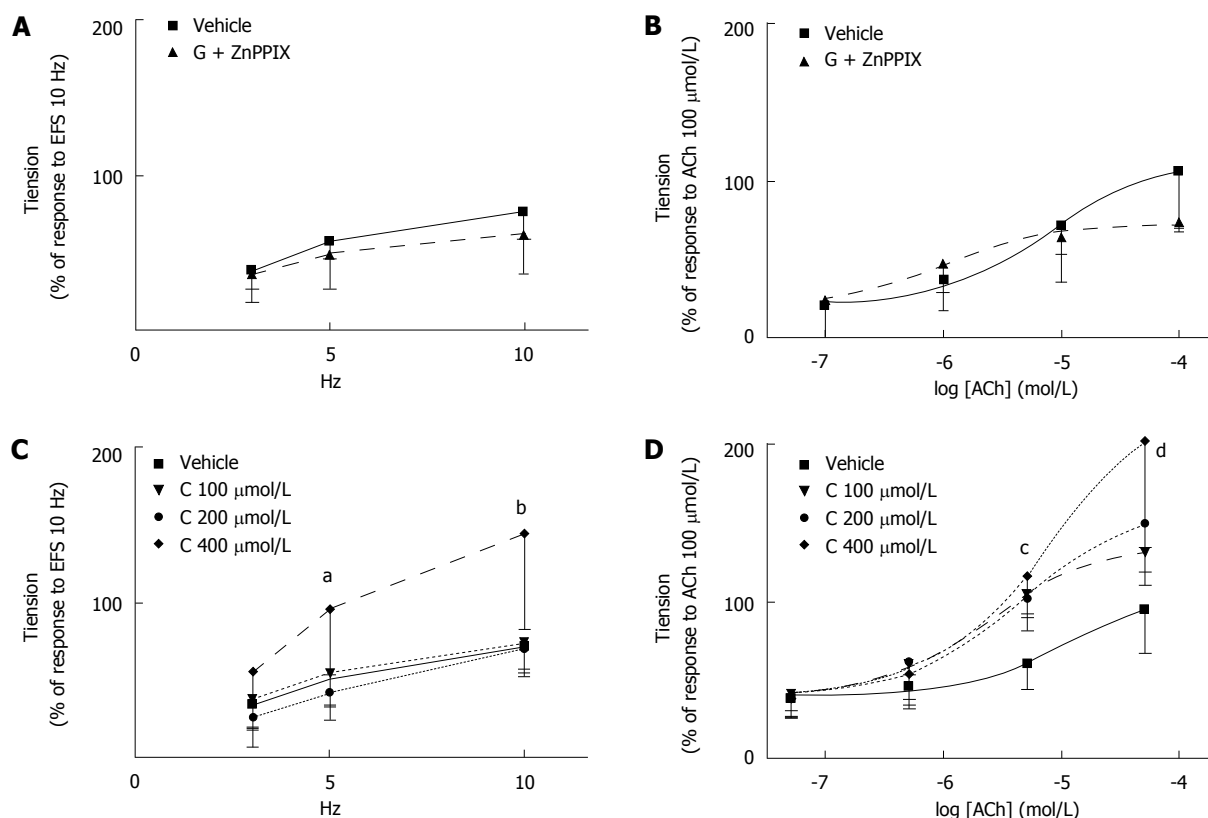


Figure 7 Effects of *in vitro* treatment with granisetron and zinc protoporphyrin and with granisetron and CORM-3 on rat colon contractile response to electrical field stimulation (EFS) and to acetylcholine (ACh). A: Co-incubation with granisetron (G) (3 μmol/L, 15 min) and zinc protoporphyrin (ZnPPiX) (10 μmol/L, 60 min) did not significantly modify the EFS-induced contraction compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.43$, $df = 1/8$, $P = 0.53$; $F_{\text{frequencies}} = 55.35$, $df = 2/16$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 1.66$, $df = 2/16$, $P = 2$; B: Co-incubation with G (3 μmol/L, 15 min) and ZnPPiX (10 μmol/L, 60 min) did not modify the myogenic contractile response to acetylcholine (ACh) (0.1–100 μmol/L) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.22$, $df = 1/8$, $P = 0.65$; $F_{\text{concentrations}} = 39.19$, $df = 3/24$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 4.06$, $df = 3/24$, $P = 0.02$; C: Co-incubation with G (3 μmol/L, 15 min) and CORM-3 (C) (400 μmol/L) increased the contractile response to EFS at 5 and 10 Hz compared to vehicle. ANOVA results: $F_{\text{treatments}} = 5.47$, $df = 3/19$, $P < 0.01$; $F_{\text{frequencies}} = 55.40$, $df = 2/38$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 3.05$, $df = 6/38$, $P = 0.04$. G (3 μmol/L, 15 min) and C (400 μmol/L) vs vehicle at 5 Hz: $^aP = 0.007$ and at 10 Hz: $^bP = 0.001$; D: Co-incubation of G (3 μmol/L, 15 min) and C (400 μmol/L) increased the contractile response to ACh 10 and 100 μmol/L compared to vehicle. ANOVA results: $F_{\text{treatments}} = 7.40$, $df = 3/19$, $P = 0.002$; $F_{\text{concentrations}} = 61.69$, $df = 3/57$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.55$, $df = 9/57$, $P = 0.027$. G (3 μmol/L, 15 min) and C (400 μmol/L) compared to vehicle at ACh 10 μmol/L: $^cP = 0.001$ and at ACh 100 μmol/L: $^dP = 0.001$. Values are expressed as the mean \pm SD of 6–8 experiments.

tions = 45.18, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.90$, $df = 3/27$, $P = 0.04$) (Figure 8B).

Co-administration of granisetron (3 μmol/L, 15 min) and CORM-3 (100–400 μmol/L) with L-NNA (100 μmol/L, 20 min) did not affect the EFS-induced contractile response at any frequency investigated (vs vehicle-treated samples) ($F_{\text{treatments}} = 0.83$, $df = 3/18$, $P = 0.49$, $F_{\text{frequencies}} = 25.51$, $df = 2/36$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 0.89$, $df = 6/36$, $P = 0.50$) (Figure 8C) and did not modify the contractile responses to exogenous ACh administration (vs vehicle-treated samples) ($F_{\text{treatments}} = 3.38$, $df = 3/17$, $P = 0.04$; $F_{\text{concentrations}} = 33.08$, $df = 3/57$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 1.47$, $df = 9/51$, $P = 0.25$, pre-planned contrast not significant) (Figure 8D).

Effects of co-administration of granisetron and L-NNA on EFS-induced and ACh-induced contractile response of colon preparations: Co-administration of granisetron (3 μmol/L, 15 min) and L-NNA (100 μmol/L, 20 min) increased the contractile response to EFS compared to vehicle-treated samples ($F_{\text{treatments}}$

= 6.73, $df = 1/11$, $P = 0.025$; $F_{\text{frequencies}} = 16.80$, $df = 2/22$, $P = 0.001$; $F_{\text{treatments} \times \text{frequencies}} = 1.26$, $df = 2/22$, $P = 0.30$) (Figure 9A).

Likewise, administration of granisetron (3 μmol/L, 15 min) and L-NNA (100 μmol/L, 20 min) increased the myogenic response to ACh compared to vehicle-treated samples ($F_{\text{treatments}} = 25.33$, $df = 1/11$, $P < 0.001$; $F_{\text{concentrations}} = 80.22$, $df = 3/33$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 15.8$, $df = 3/33$, $P = 0.001$; *t*-test for ACh 10 μmol/L: $t = 5.06$, $P = 0.000$ and for ACh 100 μmol/L: $t = 4.99$, $P = 0.000$) (Figure 9B).

DISCUSSION

This study was planned to clarify the mechanisms underlying the potential contribution of the HO/CO pathway in the constipating effects of granisetron in rats. In a previous report, we found that inhibition of HO or increased expression of HO-1 in rat duodenum was able to influence the granisetron effects on the EFS-dependent response^[9]. These findings provided a first evidence that the HO/CO pathway may play a

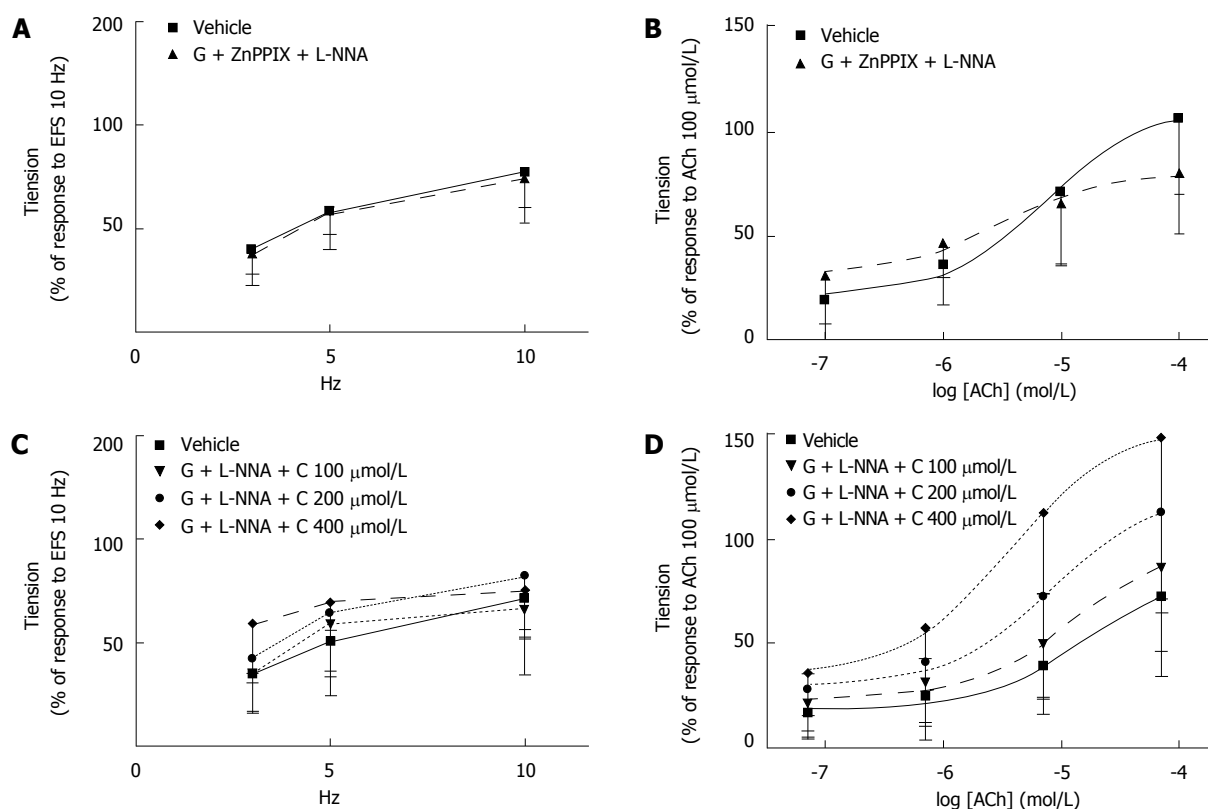


Figure 8 Effects of *in vitro* treatment with granisetron, zinc protoporphyrin and N⁶-nitro-L-Arginine and with granisetron, CORM-3 and L-NNA on rat colon contractile response to electrical field stimulation and to acetylcholine. A: Co-incubation with granisetron (G) (3 μmol/L, 15 min), ZnPPiX (10 μmol/L, 60 min) and N⁶-nitro-L-Arginine (L-NNA) (100 μmol/L, 20 min) did not affect the electrical field stimulation (EFS)-induced contractile response compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.08$, $df = 1/9$, $P = 0.79$; $F_{\text{frequencies}} = 24.89$, $df = 2/18$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 0.03$, $df = 2/18$, $P = 0.91$; B: Co-incubation with G (3 μmol/L, 15 min), zinc protoporphyrin (ZnPPiX) (10 μmol/L, 60 min) and L-NNA (100 μmol/L, 20 min) did not affect the contractile response to acetylcholine (ACh) (0.1–100 μmol/L) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.03$, $df = 1/9$, $P = 0.87$; $F_{\text{concentrations}} = 45.18$, $df = 3/27$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 3.90$, $df = 3/27$, $P = 0.04$; C: Co-incubation with G (3 μmol/L, 15 min), C (100–400 μmol/L) and L-NNA (100 μmol/L, 20 min) did not affect the EFS-induced contractile response compared to vehicle. ANOVA results: $F_{\text{treatments}} = 0.83$, $df = 3/18$, $P = 0.49$, $F_{\text{frequencies}} = 25.51$, $df = 2/36$, $P < 0.0001$; $F_{\text{treatments} \times \text{frequencies}} = 0.89$, $df = 6/36$, $P = 0.50$; D: Co-incubation with G (3 μmol/L, 15 min), CORM-3 (C) (100–400 μmol/L) and L-NNA (100 μmol/L, 20 min) did not affect the contractile response to ACh (0.1–100 μmol/L) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 3.38$, $df = 3/17$, $P = 0.04$, $F_{\text{concentrations}} = 33.08$, $df = 3/57$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 1.47$, $df = 9/51$, $P = 0.25$. Values are expressed as the mean \pm SD of 6–8 experiments.

role in the constipating activity of granisetron. However, because constipation is more closely related to abnormalities of colon motility, rather than in the duodenum^[16–19], we planned to focus directly on the colon contractile responses. Moreover, in our previous study, the role of the HO/CO pathway on rat duodenum was evaluated under NANC conditions^[9] to avoid the overwhelming effects of the main neurotransmitters at the gastrointestinal level, namely ACh and noradrenaline (NA). However, neurogenic gastrointestinal motility is strictly dependent on ACh and NA-mediated effects, and the functional relevance of NANC neurotransmission *in vivo* is still largely unknown^[30]. Thus, in this work, the assessment of colon neurogenic response to granisetron was investigated under conditions directly resembling the existing intestinal environment.

Consistent with literature data reporting constipation in patients treated with granisetron as an antiemetic therapy^[11,15], we observed an increased time to first defecation, a recognized indicator of whole-gut transit^[24,25], after acute administration of granisetron in rats. Granisetron-induced constipation was abol-

ished by *in vivo* co-administration with ZnPPiX (HO inhibitor), whereas co-administration of hemin (HO-1 inducer) did not decrease the delayed time to first defecation observed in granisetron-treated rats. These data support an active role of the HO/CO system in the constipating effect of granisetron^[9]. Interestingly, neither ZnPPiX nor hemin was able to affect rat gastrointestinal motility when administered alone *in vivo*. This is not surprising because the HO/CO pathway is likely to be a fine-tuning mechanism whose activity may enhance or limit the extension of major signals involved in the integrated control of colon motility.

Consistent with this view, and with studies reporting a substantial effect of 5-HT₃ antagonists only in the presence of high levels of 5-HT, either exogenously administered or endogenously released from enterochromaffin cells (for example, by mucosal pressure, distortion and/or chemical stimuli^[31–33]), granisetron administration *in vitro* did not significantly inhibit the contractile response to EFS and showed a borderline trend to increase the contraction mediated by ACh ($P = 0.09$). Interestingly, colon contractile responses to

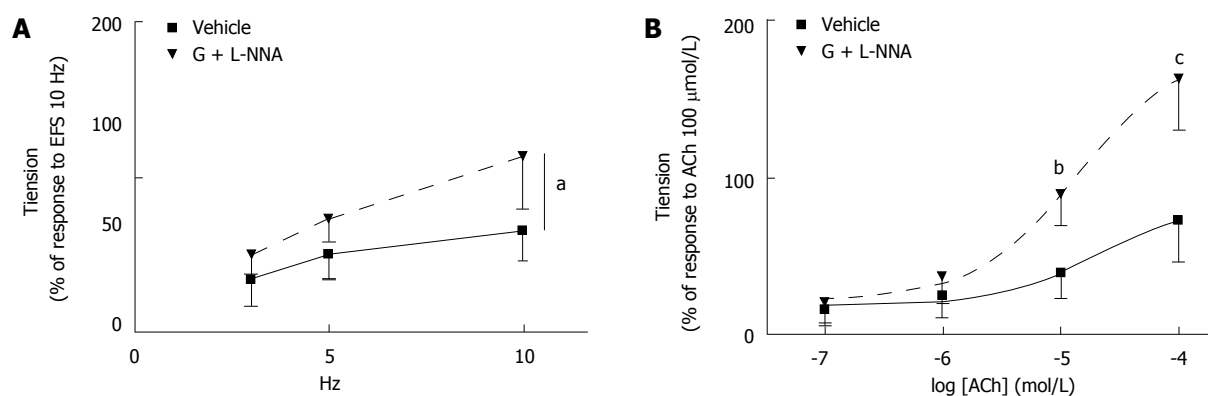


Figure 9 Effects of *in vitro* treatment with granisetron and N^6 -nitro-L-Arginine on rat colon contractile response to electrical field stimulation and to acetylcholine. **A:** Co-incubation with granisetron (G) (3 μ mol/L, 15 min) and N^6 -nitro-L-Arginine (L-NNA) (100 μ mol/L, 20 min) resulted in significantly increase of electrical field stimulation (EFS)-induced contractile responses at all frequencies used compared to vehicle. ANOVA results: $F_{\text{treatments}} = 6.73$, $df = 1/11$, $^aP = 0.025$; $F_{\text{frequencies}} = 16.80$, $df = 2/22$, $P = 0.001$; $F_{\text{treatments} \times \text{frequencies}} = 1.26$, $df = 2/22$, $P = 0.30$; **B:** Co-incubation with G (3 μ mol/L, 15 min) and L-NNA (100 μ mol/L, 20 min) resulted in significantly increased contractile response induced by acetylcholine (ACh) (10 and 100 μ mol/L) compared to vehicle. ANOVA results: $F_{\text{treatments}} = 25.33$, $df = 1/11$, $P < 0.001$; $F_{\text{concentrations}} = 80.22$, $df = 3/33$, $P < 0.0001$; $F_{\text{treatments} \times \text{concentrations}} = 15.8$, $df = 3/33$, $P = 0.001$. *T*-test for ACh 10 μ mol/L: $t = 5.06$, $^bP = 0.000$ and for ACh 100 μ mol/L: $t = 4.99$, $^cP = 0.000$. Values are expressed as the mean \pm SD of 6-8 experiments.

EFS were decreased *in vitro* by incubation with ZnPIX alone. Because ZnPIX inhibits the HO-mediated production of CO, it is plausible to infer that the EFS-dependent contraction is mediated, at least in part, by CO. This hypothesis is consistent with studies reporting an almost completely abolished inhibitory response to EFS in jejunal smooth muscle strips of mice with targeted genomic deletion of HO-2. Concomitantly, in these animals, an exogenous administration of CO restores the EFS response^[34].

CO appears to have a facilitatory effect on EFS-mediated ACh release, as suggested by the impaired ACh release observed in frog neuromuscular junctions under ZnPIX incubation^[35]. Analogous behavior was observed in our study in which the impaired contractile response to EFS obtained under ZnPIX was restored by concomitant incubation with the muscarinic antagonist atropine. This finding, together with the lack of any effect of ZnPIX on the myogenic contractile response to exogenous ACh, implies that a phasic CO production is required for physiological ACh release in rat colon.

The potential role of CO on granisetron effects, investigated *in vivo* by co-administration of hemin, was mimicked *in vitro* by co-administration of CORM-3, a CO-releasing molecule able to replicate the effects of HO-1 stimulation with hemin^[3,36]. At the highest dose used (400 μ mol/L) CORM-3 significantly increases the contractile response to both EFS (10 Hz) and exogenous ACh (100 μ mol/L). These findings suggest that one mechanism by which CO may enhance the contractile response in rat colon is by facilitating the release of endogenous ACh. In addition, CO may indirectly potentiate the ACh contractile effects, as proposed by Lim *et al.*^[37], by concurrently activating L-type calcium channels in human intestinal smooth muscle *via* a nitric oxide (NO)-dependent mechanism. The binding of NO to guanylyl cyclase with subsequent changes in cAMP and intracellular Ca^{2+} levels will eventually lead to activation of the "contractile apparatus"^[37].

When granisetron and CORM-3 were co-administered, the colon's contractile responses to both EFS and ACh were further increased, suggesting a synergistic effect between these two substances. Similarly, when granisetron and ZnPIX were co-administered, the effects of ZnPIX alone were lost. Although the exact mechanism of granisetron and HO/CO system interplay remains to be clearly established, some explanations may be proposed: one is that, as suggested by the bell-shaped curve for *in vivo* response^[38], granisetron may behave as a partial agonist at the concentrations used for the present *in vitro* and *in vivo* studies^[39,40]. In this case, the activation of 5-HT₃ receptors followed by subsequent increased release of ACh may have overcome the inhibition of ACh release secondary to ZnPIX. Concomitantly, acting as a partial 5-HT₃ agonist, granisetron may synergistically potentiate CORM-3 effects by increasing calcium influx.

Because the activation of L-type Ca^{2+} channels operated by CO is a NO-dependent mechanism, inhibition of NO production is expected to decrease the CORM-3-mediated effects. Indeed, in the presence of NO synthase inhibitor L-NNA, the potentiating effect of CORM-3 on granisetron activity was lost, confirming the necessary role of NO for the observed activities.

Because of the nature of the study, the following limitations must be considered. First, we cannot conclusively exclude that the colon response to granisetron/ZnPIX treatment might be related to changes in the serotonergic system; nevertheless, the results obtained strongly suggest that the constipating effect of granisetron is only indirectly affected by ZnPIX, which acts through reduction of EFS-induced acetylcholine release. Second, it is not clear whether the alleviation of granisetron-induced constipation might affect the antiemetic potential of this drug; studies directly evaluating this parameter would require a specific animal model and a completely different experimental approach, both of which are unavailable at this time. However, our per-

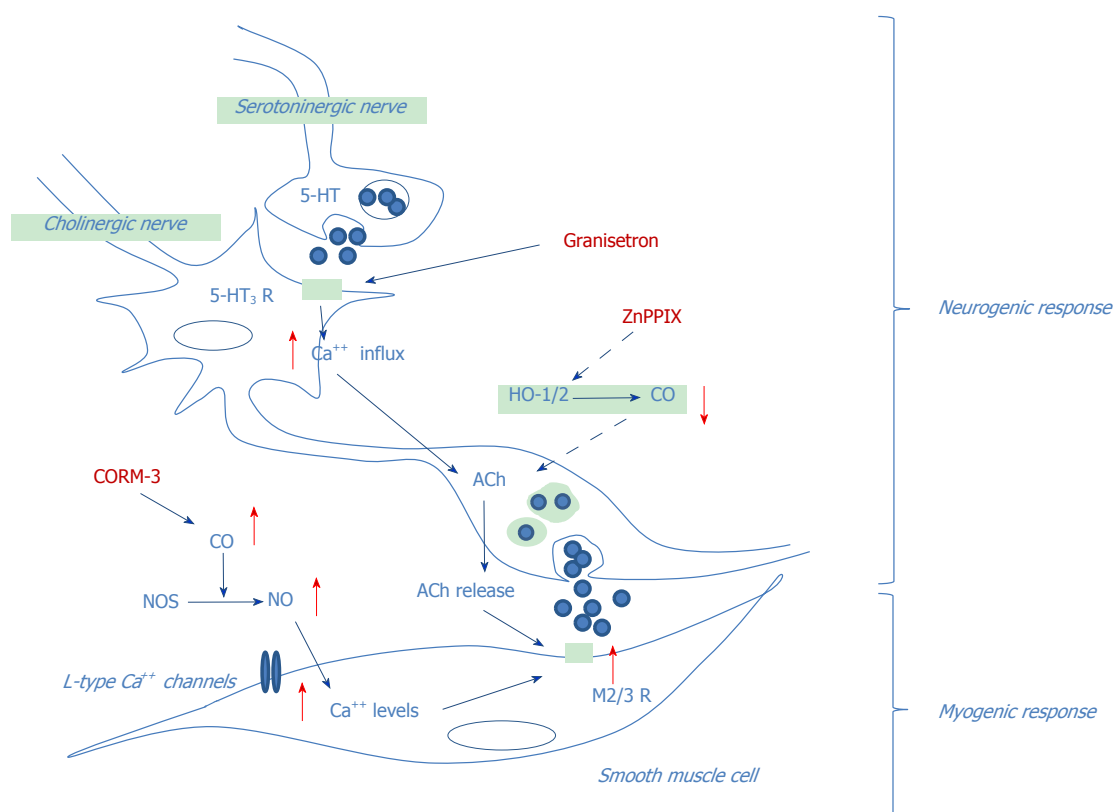


Figure 10 Potential relationship between granisetron, ZnPPiX (HO-1/2 inhibitor), and CORM-3 (CO-releasing agent) on colon neurogenic and myogenic contractile responses. Acting on 5-HT₃ receptors, granisetron may increase calcium influx, thus facilitating the release of acetylcholine (ACh) (neurogenic response), which in turn elicits a myogenic contractile response. By inhibiting the heme oxygenase (HO)-mediated carbon monoxide (CO) production, ZnPPiX may reduce the nerve terminal release of ACh, thereby counteracting granisetron effects. By releasing carbon monoxide (CO), CORM-3 may enhance the ACh-mediated myogenic contraction via a nitric oxide (NO)-dependent mechanism resulting in increased intracellular cAMP and calcium levels, with subsequent activation of L-type calcium channels and potentiation of the granisetron-mediated myogenic response.

ception is that alleviation of granisetron-induced constipation does not interfere with its antiemetic activity because this last effect relates to granisetron's ability to reach the CNS. In this regard, it has been reported that ZnPPiX does not cross the blood-brain barrier^[1,41]. Thus, it is plausible that the effects of ZnPPiX to reduce granisetron-induced constipation are related to peripheral mechanisms not involving the CTZ. Third, gastrointestinal transit (GIT) was measured by observing the time to first defecation after food ingestion; although intragastric administration of a non-absorbable, colored marker is considered the reference method to measure GIT, additional gavage administration would increase stress in animals and potentially affect the parameter evaluated. In our study, we considered the delayed GIT in rats treated with granisetron (compared to rats treated with vehicle) as a positive control to evaluate the effects of ZnPPiX and CORM-3 on the "time to first defecation" after food ingestion.

In conclusion, findings from the present study may shed light on the involvement of the HO/CO pathway in the neurogenic and myogenic contractile responses in rat colon and propose potential mechanisms underlying the interaction of granisetron and CO on colon motility (Figure 10).

Considering that granisetron is mainly used to pre-

vent chemotherapy-induced nausea and vomiting in cancer patients and that increased expression of HO-1 has been observed in several cancer types^[42], our findings suggest that HO inhibitors may be a reasonable therapeutic approach to reduce the unwanted constipating effects of granisetron.

ACKNOWLEDGEMENTS

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COMMENTS

Background

In recent decades, the role played by carbon monoxide (CO) in several biochemical processes has been increasingly recognized. Once considered only for its lethal effects, the therapeutic use of CO has been proposed after the discovery of its potential "positive" functions. Ion channels have been shown to be, among others, the target of CO; thus, it is possible that CO may modulate the effects of other signals by acting directly on the same target or indirectly on the shared pool of secondary messengers. A similar modulating activity of CO might also be plausible toward specific drugs.

Research frontiers

In a previous report, authors observed the involvement of the heme oxygenase (HO)/CO pathway in granisetron-mediated effects on duodenal motility.

Innovations and breakthroughs

Findings from the present study may shed light on the involvement of the HO/CO pathway in the neurogenic and myogenic contractile responses in rat colon and propose potential mechanisms underlying the interaction of granisetron and CO on colon motility.

Applications

Considering that granisetron is mainly used to prevent chemotherapy-induced nausea and vomiting in cancer patients and that increased expression of HO-1 has been observed in several cancer types^[42], the authors findings suggest that HO inhibitors may be a reasonable therapeutic approach to reduce the unwanted constipating effects of granisetron.

Terminology

Electrical field stimulation allows measurement of the neurogenic contractile response. In rat colon preparations, the electrical field stimulation (EFS) induces an immediate relaxation of specimens followed, at the end of EFS, by a contraction called off-contraction. This contractile response is indicative of a nervous reflex. Moreover, activation of enteric nerves by electrical field stimulation mimics the *in vivo* conditions because neurotransmitters are released by motor neurons to the neuroeffector apparatus in which interstitial cells of Cajal, neurons, glial cells and smooth muscle cells interact and induce contraction.

Peer-review

The authors present interesting data about HO/CO pathway and granisetron. The authors report detailed data and proposed potential mechanisms underlying the interaction of granisetron and CO. Overall, it is an important study, and should be considered for publication.

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Basic Study

Genetic polymorphism in *CD14* gene, a co-receptor of TLR4 associated with non-alcoholic fatty liver disease

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Abstract

AIM

To evaluate the pathogenic role of toll-like receptor (TLR) gene polymorphisms in patients with non-alcoholic fatty liver disease (NAFLD).

METHODS

Two hundred and fifty subjects (NAFLD = 200, healthy volunteers = 50) underwent polymerase chain reaction and restriction fragment length polymorphism to assess one polymorphism in the toll-like receptor 2

(*TLR2*) gene (A753G), two polymorphisms in the *TLR4* gene (TLR4 Asp299Gly and Thr399Ile allele), and two polymorphisms in the cluster of differentiation 14 (*CD14*) (C-159T and C-550T) gene, a co-receptor of TLR4. Association of *TLR* gene polymorphisms with NAFLD and its severity was evaluated by genetic models of association.

RESULTS

On both multiplicative and recessive models of gene polymorphism association, there was significant association of CD14 C (-159) T polymorphism with NAFLD; patients with TT genotype had a 2.6 fold increased risk of developing NAFLD in comparison to CC genotype. There was no association of TLR2 Arg753Gln, TLR4 Asp299Gly, Thr399Ile, and CD14 C (-550) T polymorphisms with NAFLD. None of the TLR gene polymorphisms had an association with histological severity of NAFLD.

CONCLUSION

Patients with CD14 C (-159) T gene polymorphism, a co-receptor of TLR4, have an increased risk of NAFLD development.

Key words: Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Toll-like receptors; Obesity; Cirrhosis; Insulin resistance; Bacterial overgrowth

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Core tip: Our study demonstrated that non-alcoholic fatty liver disease (NAFLD) patients with TT genotype of C (-159) T polymorphism in cluster of differentiation 14 promoter gene have a higher risk of NAFLD development. However, this polymorphism did not affect liver disease severity. We found no association of toll-like receptor (TLR) 2 ARG753, TLR4 (Asp299Gly), TLR4 (Thr399Ile), and CD 14 C/T 550 polymorphisms with the risk of NAFLD development.

Kapil S, Duseja A, Sharma BK, Singla B, Chakraborti A, Das A, Ray P, Dhiman RK, Chawla Y. Genetic polymorphism in *CD14* gene, a co-receptor of TLR4 associated with non-alcoholic fatty liver disease. *World J Gastroenterol* 2016; 22(42): 9346-9355 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9346.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9346>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum condition, ranging from simple steatosis to its progressive form of non-alcoholic steatohepatitis (NASH), and has emerged as an important cause of the otherwise unexplained increase in hepatic transaminases, cryptogenic cirrhosis, and cryptogenic

hepatocellular carcinoma (HCC)^[1-3]. Familial studies and inter-ethnic variation in susceptibility to the disease suggest that genetic factors are important in the occurrence and determining the risk of progressive NAFLD^[4]. Studies suggests a strong association of NAFLD with patatin-like phospholipase domain containing 3 gene polymorphism^[5-7], as well as inconclusive association with apolipoprotein C-III^[8,9] and human hemochromatosis gene mutations^[10].

Emerging data suggest the role of small intestinal bacterial overgrowth (SIBO) and gut-derived endotoxins in the pathogenesis of NAFLD *via* inducing the release of proinflammatory cytokines, including tumor necrosis factor- α (TNF- α) from hepatic Kupffer cells.

Toll-like receptors (TLRs) are the most important family of pattern recognition receptors^[11,12]; they are the sensors for recognizing bacterial and viral components, such as lipopolysaccharides, bacterial DNA, and peptidoglycan. Of the various TLRs, toll-like receptor 2 (TLR2), TLR4, the co-receptor cluster of differentiation 14 (CD14), and TLR9 have been well studied in the pathogenesis of NAFLD. In addition to animal studies, two human studies^[13,14] have also suggested a higher risk of developing NASH in patients with NAFLD that possess *CD14* gene polymorphism.

The aim of our study was to evaluate the role of TLR polymorphisms in the causation and severity of NAFLD.

MATERIALS AND METHODS

Patients

This was a case-controlled study where 250 subjects [NAFLD ($n = 200$, males = 122, mean age 38.27 ± 10.3 years) and healthy volunteers (HVs) ($n = 50$, males = 38, mean age 36.56 ± 4.2 years)] were enrolled after informed consent. The study had the approval of the Institute's Ethics Committee. All subjects were enrolled prospectively, with the exception of 28 patients with biopsy-proven NAFLD; their records were retrieved from the existing database and were called again for consent and fresh sampling for TLR polymorphisms. Patients with NAFLD were recruited as per the standard inclusion criteria. Fifty age and gender matched HVs were recruited, as per the guidelines from the Indian Council of Medical Research, and they all had normal liver function tests, normal fasting plasma glucose, normal lipid profile, and no evidence of fatty liver on ultrasound.

Inclusion criteria for NAFLD: (1) age greater than 13 years; (2) non-alcoholic individuals, defined as either total abstainers or individuals who consumed less than 20 g of alcohol per day. History of alcohol consumption was confirmed by two family members of the patient; (3) raised serum transaminases more than one and a half times the upper limit of normal for at least 3 mo; (4) ultrasound showing

features of steatosis; (5) negative viral markers (HBsAg/Anti HCV), negative autoimmune markers [antinuclear antibody (ANA), anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and anti-mitochondrial antibody (AMA)]; (6) normal ceruloplasmin/negative Kayser-Fleischer rings; (7) normal iron work up [serum iron, total iron binding capacity (TIBC), ferritin, and transferrin saturation]; and (8) liver biopsy consistent with NAFLD (60 cases where liver biopsy was performed).

Exclusion criteria for NAFLD: (1) pregnant females; (2) patients with history of drug intake likely to cause NAFLD (e.g., corticosteroids, methotrexate, and tamoxifen); (3) jejunioileal bypass or extensive small bowel resection; (4) total parenteral nutrition at the time of liver biopsy; and (5) clinical, imaging, or liver biopsy features of liver cirrhosis.

Anthropometry

Height was determined with a measuring tape to the nearest cm. Subjects were requested to stand upright without shoes with their heels tight against the wall and eyes directed forward. Weight was measured in kilograms (kg) with a traditional spring balance, which was kept on a firm horizontal surface and the scale checked every day. Body Mass Index (BMI) was calculated using the formula: weight (kg)/height (m^2). Waist circumference was taken as the average of two measurements taken after inspiration and after expiration at the midpoint between the lowest rib and the iliac crest. Hip circumference was taken at the level of greater trochanter and waist-to-hip ratio was defined as the ratio of the waist and hip circumference.

Patients were classified as being lean, overweight, class I obese, class II obese, or centrally obese as per the Asian Pacific criteria (lean: BMI = 18-23 kg/ m^2 ; overweight: $> 23 < \text{BMI} < 25 \text{ kg}/m^2$; class I obesity: BMI ≥ 25 -30 kg/ m^2 ; class II obesity: BMI $> 30 \text{ kg}/m^2$; central obesity: waist circumference $> 90 \text{ cm}$ in males and $> 80 \text{ cm}$ in females)^[15,16].

Biochemical assessment

All patients with NAFLD underwent detailed baseline investigations, with selective investigations performed with HVs. In patients undergoing liver biopsy (32 = prospective, with 28 retrieved from the existing database), laboratory parameters were measured before the procedure. Serum bilirubin, aspartate aminotransferases, alanine aminotransferases (ALT) (Diagnosticum Rt., Budapest, Hungary), alkaline phosphatase (Reckon Diagnostics, Baroda, India), albumin, globulin (Far Diagnostics, Verona, Italy), total cholesterol, triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol (Roche Diagnostics, Indianapolis, United States) were determined as per the standard

methodology. A fasting plasma glucose of $> 126 \text{ mg/dL}$ on more than one occasion or a random plasma glucose of $> 200 \text{ mg/dL}$ in a symptomatic patient was defined as diabetes mellitus. Fasting plasma glucose of > 110 and $< 126 \text{ mg/dL}$ was defined as impaired fasting glucose and 2 h post-prandial plasma glucose between 140 and 200 mg/dL as impaired glucose tolerance. Lipid profile was determined in all patients and serum cholesterol $> 200 \text{ mg/dL}$, HDL $< 40 \text{ mg/dL}$ in males and $< 50 \text{ mg/dL}$ in females, LDL $> 130 \text{ mg/dL}$, and serum TG $> 150 \text{ mg/dL}$ was taken as abnormal. Serum iron and TIBC were measured by the colorimetric method and serum ferritin was measured using an enzyme immunoassay kit (Orgentec Diagnostika GmbH, Germany). Virological markers, such as HBV (HBsAg, HBeAg), and HCV (anti-HCV), and auto immune markers including ANA anti-smooth muscle antibodies, anti-liver-kidney microsomes, and AMA were determined using enzyme-linked immunosorbent assay (ELISA).

Imaging

All patients with NAFLD and CVH, as well as the HVs, were subjected to an abdominal ultrasound to detect and grade the degree of hepatic steatosis.

Metabolic syndrome

Metabolic syndrome was defined by the presence of ≥ 3 out of 5 modified adult treatment panel III criteria, including modified abnormal waist as per the Asia Pacific criteria, FPG $> 110 \text{ mg/dL}$ or known diabetes, hypertension (blood pressure $\geq 130/85 \text{ mmHg}$ or on antihypertensive drugs), serum TG $> 150 \text{ mg/dL}$, and HDL $< 40 \text{ mg/dL}$ in males or $< 50 \text{ mg/dL}$ in females^[17].

Histopathology

Sixty patients with NAFLD (32 recruited prospectively and 28 retrieved from database) were histologically assessed for degree of hepatic steatosis and fibrosis, and then divided into NASH, borderline NASH and no-NASH, as per the NAFLD activity score (NAS) given by the Nonalcoholic Steatohepatitis Clinical Research Network^[18].

Polymorphisms in the genes encoding for receptors (TLR2, TLR4, CD14)

Polymorphisms in the genes encoding for receptors (TLR2, TLR4, and CD14) were detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) to assess one polymorphism in the TLR2 gene (Arg753Gln), two in the TLR4 gene (TLR4 Asp299Gly and Thr399Ile allele), and two polymorphisms in the CD14 gene (C-159T and C-550T).

PBMC and genomic DNA isolation: PBMC were isolated from whole blood samples using Ficoll-Hypaque differential density gradient centrifugation procedures.

Table 1 Primer sequences, polymerase chain reaction conditions, and product sizes of different genes

Polymorphism site	Primer sequence (5'-3')	PCR conditions	Product size (bp)
TLR4 (Asp299gly)	F: GATTAGCATACTTAGACTACTACCTCCATG R: GATCAACITCTGAAAAAGCATTCAC	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 61.5 °C for 1 min 72 °C for 1 min (3) 72 °C for 7 min	249
TLR4 (Thr399Ile)	F: GGTTCGTGTTCTCAAAGTGATTTGGGAGAA R: CCTGAAGACTGGAGAGTGAGTTAAATGCT	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 64.8 °C for 1 min 72 °C for 1 min (3) 72 °C for 10 min	406
TLR2 (Arg753Gln)	F: 5'-CCTTCAAGTGTGTCCTCATAAG-3' R: 5'-GGCCACTCCAGGTAGGTCTT-3'	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 58.6 °C for 1 min 72 °C for 1 min (3) 72 °C for 10 min	289
CD14 (-550C/T)	F: GGAAGGGGAATTTTCTTTAGGC R: -GGCAGTGTCTGATGACTCA	(1) 95 °C for 10 min (2) 32 cycles of : 94 °C for 1 min 59.8 °C for 1 min 72 °C for 1 min (3) 72 °C for 7 min	368
CD14 (-159C/T)	F: ATCATCCTTTTCCACACC R: AACTCTTCGGCTGCCTCT	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 40 s 61 °C for 40 s 72 °C for 40 s (3) 72 °C for 10 min	296

Table 2 Restriction fragment length polymorphism conditions

Polymorphic site	Restriction enzyme (units used)	Incubation temperature and duration	Genotype and restriction fragment pattern (bp)
TLR4 (Asp299gly)	NcoI	37 °C for 16 h	AA: 249 AG: 249, 223, 26 GG: 223, 26
TLR4 (Thr399Ile)	HinfI	37 °C for 8 h	CC: 406 CT: 406, 377, 29 TT: 377, 29
TLR2 (Arg753Gln)	AciI	37 °C for 16 h	GG: 252 and 37 GA: 252, 37, 289 AA: 289
CD14 (-550C/T)	HaeIII	37 °C for 12 h	CC: 23, 236, 109 CT: 23, 236, 109, 259 TT: 109, 259
CD14 (-159C/T)	HaeIII	37 °C for 16 h	CC: 141, 154 CT: 141, 154, 296 TT: 296

Genomic DNA was extracted from cells in the buffy coat using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was used for detecting polymorphisms in different genes *via* PCR and RFLP.

PCR: Genomic DNA was amplified at specific regions containing the polymorphic sites using specific primer pairs flanking the respective polymorphic sites^[19,20]. A list of the PCR's conducted primer sequences, reaction

conditions, and product sizes are given in Table 1.

RFLP: PCR products were digested with appropriate restriction endonucleases to differentiate different genotypes. A list of restriction enzymes, incubation temperatures and times, and RFLP patterns of different genotypes are given in Table 2.

DNA sequencing

PCR products for all genotypes of all genes were validated commercially by DNA sequencing.

Estimation of adipocytokines and insulin resistance

Serum levels of various cytokines viz., adiponectin, TNF- α , and interleukin-1 β were estimated by (ELISA, Ray Biotech, Norcross GA.). Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) was calculated as the product of fasting insulin (μ U/mL) (Roche Diagnostics GmbH, Mannheim, Germany) and fasting plasma glucose (mmol/L) divided by 22.5. An absolute value of HOMA-IR > 1.64 was taken as abnormal^[21].

Statistical analysis

Data were analyzed for comparison between the two groups using SPSS version 15 for Windows (SPSS Inc. Chicago IL United States). Skewed clinical and biochemical data were expressed as a median (interquartile range), whereas normally-distributed

Table 3 Anthropometric and biochemical characteristics of patients with non-alcoholic fatty liver disease and healthy volunteers *n* (%)

Parameters	NAFLD (<i>n</i> = 200)	HVs (<i>n</i> = 50)	<i>P</i> value
Mean age (yr)	38.27 ± 10.3	36.56 ± 4.2	0.218
Gender	122 M/78 F	38 M/12 F	
Mean BMI (kg/m ²)	27.16 ± 4.7	22.1 ± 1.2	0.0002
Lean	35 (17.5)	46 (92)	0.0001
Overweight	39 (19.5)	3 (6)	0.02
Class I obesity	87 (43.5)	1 (2)	0.0001
Class II obesity	39 (19.5)	0 (0)	0.003
Waist (cm)	91.20 ± 9.5	78.12 ± 4.6	0.0001
Hip (cm)	91.32 (89.90-93.94)	89.18 (86.18-93.98)	0.3
Waist/hip ratio	0.99 (0.98-1.01)	0.93 (0.89-0.97)	0.016
Central obesity	135 (67.5)	0 (0)	0.0001
Mean AST (IU/L)	60.27 (46.0-78.7)	22.9 (18.75-29.50)	0.0001
Mean ALT (IU/L)	88.04 (68.9-118.9)	24 (17-29.5)	0.0001
Mean fasting sugar (mg/dL)	94.95 (87-105.8)	83.50 (75-89.25)	0.0001
Diabetes mellitus	29 (14.5)	0 (0)	0.005
Mean HDL (mg/dL)	43 (38-48.9)	52 (47.5-56)	0.0001
Mean Triglyceride (mg/dL)	156 (126-200.2)	102 (93-127.5)	0.0001
Hypertension	48 (24)	0 (0)	0.0001

NAFLD: Non-alcoholic fatty liver disease; HVs: Healthy volunteers; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL: High-density lipoprotein.

variables were expressed as mean ± SD. For continuous data, groups were tested for normal distribution using the Kolmogorov-Smirnov test (K-S test). For skewed data, the Mann-Whitney *U* test was applied for comparison between the two groups. For categorical data, the Chi-square test or Fischer exact test was applied. Genotypic association and the odds ratio with 95%CI were estimated by binary or multinomial logistic regression analysis. Haplotype analysis was carried out for *TLR4* and *CD14* gene using SHEsis software. In all cases, a *P* value less than 0.05 was considered significant.

Models and measures of association of gene polymorphisms: Recessive and dominant models were applied to determine the association of these polymorphisms with the recessive and dominant alleles; a recessive model indicates that two copies of allele A are required for a γ -fold increase in disease risk, while a dominant model indicates that either one or two copies of allele A are required for a γ -fold increase in disease risk^[22,23]. Patients with and without significant polymorphisms were compared to assess the difference between the two groups.

RESULTS

The demographic, anthropometric, and biochemical characteristics of the two groups (NAFLD and HVs) are summarized in Table 3.

Patients with NAFLD had higher BMI, higher overall and central obesity, elevated liver enzymes, and a higher prevalence of diabetes mellitus and dyslipidemia in comparison to HVs (Table 3). In the NAFLD group, 78 patients (39%) displayed the presence of metabolic syndrome.

Polymorphism analysis of *TLR2* gene

TLR2 Arg753Gln polymorphism: A PCR product size of 289 bp was subjected to restriction digestion with ACI 1 restriction enzyme at 37 °C to get the respective band size of 289 bp, 252 bp, and 37 bp, depending upon the deletion of restriction sites by mutant allele. GG was found to be the predominant genotype (89.5%), followed by GA (7.5%) and AA (3%) in NAFLD patients and GG (90%), GA (10%) and AA (0%) in HVs. There was no difference in GG [179/200 (89.5%) vs 45/50 (90%), *P* = 0.91] GA [15 (7.5%) vs 5 (10%), *P* = 0.56] and AA [6/200 (3%) vs 0/50 (0%), *P* = 0.21] genotypes in either study group. No association of TLR2 Arg753Gln polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

Polymorphism analysis of *TLR4* gene

TLR4 Asp299Gly polymorphism: TLR4 Asp299Gly polymorphism in exon 4 is an A/G polymorphism that creates a recognition site for restriction enzyme NcoI. Amplification with primer pairs yielded a 249 bp fragment. Respective band sizes of 249 bp, 223 bp, and 26 bp, depending upon the restriction sites created by mutant allele, were obtained. Digestion with enzyme gave a 249 bp fragment in the presence of AA genotype, three bands of 249 bp, 223 bp, and 26 bp in the presence of AG genotype, and two bands of 223 bp and 26 bp fragments in presence of GG genotype. The genotype frequency of AA, AG, and GG were 79%, 17%, and 4%, respectively, in the NAFLD group and 82%, 18%, and 0%, respectively, in the HV group. Distribution was in accordance with Hardy-Weinberg equilibrium. The AA, AG, and GG genotype were not statistically different in NAFLD compared to HVs (*P* = 0.15). No association of TLR4 Asp299Gly

Table 4 Distribution of CD14 -550C/T genotype, allele frequency and genetic models for CD14-550C/T *n* (%)

CD14 C (-550) T genotypes	CC	CT	TT
Cases (<i>n</i> = 200)	120 (60)	65 (32.5)	15 (7.5)
Control (<i>n</i> = 50)	34 (68)	14 (28)	2 (4)
OR	1	1.3 (0.6-2.6) <i>P</i> = 0.43	2.1 (0.4-9.7) <i>P</i> = 0.322
Genetic models			
Multiplicative model	Allele C	Allele T	
Cases	305 (76.25)	95 (23.75)	<i>P</i> = 0.21
Control	82 (82)	18 (18)	OR = 1.41 CI: 0.81-2.48
Dominant model	CC	CT + TT	
Cases	120 (60)	80 (40)	<i>P</i> = 0.29
Control	34 (68)	16 (32)	OR = 0.7 CI: 0.3-1.3
Recessive model	TT	CC + CT	
			<i>P</i> = 0.37 OR = 1.9 CI: 0.4-8.8

polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

TLR4 Thr399Ile polymorphism: Our findings demonstrated a PCR product size of 406bp for TLR4 Thr399Ile gene. After restriction digestion of PCR product with HinfI restriction enzyme at 37 °C, respective band sizes of 406 bp, 377 bp, and 29 bp were obtained depending upon the creation of restriction sites by mutant allele. CC was found to be the predominant genotype (83%), followed by CT (12.5%) and TT (4.5%) in NAFLD patients vs CC (84%), and by CT (16%) and TT (0%) in HVs.

Among TLR4 Thr399Ile polymorphisms, the frequency of CC [166/200 (83%) vs 42/50 (84%), *P* = 0.86], CT [25 (12.5%) vs 8 (16%), *P* = 0.51], and TT [(9 (4.5%) vs 0 (0%), *P* = 0.12] genotypes were not different among patients with NAFLD and HVs. There was no difference in T allele frequency between the NAFLD or HV groups (10.75% vs 8%, *P* = 0.41), and no association of TLR4 Thr399Ile polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

Haplotype analysis for TLR4 Asp299Gly and TLR4 Thr399Ile polymorphism: A haplotype comprised of a combination of alleles present on the same chromosome. Haplotype analysis revealed that haplotypes AC, AT, GC, and GT were not associated with an increased risk of NAFLD.

Polymorphism analysis of CD14 gene

CD14 C (-550) T polymorphism: CD14 C (-550) T is a C/T polymorphism that creates a recognition site for restriction enzyme Hae111. Amplification with primer pairs yielded a 368 bp fragment. Respective band sizes of 259, 236, 109, and 23 bp were obtained

depending upon the deletion of restriction sites by mutant allele. Digestion with enzyme gave 23, 236, and 109 fragments in the presence of CC genotype, four bands of 23, 236, 109, and 259 in the presence of CT genotype, and two bands of 109 bp and 259 bp fragments in the presence of TT genotype. The genotype frequency of CC, CT, and TT was 60%, 32.5%, and 7.5%, respectively, in the NAFLD group and 68%, 28%, and 4%, respectively, in the HV group (Table 3). Distribution was in accordance with Hardy-Weinberg equilibrium. The TT genotype was not significantly overrepresented in NAFLD (*P* = 0.322) compared to HVs.

Among CD14 C (-550) T polymorphisms, the frequency of CC [120/200 (60%) vs 34/50 (68%)], CT [65 (32.5%) vs 14 (28%), OR = 1.3 (0.6-2.6), *P* = 0.43], and TT [(15 (7.5%) vs 2 (4%), OR = 2.1 (0.4-9.7), *P* = 0.322] genotypes were not different between patients with NAFLD and HVs. There was no difference in T allele frequency between the NAFLD or HV groups (23.75% vs 18%, *P* = 0.21) and no association of CD14 C (-550) T polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis (Table 4).

CD14 C (-159) T polymorphism

CD14 C (-159) T polymorphism is a C/T polymorphism that creates a recognition site for restriction enzyme Hae111. Amplification with primer pairs yielded a 296 bp fragment. Respective band sizes 259, 236, 109, and 23 bp were obtained depending upon the deletion of restriction sites by mutant allele. Digestion with enzyme gave a 296 bp fragment in the presence of CC genotype, three bands of 296, 154, and 141 in the presence of CT genotype, and two bands of 154 bp and 141 bp fragments in the presence of TT genotype (Figure 1A and Table 5).

There was no difference in the CC [36 (18%) vs 10 (20%), *P* = 0.74] genotype between patients with NAFLD and HVs. Even though there was difference in the CT [70/200 (35%) vs 30 (60%), *P* = 0.001] genotypes between patients with NAFLD and HVs, the CT genotype was not associated with an increased risk of NAFLD (Table 2). Distribution was in accordance with Hardy-Weinberg equilibrium. The TT genotype [94/200 (47%) vs 10/50 (20%), *P* = 0.0005] and T allele frequency (64% vs 50%, *P* = 0.007) were significantly higher among patients with NAFLD than HVs, with significant association of C (-159) T polymorphism with NAFLD on multiplicative (*P* = 0.007) and recessive models (*P* = 0.0005). The risk of developing NAFLD with the TT genotype was 2.6 fold higher than in CC genotypes (Table 5).

DNA sequencing

The DNA sequencing data for CD14 C (-159) T polymorphism confirmed our PCR-RFLP findings, wherein

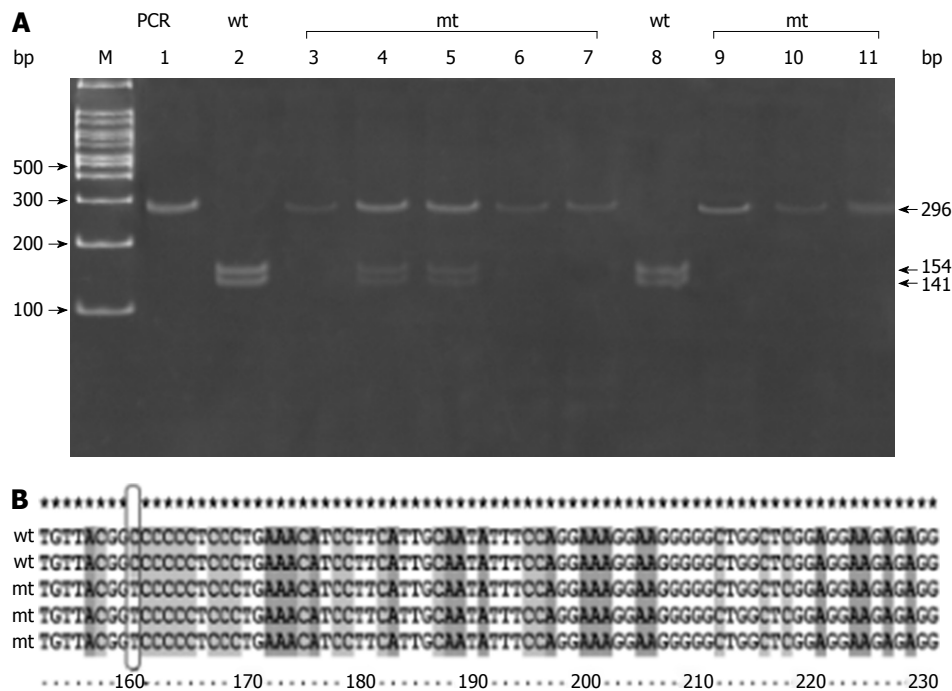


Figure 1 Polymorphism analysis of C14-159C/T. A: Representative PAGE gel picture of PCR-RFLP assay with restriction digested products. Lane M: 100 bp DNA ladder; Lanes 1-8: RE digested product, CC: 141 bp and 154 bp (Lanes 2, 8), CT: 296 bp, 141 bp, and 154 bp (Lanes 4 and 5), TT: 296 bp (Lanes 1, 3, 6, and 7); Lanes 9-11: PCR product; B: DNA sequencing showing different genotypes. Gap indicates the SNP or mutation (C→T). wt: Homozygous wild; mt: Homozygous or heterozygous mutant; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism.

Table 5 Distribution of CD14 -159C/T genotype, allele frequency and genetic models for CD14-159C/T n (%)			
CD14 C (-159) T Genotypes	CC	CT	TT
Cases (n = 200)	36 (18)	70 (35)	94 (47)
Control (n = 50)	10 (20)	30 (60)	10 (20)
OR	1	0.6 (0.2-1.4)	2.6 (1-6.7)
		P = 0.29	P = 0.043
Genetic models			
Multiplicative model	Allele C	Allele T	
Cases	142 (35.50)	258 (64.50)	P = 0.007
Control	50 (50)	50 (50)	OR = 0.8
			CI: 1.1-2.8
Dominant model	CC	CT + TT	
Cases	36 (18)	164 (82)	P = 0.74
Control	10 (20)	16 (32)	OR = 0.7
			CI: 0.4-1.9
Recessive model	TT	CC + CT	
Cases	94 (47)	106 (53)	P = 0.0005
Control	10 (20)	40 (80)	OR = 3.5
			CI: 1.6-7.4 -8.8

we found that C allele is replaced by T allele (Figure 1B). The results of PCR-RFLP of all other studied polymorphisms were comprised of 5 samples of each genotype in patients with NAFLD, which was also confirmed by DNA sequencing (5 homozygous wild, 5 heterozygous, and 5 homozygous variant) (data not shown).

Haplotype analysis for CD14 C (-159) T and CD14 C (-550) T polymorphisms: Haplotype analysis revealed that haplotype TC had a significantly higher

($P = 0.0002$) frequency in patients with NAFLD in comparison to HVs, with an odds ratio of 2.3; 95%CI: 1.4-3.7. Analysis of individual haplotypes in the CD14 gene as a method of determining the risk of developing NAFLD revealed that the TC haplotype was more frequently seen in NAFLD. None of the other haplotypes showed any association with the risk of developing NAFLD (Table 6).

Role of CD14 C (-159) T polymorphism in determining the severity of NAFLD: Owing to the significant association between CD14 C (-159) T polymorphism and NAFLD, we compared different parameters amongst NAFLD patients with ($n = 94$) and without this polymorphism ($n = 106$). There was a significant difference in serum ALT [95 (72-130) IU/L vs 83 (68-108) IU/L, $P = 0.016$] and TNF α levels [62 (40-112) pg/mL vs 56 (34-80) pg/mL, $P = 0.04$] amongst NAFLD patients with and without CD14 C (-159) T polymorphism (Table 7). However, no difference was observed between the two groups with regard to the degree of hepatic steatosis, hepatic fibrosis, NAS score, and presence of NASH, borderline NASH, and no-NASH amongst the biopsy proven patients with NAFLD in each group.

DISCUSSION

NAFLD is one of the most predominant causes of liver disease in the world, and is considered a hepatic manifestation of metabolic syndrome. Its histology spectrum ranges from steatosis to NASH, and can

Table 6 Haplotypes for CD14 C (-159) T and C (-550) T polymorphism

Haplotypes	NAFLD (frequency)	HVs (frequency)	P value (Fisher's exact test)	OR (95%CI)
CC	23.4%	50%	1.58E-007	0.3 (0.19-0.48)
TC	52.3%	32%	0.0002	2.3 (1.4-3.7)
TT	12.2%	18%	0.12	0.6 (0.3-1.1)
CT	12.1%	0%	0.0002	Undefined

HVs: Healthy volunteers; NAFLD: Non-alcoholic fatty liver disease.

Table 7 Comparison of non-alcoholic fatty liver disease patients with and without CD14 C (-159) T polymorphism

CD14 C (-159) T polymorphism (NAFLD = 200)			
Parameters	Without polymorphism (n = 106)	With polymorphism (n = 94)	P value
TNF- α (pg/mL) (n = 200)	56 (34-80)	62 (40-112)	0.04
Adiponectin (pg/mL) (n = 200)	745 (649-893)	745 (634-928)	0.93
IL-1 β (pg/mL) (n = 200)	43 (32-47)	43 (25-47)	0.53
HOMA-IR (n = 200)	1.9 (1.3-2.7)	1.8 (1.4-3.8)	0.34
MS (n = 200)			
≥ 3 components	43 (40.5%)	35 (37.2%)	0.63
ALT (IU/L)	83 (68-108)	95 (-130)	0.016
NAS score (n = 60)	8	6	0.94
No NASH	11	10	
Borderline NASH	13	12	
NASH			
Severity of steatosis (n = 60)			
1	9	6	0.733
2	14	15	
3	9	7	
Severity of fibrosis (n = 60)			
0	13	11	0.90
1	16	13	
2	1	2	
3	2	2	

TNF- α : Tumor necrosis factor- α ; NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferases; NAFLD: Non-alcoholic fatty liver disease.

progress to cirrhosis and HCC^[1-3]. NAFLD progression is governed by genetic susceptibility, environmental factors, SIBO, lifestyle, and features of metabolic syndrome. Gene expression and genome-wide association studies have identified novel disease pathways and polymorphisms in genes that may be potential biomarkers of NAFLD development and progression. Pathways that include SIBO and toll-like receptor signaling seem to be one of the contributors of NAFLD development. The primary focus of our study was to analyze the polymorphisms of TLR2, TLR4, and CD14 genes in NAFLD patients and to assess their contribution to the causation and severity of the disease.

The overgrowth of bacterial components is recognized by pathogen-associated molecular patterns, including

TLRs. Toll-like receptor 2 (TLR2) are receptors for gram-positive bacterial components. In humans, due to a single nucleotide gene polymorphism at position 753, arginine is replaced by glutamine and the G allele replaced by A allele diminishes the ability of TLR2 to respond to bacterial cell wall components^[24,25]. Although there are animal studies to show the protective role for TLR2-mediated signals in liver injury and occurrence of NASH with TLR2 deficiency^[26,27], ours is the first human study to demonstrate the absence of an association of TLR2 Arg753Gln polymorphism with the risk of developing NAFLD.

In humans, TLR4 is the principal receptor for bacterial endotoxin recognition and functional variants in the gene confer endotoxin hyporesponsiveness^[28]. The missense mutation (Asp299Gly) in the fourth exon of the TLR4 gene alters the extracellular domain of this receptor. An additional missense polymorphism (Thr399Ile) in the extracellular domain of the TLR4 receptor co-segregates with the Asp299Gly substitution in more than 95% of the Caucasian population^[29]. There are conflicting reports on the effects of the Asp299Gly polymorphism on endotoxin responsiveness *in vitro*^[30-34]; however, the authors of several clinical reports associated this polymorphism with the risk of gram-negative infections^[35,36] or severe respiratory syncytial viral infection^[37], as well as such chronic disorders as asthma^[38], arteriosclerosis^[39], and diabetic neuropathy^[40]. We did not observe any association of TLR4 A299G and TLR4 C399T gene polymorphism with the risk of developing NAFLD or NASH. In addition, we did not find any association of haplotypes for TLR4 gene with NAFLD. Our results are in accordance with a study by Day *et al.*^[13], in which biopsy-proven patients with NAFLD were genotyped for Asp299Gly single nucleotide polymorphism (SNP) in the TLR4 gene and no association was found between the susceptibility of NASH and the Asp299Gly TLR4 SNP. A recent study by Kiziltaş *et al.*^[41] demonstrated that subjects with a heterozygous mutation in genotype 299 (Asp299Gly) were significantly lower in the NAFLD group than in the control group. The authors concluded that this mutation may have had a protective role against the genesis of NAFLD.

We found a significant association of C (-159) T polymorphism with NAFLD on multiplicative and recessive models. Patients with NAFLD with C (-159) T polymorphism had a significantly higher prevalence of TT genotype with significantly high levels of TNF- α ($P = 0.04$) and ALT ($P = 0.01$) than those without this polymorphism. Patients with TT genotype had a 2.6 fold higher risk of developing NAFLD in comparison to the CC genotype of CD14 C (-159) T polymorphism. However, this polymorphism did not affect disease severity, as there was no difference in NAS score and the prevalence of NASH or borderline NASH amongst those with and without polymorphism. Brun *et al.*^[14] found that TT genotype distribution was significantly higher in NASH patients than in control subjects,

while subjects carrying TT genotype had higher TNF- α levels than CT and CC genotypes. Another study demonstrated that “high” activity of TT genotype of C-159T SNP in the CD14 promoter gene was associated with NASH, and that patients with CD14 polymorphism had higher levels of serum TNF- α levels in comparison to those without C-159 T SNP^[13].

In contrast to C (-159) T polymorphism, we did not find any association of CD14 C (-550) T polymorphism with NAFLD. Ours is the first study to demonstrate the lack of an association of CD14 C (-550) T polymorphism with the risk of developing NAFLD. However, haplotype analysis of genotypes for CD14 revealed that TC genotype had an increased risk of NAFLD. Haplotype study is now gaining attention because multiple linked SNPs have the potential to provide significantly more power for genetic analysis than individual SNPs^[42]. There was no previously performed haplotype analysis for CD14 gene in patients with liver disease, with ours being the first study to demonstrate its utility in patients with NAFLD.

In conclusion, our study demonstrated that NAFLD patients with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development. However, this polymorphism did not affect the severity of liver disease. We did not find any association between TLR 2 ARG753, TLR 4 (Asp299Gly), TLR4 (Thr399Ile), or CD 14 C/T 550 polymorphisms and the risk of NAFLD development. Studies with a larger cohort of patients are required to confirm the results.

COMMENTS

Background

Environmental and genetic factors predispose individuals to the development of non-alcoholic fatty liver disease (NAFLD). In this study, the authors demonstrated that cluster of differentiation 14 (CD14) polymorphism could predict the development of NAFLD.

Research frontiers

NAFLD is the one of the manifestation of the obesity-related complications and incidence of NAFLD-related hepatocellular carcinoma increasing worldwide. It is therefore very important to understand the molecular mechanism underlying the pathogenesis of NAFLD.

Innovations and breakthroughs

Individuals with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development.

Applications

Individuals with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development. It can be considered a marker for identifying a population at risk of NAFLD progression.

Peer-review

Individuals with CD14 C (-159) T polymorphism have a higher risk of NAFLD development.

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Basic Study

Increased CD4⁺CD45RA⁻FoxP3^{low} cells alter the balance between Treg and Th17 cells in colitis mice

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Abstract

AIM

To investigate the role of regulatory T cell (Treg) subsets in the balance between Treg and T helper 17 (Th17) cells in various tissues from mice with dextran sulfate sodium-induced colitis.

METHODS

Treg cells, Treg cell subsets, Th17 cells, and CD4⁺CD25⁺FoxP3⁺IL-17⁺ cells from the lamina propria of colon (LPC) and other ulcerative colitis (UC) mouse tissues were evaluated by flow cytometry. Forkhead box protein 3 (FoxP3), interleukin 17A (IL-17A), and RORC mRNA levels were assessed by real-time PCR, while interleukin-10 (IL-10) and IL-17A levels were detected with a Cytometric Beads Array.

RESULTS

In peripheral blood monocytes (PBMC), mesenteric lymph

node (MLN), lamina propria of jejunum (LPJ) and LPC from UC mice, Treg cell numbers were increased ($P < 0.05$), and FoxP3 and IL-10 mRNA levels were decreased. Th17 cell numbers were also increased in PBMC and LPC, as were IL-17A levels in PBMC, LPJ, and serum. The number of FrI subset cells (CD4⁺CD45RA⁺FoxP3^{low}) was increased in the spleen, MLN, LPJ, and LPC. FrII subset cells (CD4⁺CD45RA⁺FoxP3^{high}) were decreased among PBMC, MLN, LPJ, and LPC, but the number of FrIII cells (CD4⁺CD45RA⁺FoxP3^{low}) and CD4⁺CD25⁺FoxP3⁺IL-17A⁺ cells was increased. FoxP3 mRNA levels in CD4⁺CD45RA⁺FoxP3^{low} cells decreased in PBMC, MLN, LPJ, and LPC in UC mice, while IL-17A and RORC mRNA increased. In UC mice the distribution of Treg, Th17 cells, CD4⁺CD45RA⁺FoxP3^{high}, and CD4⁺CD45RA⁺FoxP3^{low} cells was higher in LPC relative to other tissues.

CONCLUSION

Increased numbers of CD4⁺CD45RA⁺FoxP3^{low} cells may cause an imbalance between Treg and Th17 cells that is mainly localized to the LPC rather than secondary lymphoid tissues.

Key words: Ulcerative colitis; Regulatory T cells; Treg cells subsets; T helper 17 cells

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Core tip: The exact etiology and pathology of ulcerative colitis (UC) remains unknown. Here we investigated the role of regulatory T cell (Treg) subsets in the balance between Treg and T helper 17 (Th17) cells in various tissues from mice with dextran sulfate sodium-induced colitis. In this study we found that increased numbers of CD4⁺CD45RA⁺FoxP3^{low} cells may cause an imbalance between Treg and Th17 cells, which was mainly localized to the lamina propria of colon rather than secondary lymphoid tissues. Based on our findings, lamina propria-resident Treg cells appear to play important roles in shaping local peripheral tolerance and maintaining intestinal homeostasis, and an imbalance of Treg and Th17 cells in the lamina propria of the colon is critical for UC pathogenesis.

Ma YH, Zhang J, Chen X, Xie YF, Pang YH, Liu XJ. Increased CD4⁺CD45RA⁺FoxP3^{low} cells alter the balance between Treg and Th17 cells in colitis mice. *World J Gastroenterol* 2016; 22(42): 9356-9367. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9356.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9356>

INTRODUCTION

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) that affects the colon and is confined to the mucosa and superficial submucosa^[1]. UC symptoms include diarrhea, abdominal pain, and rectal bleeding,

which can all seriously affect quality of life, and the disease is often marked alternating phases of clinical relapse and remission. Although the exact etiology and pathology of UC remains unknown^[2], there is increasing evidence that an aberrant immune response is involved in this disease^[3].

Acquired immunity plays a vital role in UC pathogenesis, where in T helper cell-type (Th) 1 and Th2 immune responses, as well as alternate subsets of T cells, such as regulatory T cells (Treg) and T helper 17 (Th17) cells, contribute to IBD^[4,5].

Regulatory T cells belong to a functionally specialized subset of CD4⁺ T cells that maintains immune tolerance and homeostasis *via* cell-cell interactions and secretion of interleukin-10 (IL-10) or other anti-inflammatory cytokines that inhibit activation of effector T cells^[6,7]. Notably, Treg cells may play a crucial role in inhibiting intestinal inflammation, maintaining immune tolerance, and providing protection from colitis^[8]. A study by Sakaguchi *et al.*^[9] demonstrated that Treg cells can be divided into three different functional subsets: Resting Tregs, FrI (rTreg or CD45RA⁺Foxp3^{low}); activated Tregs, FrII (aTreg or CD45RA⁺Foxp3^{high}); and non-suppressive Tregs, FrIII (CD45RA⁺Foxp3^{low}). CD4⁺CD45RA⁺Foxp3^{low} cells are resting Treg cells that upon activation become CD4⁺CD45RA⁺Foxp3^{high} cells, which are the major suppressive cells that can affect immunologic function when levels of this subtype decrease. Meanwhile, CD4⁺CD45RA⁺Foxp3^{low} cells secrete interleukin-17 (IL-17) and have the potential to become Th17 cells, a newly discovered CD4⁺ T cell subset that lacks immunosuppressive function and is characterized by interleukin 17A (IL-17A), IL-17F, IL-22, IL-21 secretion^[10].

Th17 cells show pleiotropic activities and functions that promote immune responses *via* the adaptive and innate immune systems. Like sentinel cells, Th17 cells help maintain epithelial barrier function in healthy intestines. However, in the presence of chronic intestinal inflammation, Th17 cells present IL-23 and show full pathogenic and antibacterial functions^[11]. Aberrant numbers of Th17 cells have been reported to occur in colonic LP of the ileum and colon in conventionally raised mice, and these cells are highly infiltrated in inflamed areas in colitic mice^[12].

Furthermore, other studies reported that CD4⁺ T cells can demonstrate enhanced "plasticity" between T-cell subsets, such as the IL-17 and Foxp3 double-expressing (DE) CD4⁺ T cell population, which is a crossover transition between Treg and Th17 cells^[13]. In IBD patients, the population of circulating IL-17 and Foxp3 DE CD4⁺ T cells is increased. Furthermore, the finding that IL-17 and Foxp3 DE CD4⁺ T-cell populations co-express related orphan receptor- γ t (ROR γ t) and Foxp3 suggests that Treg cells can convert to Th17 cells that have decreased suppressive function that is characteristic of CD4⁺Foxp3⁺ T lymphocytes^[14]. Indeed, in our earlier study of scleroderma patients,

we identified a CD4⁺CD45RA⁺Foxp3^{low} cell subset that had no suppressive function and co-expressed ROR γ T and Foxp3^[15].

Here we examined Treg, Treg subsets, and Th17 cells in tissues from UC mice. We found abnormal proportions of these cells and a cell population that co-expressed FoxP3 and RORC mRNA, which may represent a crossover transition of Th17 and Treg cells that is related to an imbalance of Treg cells and Th17 cells in dextran sulfate sodium (DSS)-induced colitis.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice (aged 6–8 wk; 20–22 g) were obtained from the Center for Animal Resource and Development (Weitonglihua Company, Beijing, China). All mice were maintained on a 12 h/12 h light/dark cycle under specific pathogen-free conditions. All animal procedures and stress protocols were approved by the Institutional Animal Care and Committee of Beijing Chaoyang Hospital, Capital Medical University.

Mouse model of colitis

The healthy control (HC) mice drank distilled water for 14 d, while the UC mice drank distilled water supplemented with 2.5% w/v (DSS, MW = 40000–50000, MP Biomedical, United States) for 7 d followed by 7 d of drinking water alone. The mice were sacrificed on the 14th d. DSS-induced colitis was characterized by higher disease activity index that included changes in body weight, stool consistency, and the presence of blood in the stool^[16].

Histopathological evaluation

Histopathological evaluation was performed as described previously^[17]. Mouse colons were extracted immediately after sacrifice and examined for macroscopic damage. The resected colons were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for evaluation.

Antibodies and reagents

Anti-mouse rat antibodies (conjugated with FITC, PE, PE-Cyanine7, PE-CF594, PerCP, APC, Alexa Fluor[®] 488, and BV510 as indicated) used in this study were: CD4-PerCP (L3T4, BD Pharmingen[™], United States), CD25-APC (IL-2 Receptor α chain, BD Pharmingen[™], United States), FoxP3-PE-Cyanine7 (JM2, eBioscience, United States), CD45RA-PE (BD Pharmingen[™], United States), IL-17-Alexa Fluor[®] 488 (BD Pharmingen[™], United States), CD3e-PE-CF594 (CD3 ϵ chain, BD Horizon[™], United States), CD8a-BV510 (Ly-B, BD Horizon[™], United States). In all experiments, a control antibody of the respective IgG isotype was included. Leukocyte Activation Cocktail (BD Pharmingen[™], United States), RNeasy Micro Kit (QIAGEN, Germany), FastQuant RT Kit (TIANGEN, China), SuperReal PreMix Plus (TIANGEN,

China), and the Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences, United States) were used according to the manufacturer's instructions.

Flow cytometry cell analysis

Peripheral blood (PBMC), spleen, mesenteric lymph node (MLN), lamina propria of jejunum (LPJ), and lamina propria of colon (LPC) from UC and HC mice were collected and mononuclear cells were prepared. The cells were then incubated with CD4, CD25, and CD45RA antibodies in the dark at room temperature for 15 min. Intracellular FoxP3 staining was subsequently performed according to the manufacturer's instructions. Intracellular cytokine production was detected after stimulation of 1×10^6 cells with 2 μ L Leukocyte Activation Cocktail (BD Pharmingen[™], United States) for 4.5 h. Cells were then incubated with CD4, CD25, and CD45RA and stained with antibodies against FoxP3 and IL-17 after fixation and permeabilization (eBioscience, United States). Images of stained cells were acquired using a Gallios flow cytometer (Beckman Coulter, United States) and analyzed with Kaluza v1.20 software.

Cell sorting

Mononuclear cells extracted from PBMC, spleen, MLN, and jejunum and colon LP were stained with CD4, CD25, and CD45RA antibodies before sorting with a FACS AriaII flow cytometer (BD Biosciences, United States).

Real-time PCR

Total RNA was extracted from $2\text{--}10 \times 10^3$ sorted Treg cells and CD4⁺CD45RA⁺Foxp3^{low} cells from UC and HC mice using an RNeasy Micro Kit (QIAGEN). The RNA was then reverse-transcribed to obtain cDNA (FastQuant RT Kit, TIANGEN). Real-time PCR was performed with a SYBR green assay (SuperReal PreMix Plus, TIANGEN) using the ABI 7500 system (Applied Biosystems) with a GAPDH-Primer (PMM04) and other primers listed in Table 1.

Cytokine detection

IL-10 and IL-17A levels secreted by mononuclear cells from PBMC, spleen, MLN, LPJ, and LPC samples were determined using the Mouse Th1/Th2/Th17 Cytokines kit (BD Biosciences) according to the manufacturer's instructions. Serum IL-17A levels were also assessed using the same method. In addition, IL-10 and IL-17A levels were examined using a BD FACScanto II flow cytometer (BD Biosciences, United States).

Statistical analysis

Data were analyzed with the SPSS v18.0 statistics package (IBM, United States). Variables were summarized as counts and percentages or as medians and ranges. Independent samples *t*-test and nonparametric Mann-Whitney *U* tests were used to compare data

Table 1 Real-time polymerase chain reaction primer sequences

Primers	Forward (5'-3')	Reverse (5'-3')
GAPDH mRNA	GGTGTCTCTCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC
FoxP3 mRNA	CTGCCTTGGTACATTCTGTA	CAGATGTTGTGGGTGAGTGC
IL-17 mRNA	CTGTGTCAATGCGGAGGGAA	CGACCCCTGAAAGTGAAGGGG
RORC mRNA	ACGGCCAACTTACTCTTGGGA	AGAAACTGGGAATGCAGTGG

between groups. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Treg cell numbers in DSS colitis Mice increased concurrently with lamina propria functional defects

To investigate the roles of Treg cells in DSS colitis mice, we compared the levels of Treg, FoxP3 mRNA, and IL-10 from different samples including PBMC, MLN, spleen, LPJ, and LPC isolated from HC and DSS colitis mice. Treg levels increased in PBMC, MLN, LPJ, and LPC samples from DSS colitis mice ($P < 0.0001$, $P = 0.0399$, $P = 0.0151$, $P = 0.0001$, respectively) (Figure 1A, B). In contrast, FoxP3 mRNA expression ($P = 0.0016$, $P = 0.0062$, $P = 0.0291$, $P = 0.0325$, respectively) and IL-10 levels ($P = 0.0091$, $P = 0.0353$, $P = 0.0002$, $P = 0.0012$, respectively) in Treg cells from DSS colitis mice decreased relative to the HC (Figure 1C, D). Meanwhile, spleen tissue from DSS colitis and HC groups showed no difference in the number of Treg cells, FoxP3 mRNA expression, and IL-10 levels ($P > 0.05$) (Figure 1A-D).

In LPC from DSS colitis mice, Treg cell numbers were increased compared to that of MLN, spleen, LPJ, and PBMC ($P = 0.0005$, $P = 0.0002$, $P = 0.0002$, $P = 0.0001$, respectively), while MLN, spleen, PBMC, and LPJ showed no difference in Treg numbers ($P > 0.05$) (Figure 1E). Similarly, in HC mice Treg cell numbers in LPC were increased compared to those in spleen, MLN, PBMC, and LPJ ($P = 0.0105$, $P = 0.0006$, $P < 0.0001$, $P = 0.0002$, respectively), with the latter two samples showing similar numbers with no significant differences ($P > 0.05$) (Figure 1E).

Th17 cell numbers increased in PBMC and LPC from DSS colitis mice

Given the contribution of Th17 cells to inflammatory responses, we next assessed Th17 numbers in DSS colitis mice. There were increased numbers of Th17 cells in PBMC and colon compared to HC ($P = 0.0338$, $P = 0.0004$), although spleen, MLN, and showed no differences in Th17 cell numbers between HC and DSS colitis mice ($P > 0.05$) (Figure 2A, B). In culture supernatants of PBMC, LPJ, and serum, IL-17A levels were increased compared with HC ($P = 0.0063$, $P = 0.0064$, $P = 0.0016$, respectively), while those of spleen, MLN, and LPC were similar ($P > 0.05$) (Figure 2C).

In DSS colitis mice, Th17 cell numbers in LPC were also increased compared to MLN, spleen, and PBMC

($P < 0.0001$, respectively), and LPC and LPJ had no differences ($P > 0.05$) (Figure 2D). Meanwhile, in HC mice Th17 cell numbers in both LPJ were increased compared with LPC, MLN, spleen, and PBMC ($P < 0.0001$), as were Th17 cell numbers in LPC relative to those for MLN, spleen, and PBMC ($P < 0.0001$), but MLN, spleen, and PBMC showed no differences among samples ($P > 0.05$) (Figure 2D).

CD4⁺CD45RA⁺FoxP3^{low} cell numbers increased and CD4⁺CD45RA⁺FoxP3^{high} cell numbers decreased in LPC of DSS colitis mice

In samples from DSS colitis mice the number of CD4⁺CD45RA⁺FoxP3^{low} was increased in spleen, MLN, LPJ, and LPC ($P = 0.0020$, $P = 0.0050$, $P = 0.0010$, $P < 0.0010$, respectively), but not in PBMC ($P = 0.1170$) (Figure 3A). Meanwhile, CD4⁺CD45RA⁺FoxP3^{low} cell numbers in colon LP were also obviously increased compared to MLN, spleen, PBMC, and LPJ ($P < 0.0001$, $P < 0.0001$, $P = 0.0013$, $P < 0.0001$, respectively), which all showed similar levels ($P > 0.05$) (Figure 3E). In HC mice, CD4⁺CD45RA⁺FoxP3^{low} cell numbers in LPC were obviously increased compared to MLN, spleen, PBMC, LPJ ($P = 0.0033$, $P = 0.0081$, $P = 0.0010$, $P < 0.0001$, respectively), with spleen and LPJ showing similar levels ($P > 0.05$).

In DSS colitis mice, the number of CD4⁺CD45RA⁺FoxP3^{high} in PBMC, MLN, LPJ, and LPC samples was lower than that for HC mice ($P = 0.0060$, $P = 0.0000$, $P = 0.0000$, $P = 0.0250$, respectively), but spleen tissues were similar ($P = 0.3980$) (Figure 3A). CD4⁺CD45RA⁺FoxP3^{high} cell numbers in LPC from UC mice were also increased compared to those for spleen, LPJ, MLN, and PBMC ($P < 0.0001$, $P = 0.0003$, $P < 0.0001$, $P < 0.0001$, respectively), with the latter two samples showing similar values ($P > 0.05$) (Figure 3E). In HC mice, CD4⁺CD45RA⁺FoxP3^{high} cell numbers in LPC were increased relative to spleen, LPJ, MLN, and PBMC ($P = 0.0026$, $P = 0.0322$, $P = 0.0002$, $P = 0.0002$, respectively), with MLN and PBMC again having similar numbers ($P > 0.05$).

CD4⁺CD45RA⁺FoxP3^{low} cell numbers were increased among multiple PBMC, MLN, LPJ, and LPC samples from DSS colitis mice ($P = 0.0010$, $P = 0.0330$, $P = 0.0420$, $P < 0.0010$, respectively), but not in spleen ($P = 0.248$) (Figure 3A). In both DSS colitis and HC mice, CD4⁺CD45RA⁺FoxP3^{low} cell numbers in LPC were increased relative to those for MLN, spleen, LPJ, and PBMC ($P < 0.0001$, $P = 0.0005$, $P < 0.0001$, $P < 0.0001$, respectively in DSS colitis; $P = 0.0002$, $P = 0.0175$, P

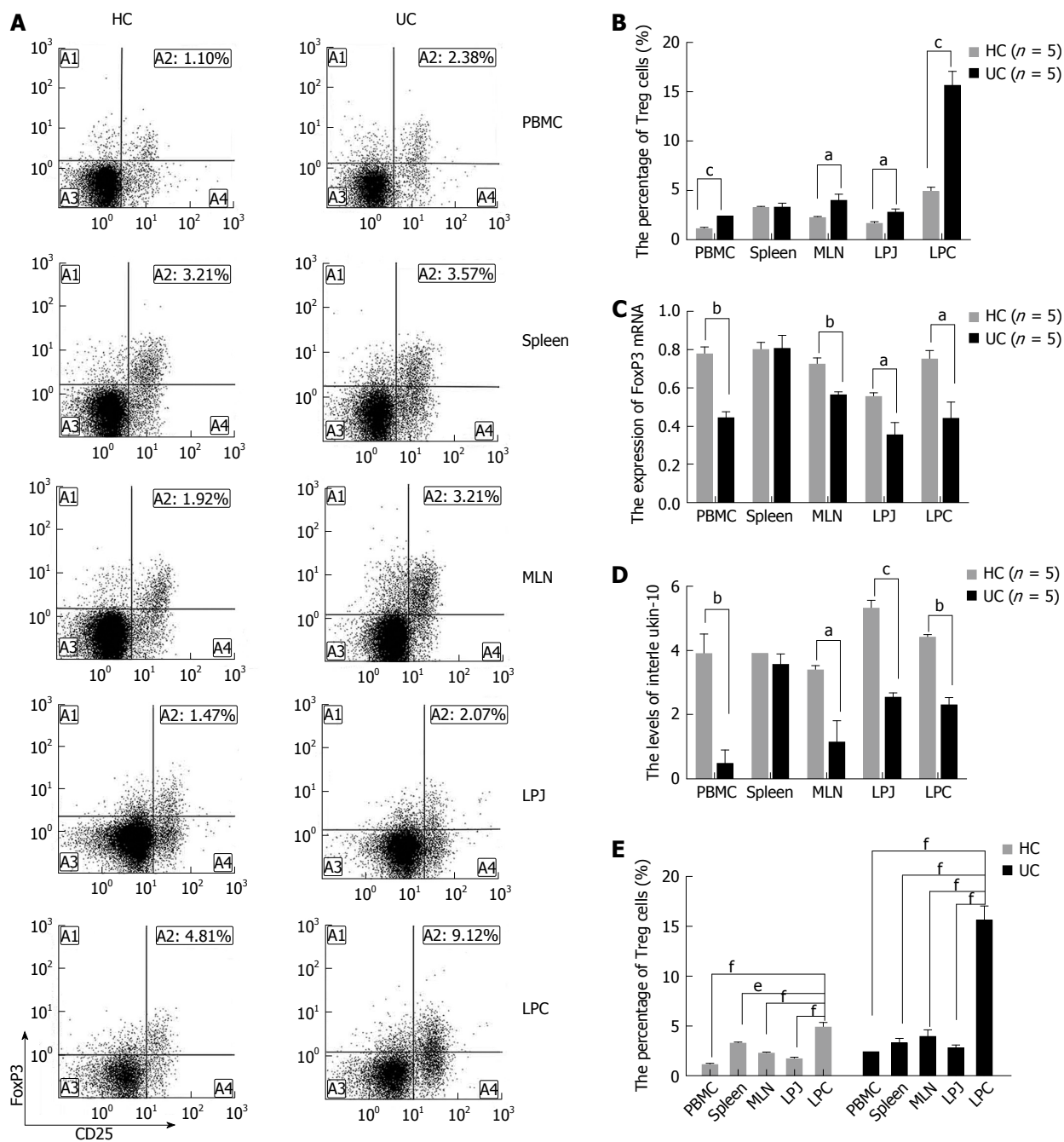


Figure 1 Treg cell features in multiple samples including peripheral blood monocytes, spleen, mesenteric lymph node, lamina propria of jejunum and colon. **A:** Representative FACS analysis of Treg cells from HC and UC mice samples gated for CD4⁺ T cells; **B:** Treg cell percentages in HC and UC samples; **C:** Expression of FoxP3 mRNA extracted from Treg cells in HC and UC samples; **D:** Levels of IL-10 secreted from mononuclear cells from HC and UC peripheral blood monocytes (PBMC), spleen, mesenteric lymph node (MLN), LPJ, and LPC samples; **E:** Treg cell distribution in HC and UC samples. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^c*P* < 0.001 vs control, ^d*P* < 0.05 vs LPC, ^e*P* < 0.01 vs LPC, ^f*P* < 0.001 vs LPC. HC: Healthy control mice; UC: Ulcerative colitis mice; LPJ: Lamina propria of jejunum; LPC: Lamina propria of colon.

= 0.0003, *P* < 0.0001, respectively in HC), and MLN, spleen, and LPJ showed no difference between DSS colitis and HC mice (*P* > 0.05) (Figure 3E).

We also evaluated levels of FoxP3, IL-17A, and RORC mRNA in CD4⁺CD45RA-FoxP3^{low} cells isolated from HC and DSS colitis mice. Compared to HC mice, in DSS colitis mice FoxP3 mRNA expression was decreased among PBMC, MLN, LPJ, and LPC samples (*P* = 0.0286, *P* = 0.0284, *P* = 0.0121, *P* = 0.0002) (Figure 3B). IL-17A and RORC mRNA from DSS colitis mice

was increased among the above samples compared to HC mice (IL-17 mRNA: *P* = 0.0150, *P* = 0.0278, *P* = 0.0247, *P* = 0.0357; RORC mRNA: *P* = 0.0369, *P* = 0.0128, *P* = 0.0101, *P* = 0.0016, respectively), but we saw no difference in spleen values (*P* > 0.05) (Figure 3C and D).

CD4⁺CD25⁺FoxP3⁺IL-17A⁺ cell numbers were higher in LPC and LPJ from DSS colitis mice

The number of CD4⁺FoxP3⁺IL-17A⁺ cells was higher in

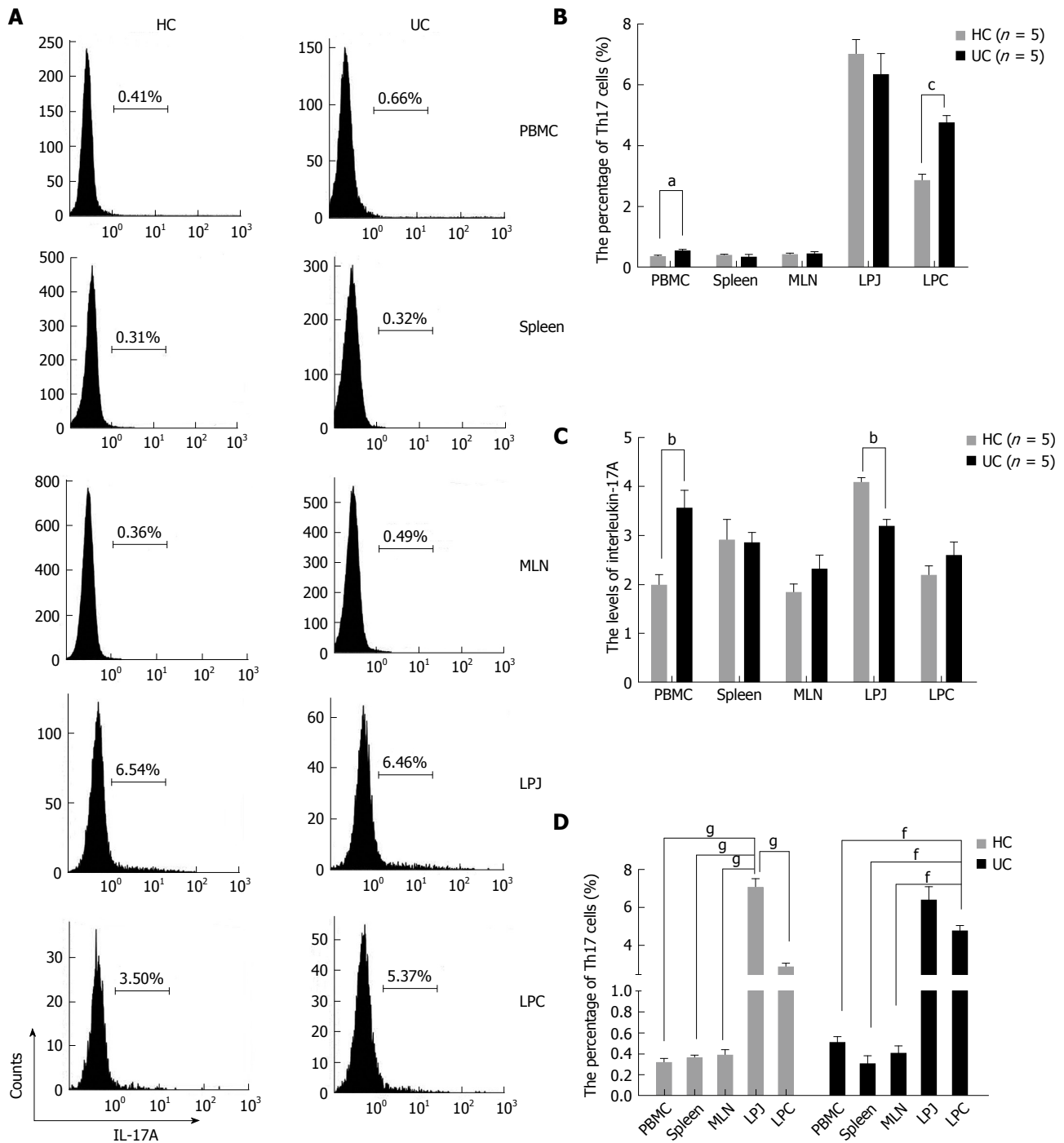


Figure 2 T helper 17 cell characteristics in healthy control mice and ulcerative colitis mice peripheral blood monocytes, spleen, mesenteric lymph node, lamina propria of jejunum and colon samples. A: FACS analysis of Th17 cells in HC and UC samples gated for CD4⁺ T cells; B: Percentages of Th17 cells in different HC and UC samples; C: Levels of IL-17A secreted by mononuclear cells extracted from HC and UC peripheral blood monocytes (PBMC), mesenteric lymph node (MLN), LPJ, and LPC samples; D: Treg cell distribution in HC and UC samples. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^c*P* < 0.001 vs control, ^d*P* < 0.05 vs LPC, ^e*P* < 0.01 vs LPC, ^f*P* < 0.001 vs LPC, ^g*P* < 0.001 vs LPJ. HC: Healthy control mice; UC: Ulcerative colitis mice; LPJ: Lamina propria of jejunum; LPC: Lamina propria of colon.

DSS colitis mice among multiple samples containing PBMC, MLN, LPJ, and LPC (*P* = 0.0161, *P* = 0.0037, *P* < 0.0001, *P* = 0.0001, respectively), but not in spleen (*P* > 0.05) (Figure 4A and B). In DSS colitis mice LPC, the levels of CD4⁺CD25⁺FoxP3⁺IL-17A⁺ cells were obviously increased compared with spleen, MLN, and PBMC (*P* = 0.0004, *P* < 0.0001, *P* < 0.0001, respectively), while

there were no differences between MLN and PBMC (*P* > 0.05), or LPJ and LPC (*P* > 0.05). In HC mice, CD4⁺CD25⁺FoxP3⁺IL-17A⁺ cell numbers were increased in LPC compared to those for LPJ, MLN, and PBMC (*P* = 0.0193, *P* = 0.0008, *P* = 0.0014, respectively), but the numbers in spleen and LPC were similar (*P* > 0.05), and there were no differences between MLN and PBMC

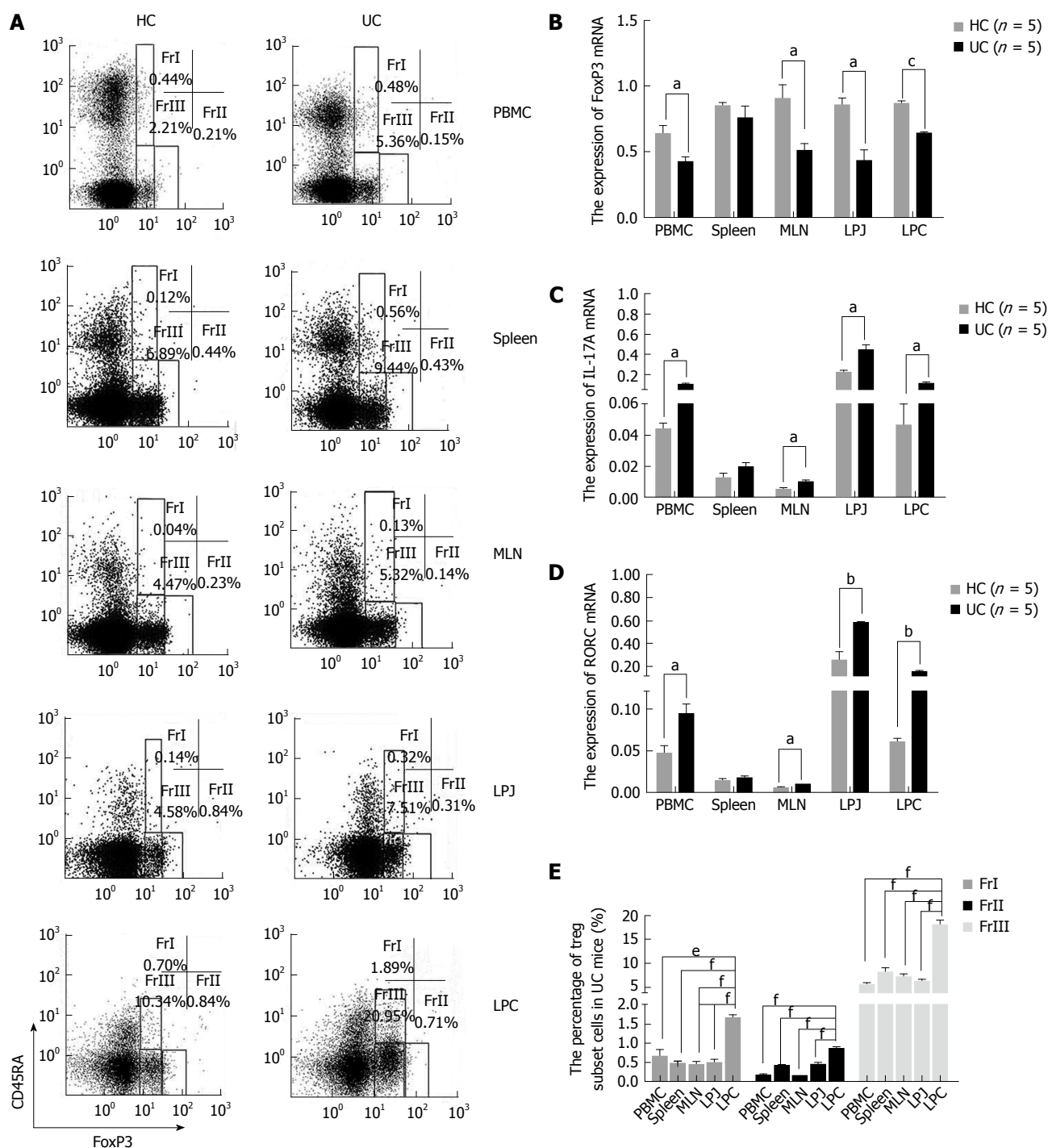


Figure 3 Treg cell subset characteristics in healthy control mice and ulcerative colitis mice peripheral blood monocytes, spleen, mesenteric lymph node, lamina propria of jejunum, and colon samples. **A:** FACS analysis of Treg cell subsets in HC and UC samples gated for CD4⁺ T cells; **B:** FoxP3 mRNA expression by FrIII cells from different HC and UC samples; **C:** Expression levels of IL-17A mRNA by FrIII cells in HC and UC samples; **D:** RORC mRNA expression by FrIII cells from HC and UC samples; **E:** Treg cell subset distribution in UC samples. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^c*P* < 0.001 vs control, ^d*P* < 0.05 vs LPC, ^e*P* < 0.01 vs LPC, ^f*P* < 0.001 vs LPC. PBMC: Peripheral blood monocytes; MLN: Mesenteric lymph node; FrI cells: CD4⁺CD45RA⁺FoxP3^{low}; FrII cells: CD4⁺CD45RA⁺FoxP3^{high}; FrIII cells: CD4⁺CD45RA⁺FoxP3^{high}; HC: Healthy control mice; UC: Ulcerative colitis mice; LPJ: Lamina propria of jejunum; LPC: Lamina propria of colon.

(*P* > 0.05) (Figure 4D).

When we excluded CD4⁺CD25⁺FoxP3⁺IL-17A⁺ cells from the total number of Treg cells, our results showed that CD4⁺FoxP3⁺IL-17A⁺ cell numbers were similar between multiple PBMC, spleen, MLN, LPJ, and LPC samples from HC and DSS colitis mice (*P* > 0.05) (Figure 4A and C).

DISCUSSION

Treg cells are anti-inflammatory cells that secrete the anti-inflammatory cytokine IL-10 and inhibit effector T cell proliferation through cell-cell interactions^[18,19]. Upon Treg-specific IL-10 ablation, mice spontaneously develop signs of colitis, illustrating the key role of

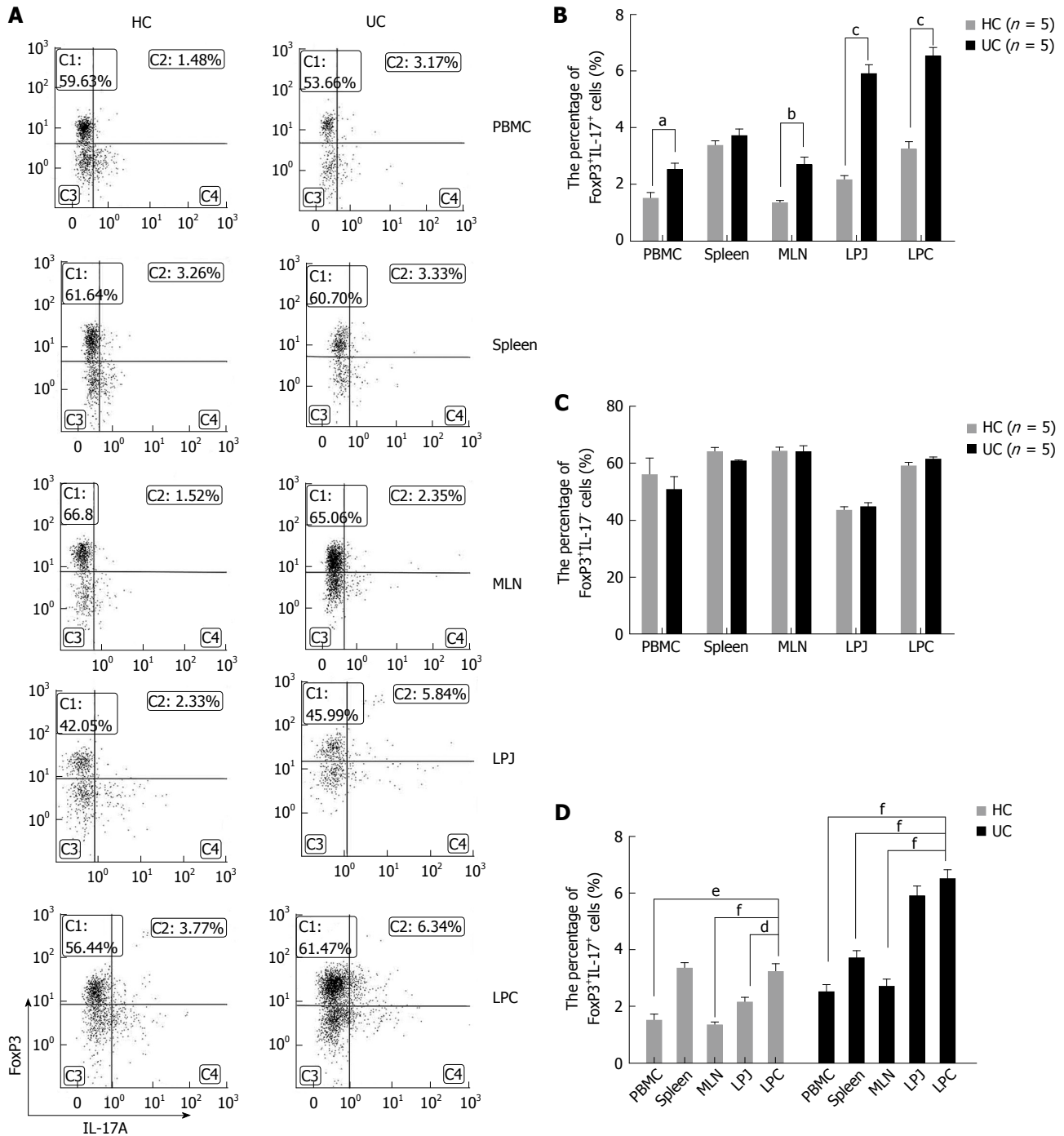


Figure 4 FoxP3⁺IL17A⁺ and FoxP3⁺IL17A⁻ cell characteristics in healthy control mice and ulcerative colitis mice samples of peripheral blood monocytes, spleen, mesenteric lymph node, lamina propria of jejunum, and colon. **A:** FACS analysis of FoxP3⁺IL17A⁺ and FoxP3⁺IL17A⁻ cells in HC and UC samples gated for CD4⁺CD25⁺ T cells; **B:** Percentage of FoxP3⁺IL17A⁺ cells in HC and UC samples; **C:** Percentages of FoxP3⁺IL17A⁻ cells in HC and UC samples; **D:** FoxP3⁺IL17A⁺ cell distribution in different HC and UC samples. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^c*P* < 0.001 vs control, ^d*P* < 0.05 vs LPC, ^e*P* < 0.01 vs LPC, ^f*P* < 0.001 vs LPC. PBMC: Peripheral blood monocytes; MLN: Mesenteric lymph node; Frl cells: CD4⁺CD45RA⁺FoxP3^{low}; FrlII cells: CD4⁺CD45RA⁺FoxP3^{high}; FrlIII cells: CD4⁺CD45RA⁺FoxP3^{low}; HC: Healthy control mice; UC: Ulcerative colitis mice; LPJ: Lamina propria of jejunum; LPC: Lamina propria of colon.

Treg-derived IL-10 in maintaining intestinal health^[20]. Meanwhile, high levels of the transcription regulation factor Forkhead box protein 3 (FoxP3) that is encoded by FoxP3 mRNA^[21] enhance the immunosuppressive function of Treg cells^[22]. In our study, we established the 2.5% DDS-induced colitis, among the common models of mice colitis: 2,4,6-trinitro benzene sulfonic acid, oxazolone and dextran sodium sulfate (DSS)

colitis. Of which the DSS-induced colitis was sample and repetitive^[16]. Moreover it resembles the clinical course of human UC occurs frequently in the chronic phase of DSS-induced colitis^[23]. Here we observed an increase in Treg cell numbers in UC mice, but this increase likely does not inhibit inflammatory responses given results from previous studies showing the presence of excessive inflammation in the intestines

of UC patients^[24,25]. Instead, the decreased expression of IL-10 and FoxP3 mRNA in Treg cells from UC mice suggested that Treg cells in these animals may have functional defects that contribute to UC morbidity.

In response to various cytokines such as IL-6 and TGF- β , CD4⁺ cells differentiate to Treg or Th17 cells, which have opposite functions and thus a balance between these populations is essential. Similar to Treg cells in the UC mice, Th17 cell numbers increased in both PBMC and LPC samples from UC mice, and IL-17A cytokine levels in PBMC and serum were concurrently increased. These results suggest the presence of a hyperactive inflammatory response in the colon mucosa of UC animals^[26-28], and also that impaired Treg cell function as well as an imbalance between Treg and Th17 cell populations could be involved in inappropriate immune responses in UC.

Aberrant immune responses that can result from Treg and Th17 imbalances are a feature of autoimmune diseases^[29,30], although the underlying mechanisms that promote these imbalances are unclear. On the one hand, there is heterogeneity in among the Treg cell population. Thus, we explored whether Treg subsets contribute to immune imbalance seen in UC mice. In our study, the number of CD4⁺CD45RA⁺FoxP3^{high} cells, which are activated Treg cells that display immunosuppressive capacity, was decreased in UC. CD4⁺CD45RA⁺FoxP3^{high} cells were previously shown to have higher levels of IL-10 transcription^[31], and together with the decreased expression of FoxP3 mRNA and IL-10 in Treg cells observed here, supports that the low levels of CD4⁺CD45RA⁺FoxP3^{high} cells contribute to immunosuppressive function of Treg cells in UC.

CD4⁺CD45RA⁺FoxP3^{low} cells are resting Treg cells that act as a reserve of cells that can be activated and differentiate into CD4⁺CD45RA⁺FoxP3^{high} cells. There is a tight balance between the continuous development of CD4⁺CD45RA⁺FoxP3^{high} cells from activated and proliferating CD4⁺CD45RA⁺FoxP3^{low} cells and CD4⁺CD45RA⁺FoxP3^{high} cell death after exerting suppressive effects^[9,15]. Here we observed an increase in CD4⁺CD45RA⁺FoxP3^{low} cells alongside the decrease in CD4⁺CD45RA⁺FoxP3^{high} cells. From these results we inferred that UC mice have defects in CD4⁺CD45RA⁺FoxP3^{low} to CD4⁺CD45RA⁺FoxP3^{high} cell conversion that could affect CD4⁺CD45RA⁺FoxP3^{high} cell replenishment.

On the other hand, we observed a concurrent increase in CD4⁺CD45RA⁺FoxP3^{low} cells and decrease in CD4⁺CD45RA⁺FoxP3^{high} cells. Earlier studies suggested that FoxP3 expression determines Treg lineage and is required for the suppressive function of these cells^[32]. FoxP3 expression downregulates expression of the retinoic acid receptor-ROR γ t, which is the lineage-defining transcription factor for Th17 cells, and in turn contributes to the inhibition of Th17 differentiation^[33,34]. However, a more recent study by Voo *et al.*^[35] documented FOXP3/RORC double-positive cells in peripheral blood and in lymphoid organs from healthy

human volunteers. Moreover, FoxP3⁺IL-17A⁺ double-positive cells appear to be the crossover cells that can differentiate into Th17 cells in response to lower amounts of TGF- β together with the presence of IL-6 or IL-21^[36,37]. These FoxP3⁺IL-17A⁺ double-positive cells also exist in several autoimmune diseases, including allergic rhinitis, psoriasis, and IBD^[38], and several studies demonstrated that FOXP3⁺IL-17⁺ cells can indeed differentiate into Th17 cells in the periphery^[35,39]. Meanwhile, FrIII cells can differentiate into Th17 cells that have no immunosuppressive function^[9]. Our results also showed that, relative to HC mice, UC mice have increased numbers of both CD4⁺CD45RA⁺FoxP3^{low} cells and FoxP3⁺IL-17A⁺ double-positive cells, especially in LPC and LPJ samples. Furthermore, we showed that these CD4⁺CD45RA⁺FoxP3^{low} cells co-expressed FoxP3 and RORC mRNA, which, together with the increased expression levels of IL-17A mRNA, confer on these cells the characteristics of FOXP3⁺IL-17⁺ double-positive cells. Thus, we inferred that CD4⁺CD45RA⁺FoxP3^{low} cells acquired the ability to express RORC in the periphery, and have the potential to differentiate into Th17 cells that lack suppressive activity.

CD4⁺ Treg cells are composed of two major populations: Thymus-derived Treg cells, or natural Tregs (nTregs), and peripherally derived Treg cells, or induced Tregs, which are generated in the periphery^[40,41]. To extend the study of functional Treg subsets into non-lymphoid tissues, unique tissue-resident Treg populations have been identified and characterized, such as Treg in visceral adipose tissue^[42], in skin^[43], in skeletal muscle^[44], in lung^[45], liver^[46], and pancreas^[47]. Recent evidence suggests that environmental signals found in peripheral non-lymphoid tissues promote the development of tissue-specific Treg cell subsets. Although the proportion of tissue-specific Treg cell subsets within tissues is difficult to determine due to differences between inflammatory and steady-state conditions^[48] accumulating evidence suggests that Treg cells are highly responsive to their local environment. The gut contains a large reservoir of both secondary lymphoid tissue-resident Treg cells and non-lymphoid tissue-resident Treg cells^[49]. The development of lamina propria-resident Treg cells differs from that for secondary lymphoid tissue Treg cells^[50,51], in that lamina propria Treg cells respond to unique environmental signals that are generated in response to products of commensal bacteria^[52,53]. As such, we further evaluated differences in Th17, Treg, and Treg cell subtypes in LPC and LPJ, spleen, MLN, and PBMC in UC and HC mice. In this study, there were more Treg in the colon relative to other tissues, while the jejunum showed the highest number of Th17 cells, even in UC mice. Round *et al.*^[52] also found that in B6 mice, distribution of Th17 cells is duodenum > jejunum > ileum > colon, while Treg cells were most abundant in the colon and scarce in the duodenum. This regional difference is associated with the T cell/APC ratio, especially CD11c(+)CD11b(+)CD103(+) dendritic

cells. Our results showed that all Tregs and the three Treg subsets were more frequent in the LPC relative to other secondary lymphoid tissue and PBMC in both UC and HC mice. In addition, the number of Treg cells in spleen tissue from both UC and HC mice was similar, suggesting that lamina propria-resident Treg cells rather than secondary lymphoid tissue Treg cells are involved in UC pathogenesis. Since UC is an organ-specific autoimmune disease, the Treg cells in PBMC likely would not reflect the characteristics of UC. Moreover, relative to HC, the number of CD4⁺CD45RA⁺FoxP3^{high} cells in the LPC and LPJ of UC mice was decreased, although the numbers were similar in spleen samples. The increased number of CD4⁺CD45RA⁺FoxP3^{low} and CD4⁺CD25⁺FoxP3⁺IL-17⁺ cells mainly occurred in the LPC in UC mice. As such, we inferred that the abnormal differentiation of active Treg cells occurred in local tissues and not secondary lymphoid tissues.

In summary, decreased numbers of CD4⁺CD45RA⁺FoxP3^{high} cells together with a reserve of abnormal of CD4⁺CD45RA⁺FoxP3^{low} cells and increased numbers of non-suppressive CD4⁺CD45RA⁺FoxP3^{low} cells, could present a potential source of Th17 cells that lack suppressive capacity and are an important characteristic of UC mice. These characteristics may contribute to an imbalance between Treg and Th17 cells and the increased numbers of functional Treg cells. Thus, lamina propria-resident Treg cells appear to play important roles in shaping local peripheral tolerance and maintaining intestinal homeostasis, and an imbalance of Treg and Th17 cells in the lamina propria of the colon is critical for UC pathogenesis.

COMMENTS

Background

Ulcerative colitis (UC) is a type of inflammatory bowel disease that affects the colon and is confined to the mucosa and superficial submucosa. UC symptoms include diarrhea, abdominal pain, and rectal bleeding, which can all seriously affect quality of life, and the disease is often marked alternating phases of clinical relapse and remission.

Research frontiers

The exact etiology and pathology of UC remains unknown, there is increasing evidence that an aberrant immune response is involved in this disease. Morbidity often involves an imbalance between T helper 17 (Th17) cells and regulatory T cells (Treg).

Innovations and breakthroughs

In this study, the authors investigated the role of Treg cell subsets in the balance between Treg and Th17 cells in various tissues from mice with dextran sulfate sodium (DSS)-induced colitis.

Applications

It is believed that this study will be of great interest to scientists and critical pathogenesis for UC, and as well as clinicians studying UC.

Terminology

Regulatory T cells belong to a functionally specialized subset of CD4⁺T cells, which can be divided into three different functional subsets: Resting Tregs, FrI (rTreg or CD45RA⁺Foxp3^{low}); activated Tregs, FrII (aTreg or CD45RA⁺Foxp3^{high});

and non-suppressive Tregs, FrIII (CD45RA⁺Foxp3^{low}). FrI cells are resting Treg cells that upon activation become FrII cells, which are the major suppressive cells. Meanwhile, FrIII cells secrete interleukin-17 (IL-17) and have the potential to become Th17 cells, a newly discovered CD4⁺T cell subset that lacks immunosuppressive function and is characterized by interleukin 17A, IL-17F, IL-22, IL-21 secretion.

Peer-review

The authors demonstrated that increased numbers of CD4⁺CD45RA⁺FoxP3^{low} cells may cause an imbalance between Treg and Th17 cells that is mainly localized to the lamina propria of colon rather than secondary lymphoid tissues. The present study was well organized and well investigated. To improve the quality of this paper, the authors should revise it according to the following suggestions: (1) the authors used a DSS colitis as a model of UC. Don't use the "UC" in the result session. It is more suitable to use "DSS colitis" instead of "UC" throughout the result session; and (2) to confirm the role of CD4⁺CD45RA⁺FoxP3^{low} cells in the pathogenesis in DSS colitis, the authors should show the time-course changes of these cells.

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Basic Study

IRF5 regulates lung macrophages M2 polarization during severe acute pancreatitis *in vitro*

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Abstract

AIM

To investigate the role of interferon regulatory factor 5 (IRF5) in reversing polarization of lung macrophages during severe acute pancreatitis (SAP) *in vitro*.

METHODS

A mouse SAP model was established by intraperitoneal (ip) injections of 20 µg/kg body weight caerulein. Pathological changes in the lung were observed by hematoxylin and eosin staining. Lung macrophages were isolated from bronchoalveolar lavage fluid. The quantity and purity of lung macrophages were detected

by fluorescence-activated cell sorting and evaluated by real-time polymerase chain reaction (RT-PCR). They were treated with IL-4/IRF5 specific siRNA (IRF5 siRNA) to reverse their polarization and were evaluated by detecting markers expression of M1/M2 using RT-PCR.

RESULTS

SAP associated acute lung injury (ALI) was induced successfully by ip injections of caerulein, which was confirmed by histopathology. Lung macrophages expressed high levels of IRF5 as M1 phenotype during the early acute pancreatitis stages. Reduction of IRF5 expression by IRF5 siRNA reversed the action of macrophages from M1 to M2 phenotype *in vitro*. The expressions of M1 markers, including IRF5 (S + IRF5 siRNA *vs* S + PBS, 0.013 ± 0.01 *vs* 0.054 ± 0.047 , $P < 0.01$), TNF- α (S + IRF5 siRNA *vs* S + PBS, 0.0003 ± 0.0002 *vs* 0.019 ± 0.018 , $P < 0.001$), iNOS (S + IRF5 siRNA *vs* S + PBS, 0.0003 ± 0.0002 *vs* 0.026 ± 0.018 , $P < 0.001$) and IL-12 (S + IRF5 siRNA *vs* S + PBS, 0.000005 ± 0.00004 *vs* 0.024 ± 0.016 , $P < 0.001$), were decreased. In contrast, the expressions of M2 markers, including IL-10 (S + IRF5 siRNA *vs* S + PBS, 0.060 ± 0.055 *vs* 0.0230 ± 0.018 , $P < 0.01$) and Arg-1 (S + IRF5 siRNA *vs* S + PBS, 0.910 ± 0.788 *vs* 0.0036 ± 0.0025 , $P < 0.001$), were increased. IRF5 siRNA could reverse the lung macrophage polarization more effectively than IL-4.

CONCLUSION

Treatment with IRF5 siRNA can reverse the pancreatitis-induced activation of lung macrophages from M1 phenotype to M2 phenotype in SAP associated with ALI.

Key words: Interferon regulatory factor 5; Macrophage polarization; Severe acute pancreatitis; SiRNA

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Core tip: We investigated the role of interferon regulatory factor 5 (IRF5) in reversing polarization of lung macrophages during severe acute pancreatitis (SAP). Treatment with IRF5 specific siRNA could reverse the pancreatitis-induced activation of lung macrophages from M1 phenotype to M2 phenotype *in vitro*. Reduced expression of IRF5 may lead to a new therapeutic approach for SAP associated with acute lung injury.

Sun K, He SB, Qu JG, Dang SC, Chen JX, Gong AH, Xie R, Zhang JX. IRF5 regulates lung macrophages M2 polarization during severe acute pancreatitis *in vitro*. *World J Gastroenterol* 2016; 22(42): 9368-9377 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9368.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9368>

INTRODUCTION

Severe acute pancreatitis (SAP) is a most serious disease especially when multiple remote organs are involved^[1,2]. The developing process of this disease is rapid and can result in a series of complications and multiple organ dysfunction (MODS) including the lung, liver, kidney, intestine and stomach^[3,4]. MODS is an essential process during SAP. MODS caused nearly half of deaths in SAP patients^[5]. Acute lung injury (ALI) is the most common extrapancreatic complication in SAP, which is associated with the high rates of morbidity and mortality. ALI is characterized by accumulation of activated neutrophils and the development of interstitial edema^[6,7].

Although accumulation of activated neutrophils in the lungs is characteristic of ALI, macrophages which reside in the pulmonary interstitium and alveoli are considered to be the most critical cells involved in development of the SAP associated with ALI^[8-10]. Macrophages are plastic cells displaying versatile functional phenotypes depending on microenvironments. M1 macrophages (M1, the classically activated macrophages) and M2 macrophages (M2, the alternatively activated macrophages) have been defined as the two extremes in a spectrum of macrophage functional phenotypes. M1 macrophages are critical effector cells that kill micro-organisms^[11,12]. In contrast, M2 macrophages are involved in the resolution of inflammation^[13]. The pro-inflammatory response must be balanced by regulatory and inhibitory effector mechanisms to protect against tissue damage caused by the effects of excessive inflammation^[14]. Lung macrophages can be subdivided into alveolar, pleural, interstitial and intravascular macrophages, and alveolar macrophages (AM) considered as the most important cell of the innate immune system^[10,15].

Distinct macrophage subtypes are not only characterized by their differences in cytokine release but also by the differential expression of key transcription factors. Krausgruber *et al.*^[16] found that transcription factor interferon regulatory factor 5 (IRF5) was a major factor in defining macrophage polarization. They found that IRF5 was the major regulator of pro-inflammatory M1 macrophage polarization. IRF5 directly induces the expression of pro-inflammatory cytokines such as IL-6, IL-12b, and IL-23a while repressing transcription of anti-inflammatory cytokines such as IL-10. Although IRF5 plays an important role in macrophage activation, it has rarely been used to track macrophages in inflammatory disease. In the present study, we used the SAP model induced by intraperitoneal (ip) injection of caerulein, and isolated murine lung macrophages from bronchoalveolar lavage (BAL) fluid to detect IRF5 expression and their polarization *in vitro*. In addition, we treated lung macrophages with IRF5 specific siRNA (IRF5 siRNA)/IL-4 to reverse macrophage polarization

from M1 phenotype to M2 phenotype and compare its effect with IL-4.

MATERIALS AND METHODS

Animals and experimental design

C57BL/6 mice, male or female, aged 8–12 wk, were obtained from the Laboratory Animal Center of Jiangsu University, Zhenjiang, Jiangsu, China. The laboratory animal experimental protocol was approved by Institutional Animal Care and Use Committee of Jiangsu University. All experiments followed the laboratory animal care principles. Rats were randomly divided into two groups: (1) SAP group: rats received ip injections of caerulein; and (2) control group: rats received ip injection of an equal volume of PBS. Each group had 15 rats. The RAW264.7 cells (mouse macrophages, M0) were placed in a 12-well plate at 10^5 /mL 24 h before intervention and IFN- γ (100 U/mL) + LPS (5 μ g/L) was used to induce M1 phenotype (M1), and IL-4 (10 μ g/L) was used to induce M2 phenotype (M2). The three types of macrophages (M0, M1 and M2) were as control compared with lung macrophages.

SAP model

SAP models were prepared according to the protocol by Tang *et al.*^[17]. The mice were deprived of food but allowed access to water 24 h prior to the start of the experiments. SAP was induced in C57BL/6 mice by ip injections of 20 μ g/kg body weight (diluting stock solution in saline) caerulein at hourly intervals for a total of seven injections.

Histological analysis

For the histological analysis, mouse pancreatic and lung tissue samples were washed thoroughly in PBS, fixed in 10% buffered formalin, and embedded in paraffin, tissue samples were cut into sections. Five μ m/L sections were stained with hematoxylin and eosin using standard procedures and examined by light microscopy. Sections were examined by an experienced morphologist, who was blinded to the sample identity, for tissue injury.

Cell preparation and AM culture

AM was purified according to the method of Small *et al.*^[18]. Briefly, anesthetized mice were sacrificed, the lungs from 36 mice were lavaged with RPMI 1640 containing 10% FCS, and BAL was mixed in a 50 mL tube. BAL fluids were then plated, and the AM was allowed to adhere for 2 h at 37 °C, 5% CO₂.

Quantity and purity of the lung macrophages

BAL cells were incubated for 30 min at 4 °C with rat-anti-F4/80 monoclonal antibody. The cells were analyzed using fluorescence-activated cell sorting (FACS). The BAL cells were seeded on plates for 2 h and non-adherent cells were washed, and the

adherent cells were scraped and fixed with 4% para-formaldehyde at 4 °C for 30 min, and then incubated for 2 h with a 1:100 dilution of the CD11b/c antibody and F4/80 antibody (Ebioscience, San Diego, United States) labeled with Alexa Fluor 488 for flow cytometry analysis^[19].

Reversion of the polarized lung macrophages in vitro by IRF5 siRNA/IL-4

Lung macrophages obtained from BAL fluid as well as rats 18 h after SAP induction. One part of polarized lung macrophages was transfected with IRF5 siRNA using a Mouse Macrophage Nucleofector[®] Kit (LONZA, Anaheim, CA, United States) according to the manufacturer's instructions. The other part of polarized lung macrophages was incubated with IL-4 (20 ng/mL) for 24 h. Macrophages were collected for further detection after incubation.

Measurement of polarization of lung macrophages

Real-time polymerase chain reaction (RT-PCR) was performed with the ABI Prism 7900HT (Applied Biosystems, Foster City, CA, United States) using the SYBR Green PCR Master Mix (Applied Biosystems) according to the protocol in our lab. The PCR primers used were as follows: GAPDH (F: GTATGACTCTA CCCACGGCAAGT; R: TTCCCGTTGATGACCAGCTT), TNF- α (F: CCGATTTGCCACTTCATACCA; R: TAGGG CAAGGGCTCTTGATG), IL-10 (F: AGAAGGACCA GCTGGACAACAT; R: CAAGTAACCCTTAAAGTCCT GCAGTA), Arg-1 (F: CCGCAGCATTAAAGGAAAGC; R: CCCGTGGTCTCTCACATTG), iNOS (F: CAGCCCT CAGAGTACAACGAT; R: CAGCAGGCACACGCAATGAT), IRF5 (F: GTTGCCTTTGACGGACCTA; R: 5'-GGCC CACTCCAGAACACCT-3'), IL-12 (F: 5'-AAAGGTGC GTTCTCGTAG; R: CAACAGCATAAGGCCAAGT).

Statistical analysis

All values were expressed as mean \pm SE. Independence *t* test was used in the comparison of two groups. *P* values < 0.05 indicate statistical significance. Analyses were performed using SPSS 17.0.

RESULTS

Histopathology and morphometry of the lung tissue during SAP

As shown in Figure 1, lung tissue and pancreas tissue from control rats showed a normal structure and no histopathological changes under light microscope (Figure 1A, C). A typical photomicrograph of pancreatic lung injury included lung edema, neutrophil infiltration, necrosis and hemorrhage (Figure 1B). The histological changes of pancreas tissue, such as the infiltration of neutrophils, macrophages, interstitial edema, hemorrhage and focal necrotic areas, were seen in the pancreas tissue of SAP group (Figure 1D). The pathological findings confirmed that we had established

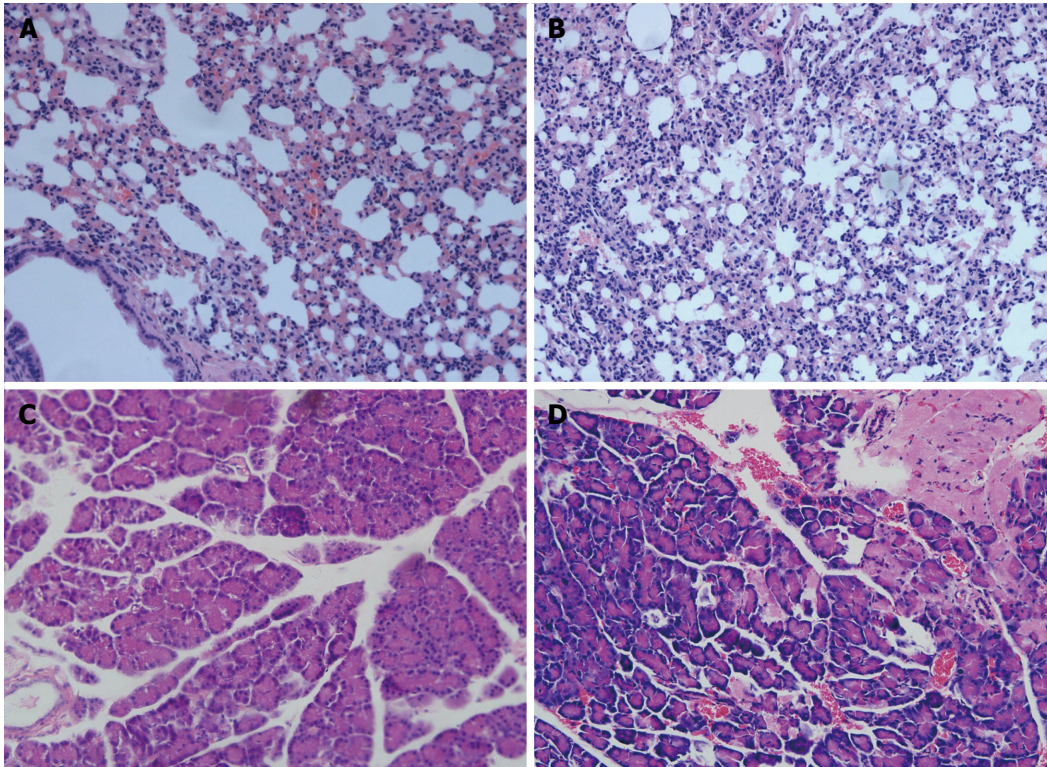


Figure 1 Histological examination of lung and pancreas stained with hematoxylin and eosin staining. A: Lung of control rats; B: Lung of acute pancreatitis (SAP) rats; C: Pancreas of control rats; D: Pancreas of SAP rats. There were no remarkable pathologic changes in control rats (A, C); Significant inflammatory cell infiltration was observed in the SAP group. The typical pathological changes of SAP associated with acute lung injury, including lung edema, necrosis, hemorrhage and neutrophil infiltration, were seen in SAP group (B). The histological changes of pancreas tissue such as the infiltration of neutrophils, macrophages, interstitial edema, hemorrhage and focal necrotic areas were seen in the pancreas tissue of SAP group (D). Original magnification $\times 200$ (A-D).

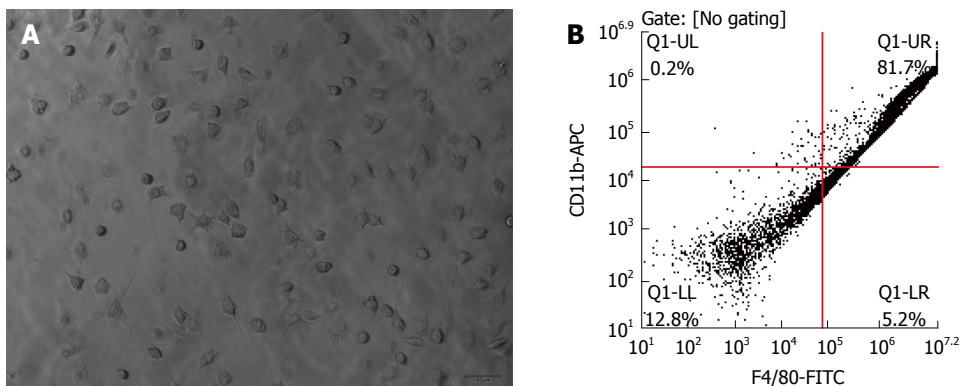


Figure 2 Identification of lung macrophages. A: Lung alveolus macrophages were identified by microscopy (original magnification $\times 200$); B: The purity of lung macrophages cells was examined by fluorescence-activated cell sorting as CD11b/c, F4/80 positive cells after primary culture for 24 h.

the mouse SAP model associated with ALI successfully.

Isolation of lung macrophages of SAP rats

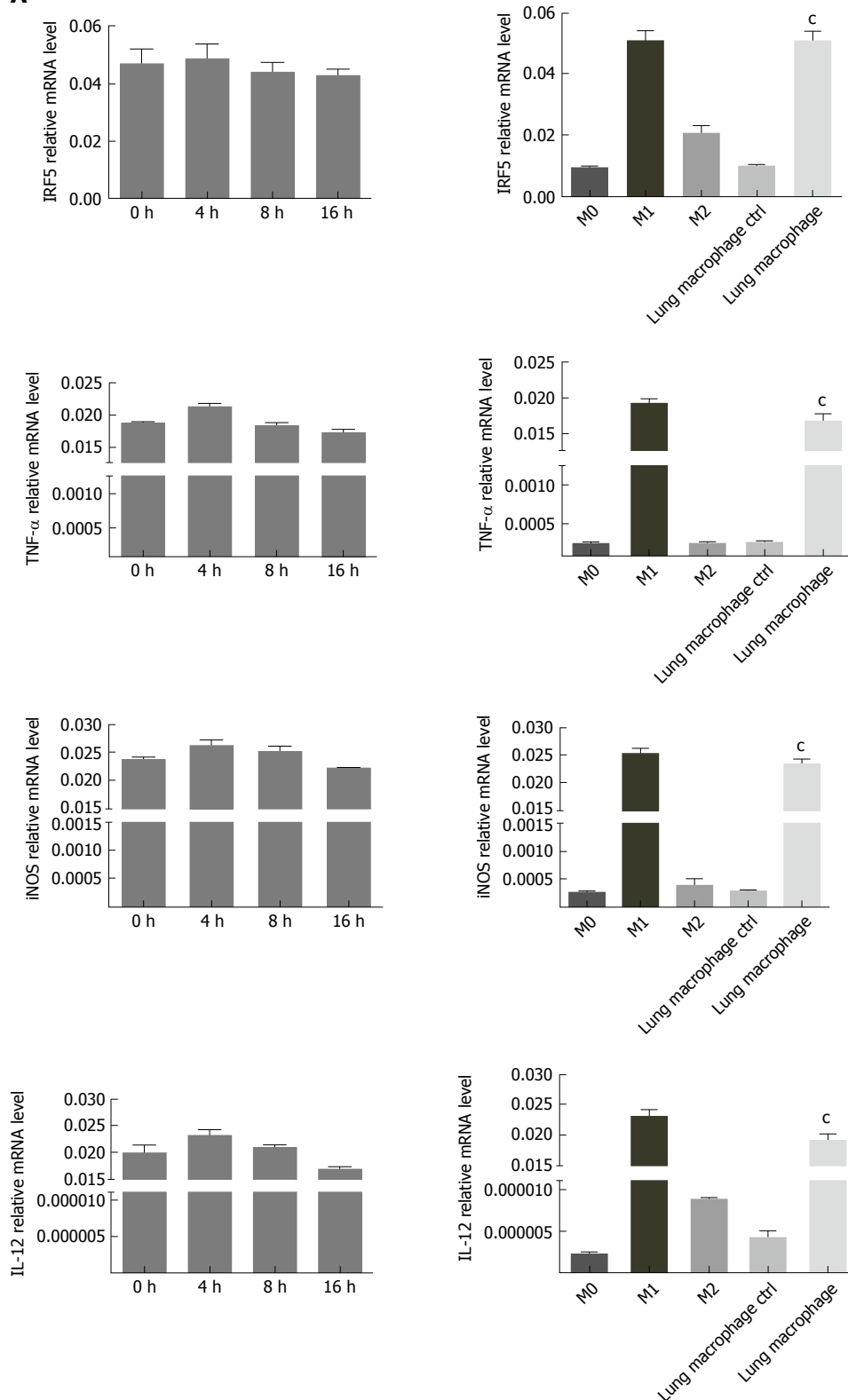
After 24 h primary culture, the lung alveolus macrophages were observed under microscope and examined by FACS for the purity and quantity. The purity of lung alveolus macrophages was approximately 81.7% (Figure 2).

Expression of M1 phenotype of lung alveolus macrophages during early phase of SAP

Lung alveolus macrophages were isolated from BAL

fluid of the control group and SAP group by FACS. *In vitro* lung macrophages from SAP group were treated with PBS for 4, 8 and 16 h respectively, and macrophages from control group were treated with PBS for 4 h. The expression of major M1 markers (IRF5, TNF- α , iNOS and IL-12) and M2 markers (IL-10 and Arg-1) were detected by RT-PCR. The polarization of lung macrophages was detected by RT-PCR. As shown in Figure 3, the mRNA levels of lung macrophage markers in SAP group were significantly higher than in the control group (M0) ($P < 0.01$). In contrast, mRNA levels of lung macrophage markers in SAP group were

A



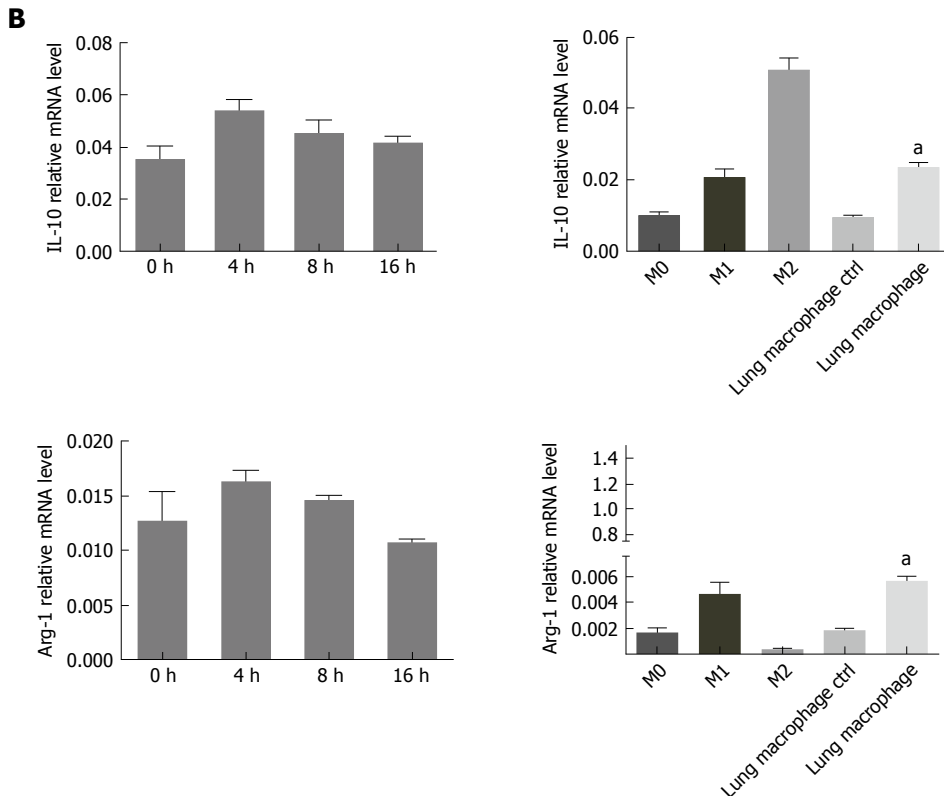


Figure 3 Expression of M1 phenotype of lung alveolus macrophages during early phase of acute pancreatitis. The expression of major M1 phenotypes [interferon regulatory factor 5 (IRF5), TNF-, iNOS and IL-12] and M2 phenotypes (IL-10 and Arg-1) were detected by real-time polymerase chain reaction (RT-PCR). The polarization of lung macrophages was detected by RT-PCR. A: mRNA levels of M1 phenotypes in the macrophages; B: mRNA levels of M2 markers in the macrophages. The statistical significance was analyzed in comparison with the CTRL groups (M0, M1). ^a $P < 0.05$, ^c $P < 0.01$ vs control.

similar to those in another control group (M1) ($P > 0.05$). The experimental data indicated a shift to M1 polarization of lung macrophages during the initiation of SAP.

Reversion of the polarization of lung macrophages in SAP group

Whether the polarization of lung alveolus macrophages could be reversed from M1 to M2 *in vitro* was studied. After treatment with PBS/IL-4/IRF5 siRNA, the expression levels of macrophage markers (M1: IRF5, TNF- α , iNOS, IL-12; M2: IL-12, Arg-1) were compared at 4 h. As shown in Figure 4, RT-PCR showed that IL-4/IRF5 siRNA could reduce expression of M1 markers significantly: IRF5 (S + IRF5 siRNA vs S + PBS, 0.013 ± 0.01 vs 0.054 ± 0.047 , $P < 0.01$), TNF- α (S + IRF5 siRNA vs S + PBS, 0.0003 ± 0.0002 vs 0.019 ± 0.018 , $P < 0.001$), iNOS (S + IRF5 siRNA vs S + PBS, 0.0003 ± 0.0002 vs 0.026 ± 0.018 , $P < 0.001$) and IL-12 (S + IRF5 siRNA vs S + PBS, 0.000005 ± 0.00004 vs 0.024 ± 0.016 , $P < 0.001$). In contrast, M2 markers were upregulated by IL-4/IRF5 siRNA treatment: IL-10 (S + IRF5 siRNA vs S + PBS, 0.060 ± 0.055 vs 0.0230 ± 0.018 , $P < 0.01$) and Arg-1 (S + IRF5 siRNA vs S + PBS, 0.910 ± 0.788 vs 0.0036 ± 0.0025 , $P < 0.001$). The results suggested that the polarization of lung macrophages could be reversed by IRF5 siRNA from

M1 to M2 as well as by IL-4.

Ability to reverse lung macrophage polarization by IRF5 siRNA/IL-4

In order to identify a better way to alter the polarization of macrophages *in vitro*, we compared the expression of M1 and M2 markers after the treatment of IL-4 or IRF5 siRNA. Gene expression in primary culture macrophages from the rats with SAP was determined by real-time PCR. As shown in Figure 5, the effect of treatment with IL-4 and IRF5 siRNA were similar in IRF5 (4 h, 8 h), IL-12 (4 h, 8 h), iNOS (4 h, 8 h), TNF- (4 h, 16 h), IL-10 (8 h, 16 h), Arg-1 (4 h) ($P > 0.05$); but in some groups, the levels of IRF5 (16 h, 24 h), TNF- (8 h, 24 h), iNOS (16 h, 24 h) and IL-12 (16 h, 24 h) were much lower, and Arg-1 (8 h, 16 h, 24 h), IL-10 (4 h, 24 h) were significantly higher after IRF5 siRNA treatment than after IL-4 treatment ($P < 0.05$). These results indicated that IRF5 siRNA could reverse the lung macrophage polarization more effectively.

DISCUSSION

SAP can cause SIRS and high morbidity. In SAP, MODS in the early phase is the main cause of high mortality^[5,20]. In the early phase, activated macrophages can release humoral mediators which may

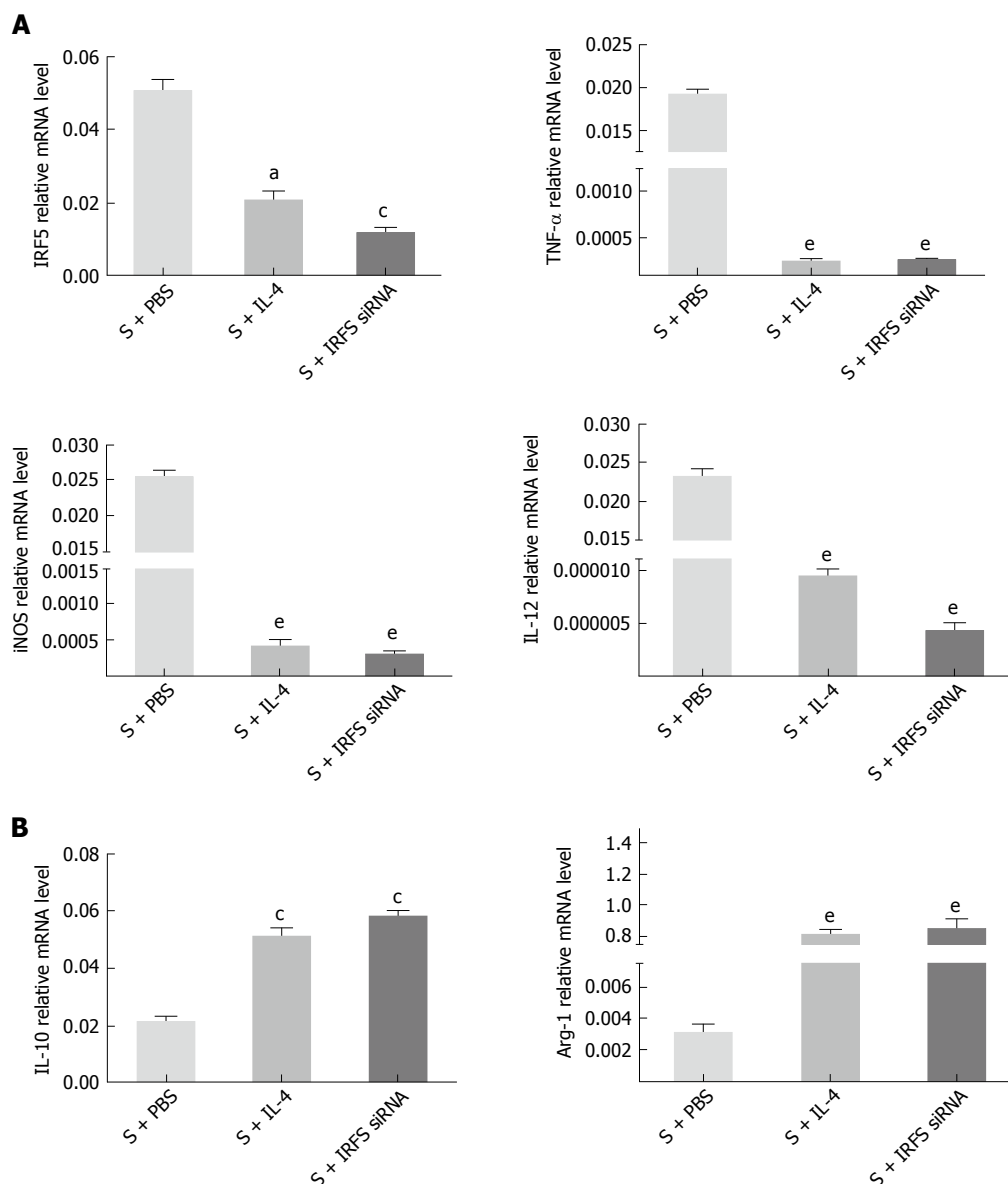


Figure 4 Reversion of the polarization of lung macrophages in acute pancreatitis group. Lung alveolus macrophages were obtained 24 h after acute pancreatitis (SAP) model establishment and respectively treated with PBS, IL-4 or interferon regulatory factor 5 (IRF5) siRNA for 8 h (S + PBS, S + IL-4, S + IRF5 siRNA group). The M1/M2 phenotypes were detected by real-time polymerase chain reaction. A: mRNA levels of M1 phenotypes in the macrophages; B: mRNA levels of M2 phenotypes in the macrophages. The statistical significance was analyzed in comparison with the CTRL group (S + PBS). Error bars indicate the mean \pm SE. ^a $P < 0.05$, ^c $P < 0.01$, ^e $P < 0.001$ vs control. S: Lung alveolus macrophages from the mouse SAP model.

lead to remote organ injury, including ALI, acute liver injury, acute kidney injury, etc. ALI is one of the most common organ failures in SAP. It is the first cause of patients' death during the early stage of SAP^[21].

A number of investigations have indicated that the severity of SAP and pancreatitis-associated lung injury were regulated by a great deal of inflammatory factors, including inflammatory cells and cytokines^[22-24]. Among many inflammatory cells, macrophages are key pro-inflammatory and anti-inflammatory cells, which accumulate in the damaged organ in SAP. Plasticity and functional polarization are hallmarks of macrophages that result in the phenotypic diversity of macrophage populations^[25,26]. Thus, targeting selected macrophage pathways is a potential therapeutic strategy for su-

ppressing macrophage-mediated lung injury.

Recently, transcriptional pathways were believed to play an important role in the polarization of macrophage. IRF5 was considered as a major factor in defining macrophage polarization^[27,28]. Modulation of IRF5 led to the conversion of one macrophage subset phenotype into the other one^[29]. High expression of IRF5 results in polarization of the macrophage phenotype toward M1, however, low expression of IRF5 leads to M2 polarization, which is the anti-inflammatory macrophage phenotype. This indicates a possible broad effect of therapy which targets the induction of IRF5 expression by macrophages^[30].

In the present study, a mouse model of SAP associated with ALI was constructed successfully

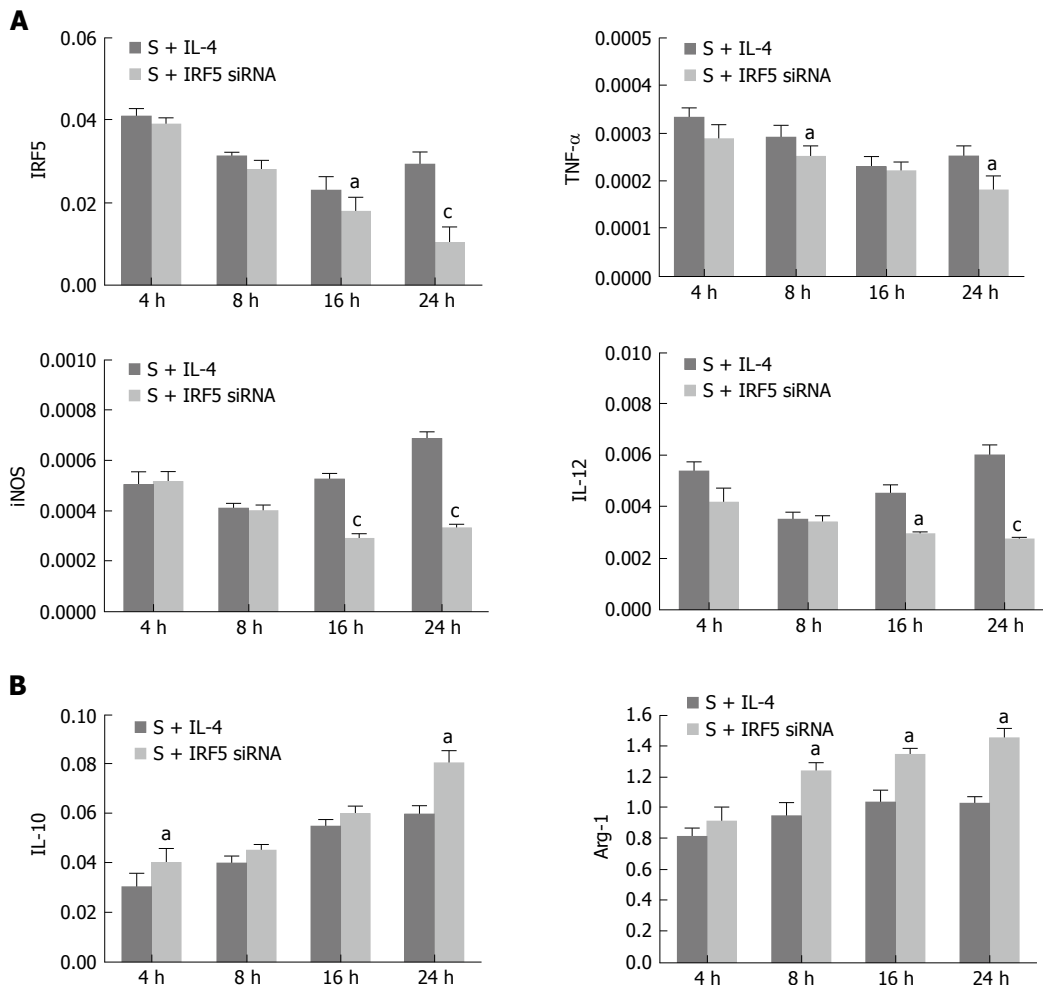


Figure 5 Comparison of the ability to reverse lung macrophage polarization by interferon regulatory factor 5 siRNA/IL-4. Lung macrophages were treated with interferon regulatory factor 5 (IRF5) siRNA/IL-4 for 4, 8, 16 and 24 h. The M1/M2 phenotypes were detected by real-time polymerase chain reaction. A: mRNA levels of M1 phenotypes in the macrophages; B: mRNA levels of M2 phenotypes in the macrophages. The statistical significance was analyzed when the two groups were compared in the same time point using indifferent *t* test. Error bars indicate the mean \pm SE. **P* < 0.05, ^c*P* < 0.01 vs control.

by ip caerulein. We found that lung macrophages were M1 polarized in the early phase of SAP. The M1 state was confirmed by the up-regulated expression of M1 phenotypes and down-regulated expression of M2 phenotypes. And the results conform to the conventional theory that macrophages can induce pro-inflammatory cytokines and amplify the degree of the inflammation during early phase of SAP^[31,32].

It has been reported that macrophage polarization could be reversed from M1 phenotype to M2 phenotype^[33]. The development and resolution of lung injury are accompanied by alteration of macrophage polarization in the lungs. We hypothesized that lung macrophages which act as M1 phenotype could aggravate inflammation, and M2 could alleviate the inflammation if we reversed the polarization of lung macrophages in SAP by IRF5 siRNA. In the present study, the lung macrophages showed M1 polarization reflected in the high expression of M1 phenotypes (IRF5, TNF- α , iNOS and IL-10). This activation was expected as lung macrophages contact early with mediators released by inflammatory pancreatic tissue.

Several studies had reported the plasticity of activated macrophages^[34,35]. Furthermore, the M2 macrophage state induced by these mediators can alleviate the inflammatory response associated with SAP. This kind of reversion may alleviate systemic inflammation and promote tissue repair during SAP.

It is interesting to know the capacity of these pancreatitis-activated lung macrophages to be reverted to an anti-inflammatory phenotype from M1 to M2 *in vitro*. We isolated lung macrophages from BAL fluid of the rats with ALI and the isolated cells were treated by IRF5 siRNA or IL-4. Our data showed that both treatments can reverse macrophage polarization from M1 phenotype to M2 phenotype. After treatment with IRF5 siRNA or IL-4, lung macrophages down-regulated the expression of M1 phenotypes such as IRF5, TNF- α , iNOS and IL-10, which are pro-inflammatory mediators. At the same time, macrophages expressed more M2 phenotypes such as IL-10, which is an important anti-inflammatory factor. Our result indicated that activated lung macrophages obtained from rats with SAP could be reversed to M2 state by IRF5 siRNA

or IL-4 treatment.

IL-4 is known to induce the alternative M2 activation of macrophages. M2 macrophage can promote the repair phenotype and counteract the effects of pro-inflammatory cytokines^[36]. However, it is reported that IL-4 administration was not enough to reverse the M1 phenotype in lung macrophages therapeutically. The reason could be related to the strongly proinflammatory environment generated in the lung. Because of the long half-life of IRF5 siRNA, it demonstrated its high capability to reverse the polarization of macrophages from M1 phenotype to the M2 phenotype. In our study, we proposed that there may be two reasons for these results: (1) IRF5 mediates inflammatory and immune responses by controlling expression of proinflammatory cytokines downstream of MyD88-dependent Toll-like receptor signaling; and (2) IRF5 regulates the expression of inflammatory gene TNF- α and IL1 β directly^[37].

In conclusion, treatment with IRF5 siRNA could reverse the pancreatitis-induced activation of lung macrophages from M1 phenotype to M2 phenotype in SAP associated with ALI. Nevertheless, targeting transcription factors for therapeutic aims is still an unknown area. Meanwhile, the molecular mechanism of macrophage polarization is still unknown. In our experiment, silencing the IRF5 regulated a range of inflammatory genes, and this method can reverse the lung macrophage polarization more effectively than IL-4 administration. IRF5 is an attractive target for SAP associated with ALI therapy. However, the molecular mechanisms of IRF5 in macrophage polarization should be further studied.

COMMENTS

Background

Severe acute pancreatitis (SAP) is a life threatening disease especially when multiple remote organs and systems are involved in the inflammation. Acute lung injury (ALI) is the most common extrapancreatic complication in SAP. Recently, several reports have demonstrated that macrophages are considered to be the most critical cells involved in development of the acute pancreatitis. M1 macrophages and M2 macrophages have been defined as the two extremes in a spectrum of macrophage functional phenotypes. Interferon regulatory factor 5 (IRF5) was a major factor in defining macrophage polarization. The expression of IRF5 in lung macrophages from an SAP murine model and whether the IRF5 specific siRNA (IRF5 siRNA) could reverse M1 phenotype of macrophages to M2 have not yet been elucidated.

Research frontiers

Recently, several reports have demonstrated that macrophages are considered to be the most critical cells involved in development of the acute pancreatitis. Macrophages are plastic cells displaying versatile functional phenotypes depending on microenvironments. Krausgruber *et al* found that transcription factor IRF5 was a major factor for defining macrophage polarization. IRF5 was the major regulator of pro-inflammatory M1 macrophage polarization.

Innovations and breakthroughs

The authors for the first time investigated the role of IRF5 in reversing polarization of lung macrophages during SAP. Treatment with IRF5 siRNA could reverse the pancreatitis-induced activation of lung macrophages from M1 phenotype to M2 phenotype *in vitro*.

Applications

Lung macrophages adopt a pro-inflammatory activation (M1 phenotype) early during acute pancreatitis. Treatment with IRF5 siRNA could reverse the pancreatitis-induced activation of lung macrophages from M1 to M2 *in vitro*. The research revealed the function of IRF5 in regulating the polarization of lung macrophages. Reducing the expression of IRF5 may lead to a new therapeutic approach for SAP associated with ALI.

Terminology

M1 and M2 have been defined as the two extremes in a spectrum of macrophage functional phenotypes. M1 macrophages are critical effector cells that kill microorganisms. In contrast, M2 macrophages are involved in the resolution of inflammation. Distinct macrophage subtypes display differential expression of key transcription factors. IRF5 was the major regulator of pro-inflammatory M1 macrophage polarization.

Peer-review

This is a very well designed, performed and written experimental study. In this study, the authors reported the role of IRF5 in regulating lung macrophages M2 polarization during SAP *in vitro*. This research reveals a novel function of IRF5 in controlling the polarization of macrophages. Therefore, modification of IRF5 expression may lead to a new therapeutic approach for SAP associated with ALI.

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Retrospective Study

Folfirinox in elderly patients with pancreatic or colorectal cancer-tolerance and efficacy

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Abstract

AIM

To study the tolerance and the efficiency of FOLFIRINOX in elderly patients diagnosed with colorectal or pancreatic cancer.

METHODS

This retrospective study included elderly patients aged over 70 years of age treated at Georges-François Leclerc Center by FOLFIRINOX for histological proved colorectal or pancreatic cancer between January 2009 and January 2015. Chemotheapy regimen consisted of oxaliplatin (85 mg/m² in over 120 min) followed by leucovorin (400 mg/m² in over 120 min), with the addition, after 30 min of irinotecan (180 mg/m² in over 90 min) then 5 fluorouracil (5FU) (400 mg/m² administred intravenous bolus), followed by 5FU (2400 mg/m² intraveinuous infusion over 46 h) repeated every 2 wk. Geriatric parameters were recorded at the beginning. Toxicities were evaluated with the Common Terminology Criteria for Adverse Events 4.03. Tumor response was evaluated by CT scan. Treatment continued until disease progression, unacceptable toxicities or patient refusal.

RESULTS

Fifty-two patients aged from 70 to 87 years were treated by FOLFIRINOX, 34 had colorectal cancer and 18 had pancreatic cancer. Most of them were in good general condition, 82.7% had a 0-1 performance status and 61.5% had a Charlson Comorbidity Index < 10. The most frequent severe toxicities were neutropenia (17 patients, $n = 32.7\%$) and diarrhea (35 patients $n = 67.3\%$); 10 of the case of neutropenia and 5 of diarrhea registered a grade 4 toxicity. Thirty-nine patients (75%) initially received an adapted dose of chemotherapy. The dosage was adjusted for 26% of patients during the course of treatment. Tumor response evaluated by RECIST criteria showed a controlled disease for 25 patients (48.1%), a stable disease for 13 and a partial response for 12 patients. Time under treatment was higher for colorectal cancer with a median time of 2.44 mo (95%CI: 1.61-3.25). Overall survival was 43.88 mo for colorectal cancer and 12.51 mo for pancreatic cancer. In univariate or multivariate analysis, none of geriatric parameters were linked to overall survival. Only the type of tumor (pancreatic/colorectal) was linked in both analysis.

CONCLUSION

For people over 70 years old, FOLFIRINOX regimen seems to induce manageable toxicities but similar, even higher, median survival rates compared to younger people.

Key words: Elderly patients; Feasibility treatment; Pancreatic cancer; Colorectal cancer; FOLFIRINOX

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Core tip: The incidence of cancer in patients over 70 years old is still increasing, especially for pancreatic and colorectal cancer. Database is missing concerning elderly patients, especially for considered aggressive chemotherapies, like FOLFIRINOX. The aim of this retrospective study was to show the feasibility of a combination chemotherapies (FOLFIRINOX) in an elderly population, by initially adapting the treatment dose, according to the patient's general condition and comorbidities. We surprisingly observed prolonged survival and manageable toxicity levels.

Guion-Dusserre JF, Bertaut A, Ghiringhelli F, Vincent J, Quipourt V, Marilier S, Tharin Z, Bengrine-Lefevre L. Folfirinox in elderly patients with pancreatic or colorectal cancer-tolerance and efficacy. *World J Gastroenterol* 2016; 22(42): 9378-9386 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9378.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9378>

INTRODUCTION

In 2016, pancreatic cancer is the fourth leading cause

of death by cancer in the United States for patients from 60 to 79 years of age. Between 2005 and 2011, the recorded survival rate at 5 years was of 8% in elderly population^[1]. In 2012, in France the incidence was of 6690 cases in patients over 70 years old^[2]. With an incidence of 134490 new cases per year in the United States, colorectal cancer is the third most common and lethal cancer in 2016^[1]. In France, it is also the third most common cancer in the general population with a majority of patients over 70 years old (58% in 2012)^[2]. These proportions will probably increase in the future. Recent studies have already proved the efficiency of a triple-drug combination of fluorouracil, oxaliplatin and irinotecan (FOLFIRINOX) in both types of cancer. For metastatic pancreatic cancer a phase 2-3 trial demonstrated a survival advantage when FOLFIRINOX was used as a first-line therapy compared to gemcitabine, but the patients involved were under 76 years old^[3]. For colorectal cancer, this chemotherapy regimen has shown efficiency in association alone or in association with bevacizumab or cetuximab as first-line therapy^[4-7] and for more advanced stages, refractory to oxaliplatin and irinotecan^[8]. It can also be used as a neo-adjuvant treatment for locally advanced or metastatic rectal cancer as part of a clinical trial^[9,10].

Concerning these cancers, elderly patients are underrepresented in trials, or are selected according to their general condition^[11,12]. Therefore, there is a lack of evidence-based data when it comes to an older population. The older cancer population is heterogeneous with differences in co-morbidities, functional statuses, geriatric syndroms and socioeconomic aspects^[13]. The G8 instrument was approved as a screening tool to identify older patients who needed a geriatric assessment^[11]. When it is carried out, it can influence the decision of oncological treatment. The patient's biological age should ideally be established through this comprehensive geriatric assessment. Age alone should not be an exclusion criteria for the use of new targeted treatments, especially for metastatic colorectal cancer^[14,15]. Few trials had already shown that for selected elderly patients, chemotherapy with 5 fluorouracil (5FU), oxaliplatin or irinotecan is feasible with manageable toxicity levels^[16-24]. In this retrospective study we report the tolerance and efficacy of FOLFIRINOX in patients over 70 years old treated in our center.

MATERIALS AND METHODS

Study design

This retrospective study was carried out at Georges-Francois Leclerc Center from January 2009 to January 2015. The use of FOLFIRINOX was evaluated and validated by the local multidisciplinary staff. This protocol has been proposed by the referent oncologist for patients who received FOLFIRINOX after 70 years with locally advanced or metastatic pancreatic cancer,

or with metastatic colorectal cancer in first-line treatment, or a more advanced stage. All treatments were validated in multidisciplinary staff. Two patients with rectal cancer who had received FOLFIRINOX as neo-adjuvant treatment as part of a clinical trial were included (GRECCAR 4).

Patients characteristics

The study included patients over 70 years old who received FOLFIRINOX, for locally advanced or metastatic pancreatic or colorectal cancer, whatever the treatment line.

We used 70 years old as cut off, because on retrospective evidence, the incidence of geriatric problems increases sharply after 70 years old in oncologic population^[25]. Almost, main oncogeriatric studies, with recommendations from the SIOG, are using the age of 70 years old as cut off for geriatric assessment and developing geriatric screening tools^[26,27].

For all patients, cancer was histologically confirmed. Patients had to have adequate bone marrow function (granulocyte count > 1500 per cubic millimeter, hemoglobin > 9.0 g/dL and platelet count > 100000 per cubic meter), liver function [total bilirubin < 3 times the upper limit of normal (ULN) and aspartate/alanine transaminases < 5 times the ULN], and renal function (creatinine < 1.2 mg/dL or creatinine clearance > 50 mL/min).

Exclusion criteria were an uncontrolled infection, pre-existing neuropathy grade ≤ 1 , a history of drug hypersensitivity, active concomitant malignancy, and concurrent severe medical conditions.

Treatment

The FOLFIRINOX regimen consisted of oxaliplatin at a dose of 85 mg per square meter, given as a 2-h intravenous infusion, immediately followed by leucovorin at a dose of 400 mg per square meter, given as a 2-h intravenous infusion, with the addition, after 30 min of irinotecan at a dose of 180 mg per square meter, given as 90-min intravenous infusion. This treatment was immediately followed by 5FU at a dose of 400 mg per square meter, administered by intravenous bolus, followed by a continuous intravenous infusion of 2400 mg per square meter over a 46-h period. This sequence was repeated every 2 wk. For metastatic colorectal cancer, chemotherapy could be associated with targeted therapies such as bevacizumab or cetuximab. Dose reductions were based on adverse events that were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. Treatment was temporarily suspended in cases of grade 3/4 hematological toxicity or grade 2 or higher non-hematological toxicity. Once the toxicity level was reduced to grade 1 or below, chemotherapy was continued at a lower dose. The treatment was suspended if the patients experienced further toxicity. Dose re-escalation was not applied

in this setting. Treatment continued until disease progression, unacceptable toxicity, or patient refusal. As a reminder, FOLFIRINOX was not necessarily administrated as first line therapy for both types of cancer.

Pretreatment and follow-up evaluation

Pretreatment evaluation included comorbidities (heart/lung/liver), usual medications, ECOG performance status, home help, autonomy, metastatic status, metastatic site (liver/lung/peritoneum/other), tumor marker level (ACE, CA 19-9), albumin levels, the use of a targeted therapy (anti-VEGF/anti-EGFR). Tumor response was evaluated using RECIST criteria. Follow-up evaluation included tumor assessment by thorax abdominal and pelvic CT-Scan, tumor marker levels, loss of autonomy, toxicities. Toxicity was graded according to the CTCAE version 4.03. The loss of autonomy was defined by home helps or a convalescence.

Statistical analysis

All patients were followed up until death or the end of data recording (31 January 2015). The time under treatment was defined as the period between the first and last cure of FOLFIRINOX, without progression during this period. Progression free survival was calculated from the date when therapy started to the date of disease progression, and Overall Survival was calculated from the date when therapy started to the date of death. Median follow-up with its 95%CI was calculated using the reverse Kaplan-Meier method. Patient or disease characteristics were examined using the χ^2 test or Fisher's exact test for qualitative variables, and the Student *t* or Mann-Whitney tests for continuous variables, as appropriate. Survival probabilities were estimated using the Kaplan-Meier method and survival curves were compared using the log-rank test.

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, United States). All tests were two sided, and *P* values < 0.05 were considered statistically significant.

RESULTS

Patients characteristics

Between January 2009 and January 2015, a total of 52 patients aged from 70 to 87 years of age were treated at the Department of Medical Oncology, Georges-Francois Leclerc Cancer Center, Dijon, France by FOLFIRINOX, with or without targeted therapy associated, for pancreatic or colorectal cancer. The main demographic and baseline characteristics of patients involved in the study are shown in Table 1.

The average age was 75, with a median of 74 years of age. The majority of patients had a good general condition, 82.7% (*n* = 43) were 0-1 performance

Table 1 Patient characteristics *n* (%)

Characteristics	Patients
Sexe	
Male	34 (67.3)
Female	18 (32.7)
Age	
< 80	9 (17.0)
≥ 80	43 (83.0)
Tumor location	
Colorectal	34 (65.4)
Pancreas	18 (34.6)
Metastasis	
≤ 1	26 (50.0)
> 1	26 (50.0)
ECOG performance status	
[0-1]	43 (82.7)
[2-3]	9 (17.3)
Comorbidities	
No	15 (28.8)
Yes	37 (71.2)
Cardiac comorbidities	
No	40 (76.9)
Yes	12 (23.1)
Pulmonary comorbidities	
No	47 (90.4)
Yes	5 (9.6)
Hepatic comorbidities	
No	52 (100.0)
Yes	0 (0.0)
Albumin level	
< 30 g/L	16 (30.8)
≥ 30 g/L	21 (40.4)
Number of usual drugs	
< 3	22 (42.3)
≥ 4	30 (57.7)
Charlson comorbidities index	
< 10	32 (61.5)
≥ 10	20 (38.5)
Initial autonomy	
No	3 (5.8)
Yes	49 (94.2)

status and 94.2% (*n* = 49) had complete autonomy at home. Although 71.2% of patients had comorbidities, the majority of them did not concern vital functions (heart, lung and liver). The Charlson Comorbidity Index (CCI) for general people with a metastatic tumor and without comorbidities is 9 for people aged 70 to 79 years, and 10 for those aged 80 to 89 years. Thirty-two patients (61.5%) had a CCI < 10. The nutritional assessment showed an upper rate of albumin in 30 g/L for 40.4% of patients.

All 52 patients were assessable for toxicity, survival and radiological response using RECIST criteria.

Toxicity and feasibility

A total of 311 cycles of chemotherapy were administered (median 4.5; range: 1-20). Hematological and non hematological toxicities are listed in Table 2. Any grade 3 or 4 toxicity according to the CTCAE 4.03 was considered severe.

Regarding all toxicity grades more than 1/3 the patients suffered from asthenia (94.2%), diarrhea

Table 2 Observed toxicity according Common Terminology Criteria for Adverse Events v 4.03 (*n* = 52) *n* (%)

	CTCAE v 4.03	
	All grades	Severe ¹
Hematological		
Anemia	28 (53.8)	5 (9.6)
Neutropenia	24 (46.2)	17 (32.7)
Thrombocytopenia	14 (26.9)	3 (5.8)
Non Hematological		
Diarrhea	35 (67.3)	13 (25.0)
Nausea and vomiting	22 (42.3)	5 (9.6)
Asthenia	49 (94.2)	5 (9.6)
Peripheral Neutropenia	17 (32.7)	4 (7.7)
Hepatic toxicity	4 (7.7)	1 (1.9)

¹Grade 3-4 according to the CTCAE version 4.03. CTCAE: Common Terminology Criteria for Adverse Events.

(67.3%), anemia (52.8%), neutropenia (46.2%) and nausea/vomiting (42.3%).

When focusing on severe sides effects, neutropenia (32.7, *n* = 24) and diarrhea (25%, *n* = 13) were the most frequent (Table 2).

Concerning treatment administration, initially, a majority of patients had a reduced dose (75%, *n* = 39), particularly for irinotecan (67.3%, *n* = 35), bolus of 5FU (25%) and continuous infusion of 5FU (21.1%). Only 7 patients had a dose reduction of oxaliplatin. During treatment, 26.9% of patients had a dose adjustment (*n* = 14). The treatment was stopped for 20 patients (38.5%) because of an excessive toxicity and for 15 patients (28.8%) due to disease progression. Almost, 25% of patients could benefited from a maintenance therapy (*n* = 13) after a response or a stabilization of the disease. Most patients died from cancer, and 5 patients are still alive (only patients with colorectal cancer, including 2 with FOLFIRINOX as a neo adjuvant treatment).

Objective tumor response and survival

The assessment of the best tumor response according to RECIST criteria, showed a progression for 21.1% of patients (*n* = 11), a stable disease for 25% (*n* = 13), a partial response for 23.1% (*n* = 12). Only one patient presented a complete response. Accordingly, the objective response rate was 25% and the disease control rate was 50%. A total of 16 patients couldn't be evaluated because of an early clinical progression or death.

The median time under treatment was 2.62 mo for colorectal cancer and 2.24 mo for pancreatic cancer (Figure 1).

Overall survival was 43.38 mo (29.64-47.87) for patients with colorectal cancer and 12.51 mo (8.85-17.2) for pancreatic cancer (Figure 2).

In univariate and multivariate analysis, none of geriatric parameters were linked to overall survival or time under treatment (age, comorbidities, autonomy, ECOG performance status, CCI and medication

Table 3 Univariate and multivariate analysis by subgroup for time under treatment

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P vaule	HR	95%CI	P vaule
Tumor location			0.1204			0.4288
CR	Ref			Ref		
Pancréas	1.615	0.882 2.958		1.316	0.667 2.599	
Metastatic sites			0.0478			0.1405
0-1	Ref			Ref		
≥ 2	0.56	0.315 0.994		0.613	0.32 1.175	
Age			0.2105			0.1637
< 75	Ref			Ref		
≥ 75	0.692	0.389 1.231		0.664	0.373 1.182	
Age			0.371			
< 80	Ref					
≥ 80	1.394	0.673 2.889				
Comorbidities			0.979			
No	Ref					
Yes	1.008	0.549 1.851				
Neoadjuvant chemotherapy			0.2339			
No	Ref					
Yes	2.432	0.563 10.5				
Initial autonomy (J0)			0.9059			
No	Ref					
Yes	0.932	0.288 3.018				
Charlson comorbidities index			0.7659			
< 10	Ref					
≥ 10	0.915	0.511 1.639				
ECOG performance status			0.5046			
0-1	Ref					
≥ 2	1.281	0.618 2.655				
Sexe			0.7228			
Male	Ref					
Female	0.856	0.361 2.026				
Number of usual drugs			0.3057			
< 4	Ref					
≥ 4	1.34	0.765 2.347				

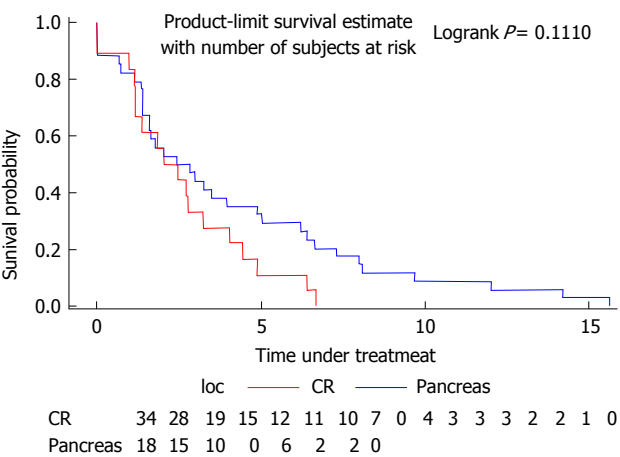


Figure 1 Time under treatment for pancreatic and colorectal cancer.

number). Only the type of tumor (pancreas or colorectal) was linked to overall survival in univariate and multivariate analysis, obviously in favor of colorectal cancer (Tables 3 and 4).

DISCUSSION

This retrospective study showed that polychemotherapies considered toxic, like FOLFIRINOX, is feasible

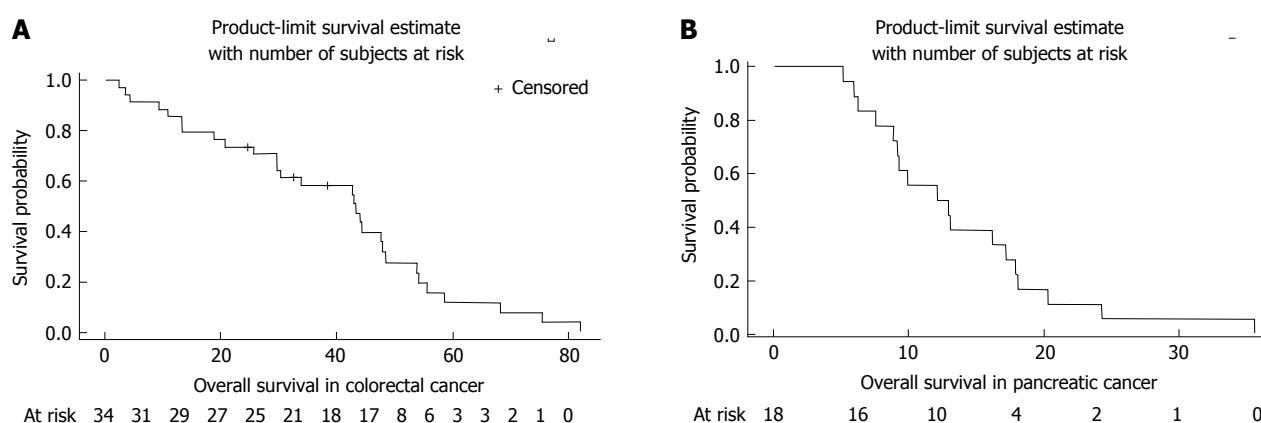
in a geriatric population with a good general condition, probably thanks to an initial dose adaptation. Age alone should not be a limiting factor for using this type of treatment. Elderly patients require a global geriatric assessment.

Our study population was healthier than a standard geriatric population with metastatic cancer. Indeed 82.7% of patients had a 0-1 performance status and 61.5% had a CCI < 10 in our study. However, for ethical reasons, a triple-drug combination cannot be given to an unhealthy patient with a performance status > 2.

A phase 2-3 French trial that compared gemcitabine to FOLFIRINOX for metastatic pancreatic cancer, had already demonstrated the efficiency of this combination^[3]. But the patients in the study were younger and had a good performance status. In this trial, overall survival was 11.1 months in FOLFIRINOX group (95%CI: 9.0-13.1). This results are comparable to the results of our study with an overall survival of 12.51 mo. Another phase 3 trial, this time comparing gemcitabine to gemcitabine plus nab-paclitaxel in pancreatic cancer, showed a median of overall survival of 8.5 mo (95%CI: 7.9-9.5) for nab-paclitaxel group, which was better than the gemcitabine alone group, knowing that the median age in this group was

Table 4 Univariate and multivariate analysis by subgroup for overall survival

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Tumor location			< 10 ⁻⁴			< 10 ⁻⁴
CR	Ref			Ref		
Pancréas	6.362	2.963	13.66	5.816	2.47	13.693
Metastatic sites			0.0634			0.9874
0-1	Ref			Ref		
≥ 2	0.555	0.298	1.034	1.006	0.498	2.032
Age			0.5721			0.7614
< 75	Ref			Ref		
≥ 75	1.184	0.659	2.124	1.096	0.607	1.976
Age			0.5365			0.274
< 80	Ref			Ref		
≥ 80	1.277	0.588	2.774	1.611	0.686	3.786
Sexe			0.1178			0.4136
Male	Ref			Ref		
Female	0.464	0.177	1.215	0.634	0.212	1.891
Comorbidities			0.0899			0.8701
No	Ref			Ref		
Yes	1.787	0.914	3.495	1.066	0.494	2.301
Initial autonomy			0.7372			
No	Ref					
Yes	1.277	0.306	5.336			
Charlson comorbidities index			0.7507			
< 10	Ref					
≥ 10	1.101	0.608	1.992			
ECOG performance status			0.6426			
0-1	Ref					
≥ 2	0.833	0.385	1.802			
Number of usual drugs			0.6116			
< 4	Ref					
≥ 4	1.169	0.64	2.132			

**Figure 2** Overall survival for colorectal cancer (A) and pancreatic cancer (B).

62^[28]. These results are in favor of administrating FOLFIRINOX in pancreatic cancer, even for elderly patients. Concerning colorectal cancer, we could observe a median overall survival of 43.38 mo (95%CI: 29.64-47.87), which is clearly higher than in other trials, involving younger patients^[4-6,29,30]. ASCO 2016 presented the first results of the phase 2 multicentric french trial METHEP-2, which tends to confirm the superiority of FOLFIRINOX associated to biotherapies versus a bi-chemotherapy for colorectal cancer with initially unresectable liver metastases^[7]. Median overall survival was of 36 mo for bi-chemotherapy, and

hasn't been reached for FOLFIRINOX group. Time under treatment for colorectal cancer in our study was low (2.62 mo), contrasting with high overall survival rates. We could suspect that either colorectal cancer progresses slowly or that tumor response obtained with FOLFIRINOX induces a prolonged control of the disease.

In important trials^[4-6,31,32], most common severe toxicities (grade ≥ 3) with this chemotherapy were neutropenia and diarrhea. In our geriatric population, severe neutropenia rates were similar to the rates observed in the literature data for younger patients,

with approximatively a third of patients who underwent neutropenia (32.7%). Seventy-five percent of patients had a reduction in the dosage of chemotherapy from the start: 67.3% for irinotecan and 25% for bolus of 5FU. It is thanks to this dose reduction that our geriatric population did not suffer more of neutropenia than a younger population. Systematically using GCSF, could clearly reduce the proportion of severe neutropenia rates. The only severe toxicity that was more frequent than in other studies, was diarrhea. Indeed 25% of our population ($n = 13$) was concerned. Systematically prescribing antidiarrheal treatments should be recommended.

Our study limits were the retrospective and monocentric plan. However, there was little missing data for patients. Also, we could consider that 52 patients is a poor recruitment, but regarding the characteristics of the population we studied (the elderly people with pancreatic/colorectal cancer, able to receive FOLFIRINOX), these numbers are clearly acceptable. Finally, carrying out a study including both pancreatic and colorectal cancer, which are two different types of cancer when it comes to evolution and prognosis, can be questionable at first. But the primary objective of our study was to evaluate the feasibility of FOLFIRINOX in an elderly population, whatever the primary tumor.

In conclusion, FOLFIRINOX is a triple-drug combination feasible in geriatric population, over 70 years of age, with manageable toxicity levels and interesting rates of overall survival, especially for colorectal cancer, compared to a younger population. Currently, a phase 2 French trial, PAMELA-70, is trying to confirm these results by evaluating the efficiency and tolerance of dose adjusted FOLFIRINOX in elderly patients with a metastatic pancreatic cancer^[33].

COMMENTS

Background

FOLFIRINOX regimen is an effective chemotherapy for locally advanced or metastatic pancreatic and colorectal cancer. However, data are lacking concerning the use of this chemotherapy for elderly people.

Research frontiers

Most studies demonstrating the efficacy of FOLFIRINOX didn't include elderly patients, aged more than 70 or 75 years old. We treated patients aged more than 70 years old, usually with an initial dose adaptation.

Innovations and breakthroughs

The median overall survival was 43.88 mo for colorectal cancer and 12.51 mo for pancreatic cancer in this geriatric population, similar, even higher, compared to younger people. Only a third (32.7%) had severe neutropenia and a quarter had severe diarrhea.

Applications

This study submit the possibility of using FOLFIRINOX for elderly people aged more than 70 years old by paying attention to main severe toxicities, as neutropenia and diarrhea.

Terminology

FOLFIRINOX regimen consisted of oxaliplatin at a dose of 85 mg per square meter, given as a 2-h intravenous infusion, immediately followed by leucovorin at a dose of 400 mg per square meter, given as a 2-h intravenous infusion, with the addition, after 30 min of irinotecan at a dose of 180 mg per square meter, given as 90-min intravenous infusion. This treatment was immediately followed by 5 fluorouracil at a dose of 400 mg per square meter, administered by intravenous bolus, followed by a continuous intravenous infusion of 2400 mg per square meter over 46-h period.

Peer-review

The manuscript by Guion-Dusserre and colleagues analyzes FOLFIRINOX in elderly patients with pancreatic (PDAC) and colorectal (CRC) cancer. This retrospective study included patients over 70 years and 52 patients were treated by FOLFIRINOX, 34 had CRC and 18 had PDAC. The authors show that FOLFIRINOX toxicities were manageable and that median survival rates were comparably good. This is a well written and clinically interesting and relevant study.

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Retrospective Study

Influence of night duty on endoscopic therapy for bile duct stones

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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 provide informed consent for this study because the analysis utilized 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.

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Abstract

AIM

To examine the influence of night duty (ND) on endoscopic therapy for biliary duct stones.

METHODS

The subjects consisted of 133 patients who received initial endoscopic therapy for biliary duct stones performed by eight endoscopists after they had been on (ND group, $n = 34$ patients) or not [day duty (DD) group, $n = 99$ patients]. Patient characteristics (age, gender, history of abdominal surgery, transverse diameter of the largest stone, number of stones), years of experience of the endoscopists, endoscopic procedures [sphincterotomy, papillary balloon dilation (EPBD), papillary large balloon dilation (EPLBD)], and outcomes of initial endoscopy (procedure time; rate of stone removal by the first endoscopist; procedure

success rate by the first endoscopist: removal of stones or endoscopic retrograde biliary drainage; rate of final stone removal; final procedure success rate; complications; hospitalization after the procedure) were compared retrospectively between the two groups. History of abdominal surgery and treatment outcomes were also compared between the groups for each of the four endoscopists who performed most of the procedures in the ND group.

RESULTS

There were no significant differences regarding the number of treatments performed by each endoscopist or the years of experience between the ND and DD groups. The frequency of endoscopic retrograde cholangiopancreatography procedures did not differ significantly between the groups. There were also no significant differences regarding patient characteristics: age, gender, history of abdominal surgery (ND 7: Billroth II 4, R-Y 3; DD 18: double tract reconstruction 1, Billroth I 3, Billroth II 6, R-Y 7, duodenoduodenostomy for annular pancreas 1), transverse diameter of largest stone, and number of stones between the two groups. Among the treatment procedures, the endoscopic sphincterotomy and EPBD rates did not differ significantly between the groups. However, EPLBD was performed more frequently in the ND group [47.1% (16/34) *vs* 19.2% (19/99)]. Regarding outcomes, there were no significant differences in the rate of stone removal, procedure success rate, complications (ND: pancreatitis 1; DD: pancreatitis 6, duodenal bleeding 1, decreased blood pressure 1, hypoxia 2), or hospitalization after the procedure. However, the procedure time was significantly longer in the ND group (71.5 ± 44.7 *vs* 54.2 ± 28.8). Among the four endoscopists, there were no significant differences in patient history of abdominal surgery, removal of stones, or procedure success rate. However, the procedure time for one endoscopist was significantly longer in the ND group.

CONCLUSION

The time required for endoscopic therapy for bile duct stones might be influenced by ND.

Key words: Night duty; Endoscopic therap; Bile duct stone; Removal of stones; Procedure time

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Core tip: Sleep deprivation affects brain activation, and therefore disturbs cognitive ability and reduces work efficiency. In the clinical field, night duty (ND) might affect surgical outcomes and the number of medical errors. In this study, we examined the influence of ND on endoscopic therapy for biliary duct stones. The procedure was significantly longer when performed by endoscopists after they had been on ND. The time required for endoscopic therapy for bile duct stones might be influenced by ND.

Sugimoto M, Takagi T, Suzuki R, Konno N, Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Hikichi T, Ohira H. Influence of night duty on endoscopic therapy for bile duct stones. *World J Gastroenterol* 2016; 22(42): 9387-9393 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9387.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9387>

INTRODUCTION

Sleep deprivation disturbs cognitive ability, and sleep is important to maintain concentration^[1]. Sleep deprivation also reduces work efficiency and changes brain activation^[2-7]. However, most reports in the clinical field indicate that sleeping hours or night duty (ND) do not influence surgery. In Ellman *et al*^[8] and Chu *et al*^[9], sleeping hours did not affect outcomes after cardiac surgery. In Sharpe *et al*^[10] complications and re-hospitalization 30 d after abdominal surgery (hernia repair, cholecystectomy, intestinal operations) did not differ significantly between doctors who were and were not on ND on the previous day. Most recently, Govindarajan *et al*^[11] reported that prior night work did not affect outcomes for gastroenterology surgeries, hysterectomy, orthopedic surgeries, lung resection, craniotomy, or angioplasty. However, in Rothschild *et al*^[12], complications were more frequent after surgery performed by surgeons with a prior sleep time of less than 6 h, and serious medical errors have been associated with interns working for more than 24 h^[13].

Endoscopic therapy requires considerable concentration, but the dependence of outcomes on sleep time or ND has not been examined. Therefore, in this study, we investigated the influence of ND on endoscopic therapy for bile duct stones.

MATERIALS AND METHODS

Study design

This study was performed as a retrospective analysis of clinical data. Written consent for endoscopic therapy was obtained from the patients. Informed consent for this study was not required. The ethics committee of Fukushima Medical University approved the study, and the details of the study are published on the homepage of Fukushima Medical University (authorization No. 2453).

Patients

Among 335 patients treated with endoscopic therapy for bile duct stones from January 2011 to December 2015 at Fukushima Medical University Hospital, 167 underwent initial endoscopic therapy and had stones confirmed *via* computed tomography or endoscopic retrograde cholangiography (Figure 1). Among these patients, data from 133 patients treated by eight endoscopists (A-H) were evaluated in this study. In

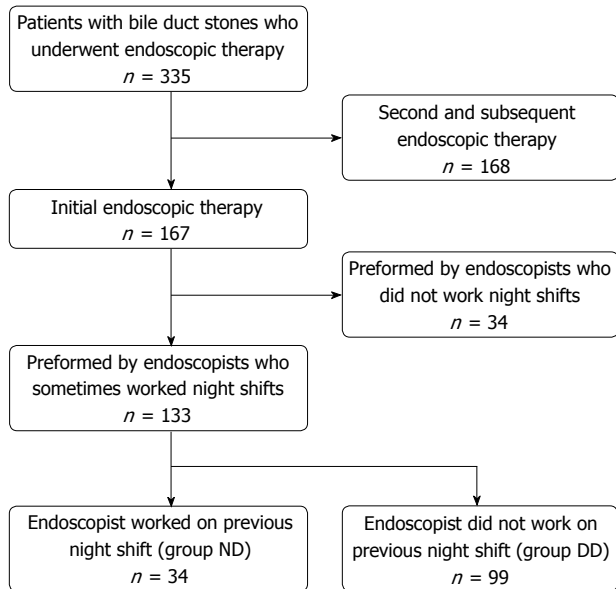


Figure 1 Disposition of patients in the study. Among 335 patients treated by endoscopic therapy for bile duct stones in five years at our hospital, 167 underwent initial endoscopic therapy. The data from 133 patients treated by eight endoscopists who sometimes worked night shifts were evaluated in this study. These 133 cases included 34 patients treated by endoscopists after they had been on night duty the previous day (ND group) and 99 treated by the same endoscopists when they had not been on night duty the previous day (DD group). ND: Night duty; DD: Day duty.

Table 1 Profile of endoscopists

Item	ND Group (n = 34)	DD Group (n = 99)	P value
Endoscopists (years of experience)			0.527
A (14-19)	5	11	
B (6-9)	7	17	
C (6-8)	8	21	
D (6-11)	5	16	
E (7-12)	2	22	
F (10-15)	3	7	
G (5-8)	3	4	
H (3-7)	1	1	
Years of experience, mean \pm SD	9.3 \pm 3.9	9.4 \pm 3.4	0.830

ND: Night duty; DD: Day duty.

total, 34 patients were treated by an endoscopist after they had been on ND the previous day (ND group), and 99 patients were treated by the same endoscopists when they had not been on ND the previous day (day duty, DD group) (Table 1). ND was defined as performing normal ward duties in the hospital overnight and sometimes examining emergency patients.

Methods

The following items were compared between the ND and DD groups: years of experience of the endoscopists, patient characteristics (age, gender, history of abdominal surgery, transverse diameter of the largest stone, number of stones), endoscopic procedure

[sphincterotomy (EST), papillary balloon dilation (EPBD), papillary large balloon dilation (EPLBD)], treatment outcomes (procedure time; removal of stones by the first endoscopist; procedure success rate by the first endoscopist: removal of stones or endoscopic retrograde biliary drainage; final rate of stone removal; final procedure success rate; complications; and hospitalization after procedure). The outcomes related to the second (or more) endoscopist were included in the study: rate of final stone removal and final procedure success rate. History of abdominal surgery and treatment outcomes (procedure time, removal of stones, procedure success rate) were also compared between the two groups for each of the four endoscopists (A-D) who treated many of the patients in the ND group. The main outcome was a comparison of treatment results to evaluate the work efficiency in the ND and DD groups.

Endoscopic retrograde cholangiopancreatography (ERCP)-related procedures were performed for patients with stable breathing and hemodynamics. Before ERCP, patients were sedated with midazolam under observation of blood pressure and oxygen saturation. However, patients in septic shock were not sedated. Most procedures were performed with a JF 260V endoscope (Olympus, Tokyo, Japan). A Q260J (Olympus) was used for double tract reconstruction in proximal gastrectomy, a PCF-Q260AI or Q260J (Olympus) was used for Billroth II (B-II) procedures, and a PCF-Q260AL (Olympus) was used for Roux-en-Y (R-Y). For R-Y after choledocojejunostomy, the bile duct-jejunum anastomosis was accessed with a SIF-Q260 (Olympus) and a sliding tube. The balloon of the sliding tube was expanded, fixed and placed, and endoscopy with a PCF-PQ260AI (Olympus) was then performed through the sliding tube.

EST was performed using Clever Cut (Olympus). In cases in which it was difficult to cannulate the biliary duct, or for patients with a history of abdominal surgery, an RX needle knife XL (Boston Scientific, Tokyo, Japan) was used. EPBD was performed if the transverse diameter of the largest stone was > 8 mm and bile duct stones were difficult to remove only by EST or if a perivaterian diverticulum was present. A Hurricane RX Biliary Balloon Dilation Catheter (Boston Scientific) was used for EPBD. EPLBD was performed if the transverse diameter of the largest stone was > 12 mm, if many bile duct stones were difficult to remove only by EST or if sufficient EST was difficult because of a parapapillary diverticulum or history of abdominal surgery. A CRE Biliary Balloon Dilation Catheter (Boston Scientific) or a Giga (Century Medical, Tokyo, Japan) was used for EPLBD. A Trapezoid RX basket catheter (Boston Scientific) and a LithoCrush V, FG-V435P (Olympus) were used as tools to crush stones.

Statistical analysis

Years of experience of endoscopists, patient age,

Table 2 Comparison of patient characteristics

	ND Group (<i>n</i> = 34)	DD Group (<i>n</i> = 99)	<i>P</i> value
Age (yr), mean ± SD	73.1 ± 13.0	72.4 ± 14.3	0.801
Gender (M/W)	21/13	59/40	0.824
History of abdominal surgery, <i>n</i> (%)	7 (20.6)	18 (18.2)	0.757
Double tract reconstruction, <i>n</i>		1	
Billroth I		3	
Billroth II	4	6	
Roux-en-Y	3	7	
Duodenoduodenostomy		1	
Transverse diameter of the largest stone (mm), mean ± SD	10.6 ± 4.6	10.3 ± 4.9	0.735
Number of stones, mean ± SD	2.8 ± 4.0	2.8 ± 3.6	1.0

ND: Night duty; DD: Day duty; Double tract reconstruction: Double tract reconstruction for proximal gastrectomy; Duodenoduodenostomy: Duodenoduodenostomy for annular pancreas.

Table 3 Comparison of endoscopic procedures, *n* (%)

	ND Group (<i>n</i> = 34)	DD Group (<i>n</i> = 99)	<i>P</i> value
EST	32 (94.1)	83 (83.8)	0.107
EPBD	0 (0)	6 (6.1)	0.163
EPLBD	16 (47.1)	19 (19.2)	0.001

ND: Night duty; DD: Day duty; EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilation; EPLBD: Endoscopic papillary large balloon dilation.

transverse diameter of the largest stone, number of stones, and hospitalization after the procedure were compared by Student's *t* tests. The number of procedures performed by each endoscopist in the ND and DD groups, patient gender, history of abdominal surgery, EPLBD, rate of stone removal by the first endoscopist, rate of final stone removal and procedure success rate by the first endoscopist were compared by the χ^2 test. Procedure time was compared with the Welch *t* test. EST, EPBD, complications, final procedure success rate and items for each endoscopist (history of abdominal surgery, removal of stones rate, procedure success rate) were compared by the Fisher exact probability test. The procedure time for each endoscopist was compared by the Mann-Whitney *U* test. *P* < 0.05 was considered to be significant. All analyses were performed using Statcel 3 (OMS Edition, Saitama, Japan).

RESULTS

There were no significant differences regarding the number of treatments performed by each endoscopist or in the years of experience between the ND and DD groups (Table 1). The frequency of ERCP did not differ significantly between the groups. There were

Table 4 Comparison of treatment outcomes *n* (%)

	ND Group (<i>n</i> = 34)	DD Group (<i>n</i> = 99)	<i>P</i> value
Procedure time (min), mean ± SD	71.5 ± 44.7	54.2 ± 28.8	0.043
Rate of stone removal by first endoscopist	13 (38.2)	52 (52.5)	0.150
Procedure success rate by first endoscopist	18 (52.9)	57 (57.6)	0.638
Rate of final stone removal	24 (70.6)	66 (66.7)	0.67
Final procedure success rate	33 (97.1)	90 (90.9)	0.22
Complications	1 (2.9)	11 (11.1)	0.136
Pancreatitis	1	6	
Duodenal bleeding		2	
Decreased blood pressure		1	
Hypoxia		2	
Hospitalization after procedure (d), mean ± SD	7.1 ± 7.6	6.6 ± 6.6	0.715

ND: Night duty; DD: Day duty; Procedure success rate: Removal of stones or biliary stenting.

also no significant differences in patient characteristics regarding age, gender, history of abdominal surgery (ND 7: Billroth II 4, R-Y 3; DD 18: double tract reconstruction 1, Billroth I 3, Billroth II 6, R-Y 7, duodenoduodenostomy for annular pancreas 1), transverse diameter of the largest stone, and number of stones between the two groups (Table 2).

Among the treatment procedures, the rates of EST and EPBD did not differ significantly between the groups, but EPLBD was performed more frequently in the ND group [47.1% (16/34) vs 19.2% (19/99)] (Table 3). Regarding outcomes, there were no significant differences in the rate of stone removal and procedure success rate, complications (ND: pancreatitis 1; DD: pancreatitis 6, duodenal bleeding 1, decreased blood pressure 1, hypoxia 2), or hospitalization after the procedure (Table 4). However, the procedure time was significantly longer in the ND group (71.5 ± 44.7 vs 54.2 ± 28.8).

For each of the four endoscopists A-D, there were no significant differences in patient history of abdominal surgery, rate of stone removal, and procedure success rate. However, the procedure time for endoscopist D was significantly longer in the ND group (Table 5).

DISCUSSION

In this report, we examined the influence of ND on endoscopic therapy for bile duct stones. The rate of EPLBD and the procedure time were significantly greater for endoscopists after ND. The procedure time was also longer in the ND group for one endoscopist.

Although more EPLBD procedures were performed in the ND group, the patients who met the criteria for EPLBD described in the Materials and Methods were not significantly different between the ND group and the DD group according to the results of χ^2 tests (Table

Table 5 Comparison of treatment outcomes for each endoscopist *n* (%)

	ND Group	DD Group	<i>P</i> value
Endoscopist A			
<i>n</i>	5	11	
Procedure time (min), median \pm SD	90.0 \pm 80.8	90.0 \pm 39.7	0.910
Rate of stone removal	3 (60.0)	6 (54.5)	0.635
Procedure success rate	5 (100)	9 (81.8)	0.458
Endoscopist B			
<i>n</i>	7	17	
Procedure time (min), median \pm SD	40.0 \pm 20.4	50.0 \pm 26.0	0.589
Rate of stone removal	5 (71.4)	11 (64.7)	0.572
Procedure success rate	5 (71.4)	12 (70.6)	0.607
Endoscopist C			
<i>n</i>	8	21	
Procedure time (min), median \pm SD	75.0 \pm 39.6	50.0 \pm 27.1	0.113
Rate of stone removal	3 (37.5)	7 (33.3)	0.745
Procedure success rate	4 (50.0)	10 (47.6)	0.617
Endoscopist D			
<i>n</i>	5	16	
Procedure time (min), median \pm SD	60 \pm 31.5	40 \pm 14.7	0.017
Rate of stone removal	1 (20)	8 (50)	0.258
Procedure success rate	1 (20)	10 (62.5)	0.126

ND: Night duty; DD: Day duty; Procedure success rate: Removal of stones or biliary stenting.

6). Fewer EPLBD procedures were performed in the DD group for several reasons. However, it has been shown that EPLBD shortens the procedure by allowing easier removal of stones or at least does not extend the procedure time^[14,15]. Based on these earlier reports, we suggest that EPLBD did not contribute to the longer procedure time in the ND group.

The cause of the longer procedure time for endoscopists after ND might be the influence of sleep deprivation or lower sleep quality on work efficiency. The attention, vigilance, and driving tasks of residents during heavy night call rotations were equivalent to those for residents with a 0.04 to 0.05 g % blood alcohol concentration during a light call rotation^[16]. Sanches *et al*^[17] also found that the psychomotor performance of young doctors on night shifts was lower than that of young doctors who were not assigned night work. Thus, ND may influence the procedure time of endoscopic therapy for bile duct stones.

In this study, there was no significant difference in complications between procedures performed by endoscopists who had and had not been on prior ND, but an extended procedure time has been reported to be a risk factor for post-ERCP pancreatitis^[18,19]. In addition, Pan *et al*^[20] found that a cannulation challenge to the common bile duct within 10 min gave the best results in trials by trainees. Therefore, if a procedure is slow by an endoscopist working after ND, it might be advisable to change the endoscopist.

There are several limitations to this study, including its retrospective design and the small number of cases

Table 6 Reasons for not performing endoscopic papillary large balloon dilation

	ND Group (<i>n</i> = 34)	DD Group (<i>n</i> = 99)	<i>P</i> value
Patients with EPLBD indication, <i>n</i> (%)	20 (58.8)	44 (44.4)	0.15
Patients in whom EPLBD was performed (shown in Table 3)	16	19	
Reasons for not performing EPLBD			
AOSC	1	5	
Narrow lower bile duct	1	1	
Biliary stricture	1	0	
Minor bleeding of Vater's papilla	1	0	
No insurance coverage for EPLBD	0	5	
96 years old and performance status 3	0	1	
Gallstone pancreatitis	0	3	
Antithrombotic drug therapy	0	4	
Difficulty identifying the biliary anastomotic region	0	2	
Smaller stones on visual inspection	0	2	
Difficulty identifying the Vater papilla	0	1	
Double tract reconstruction	0	1	

EPLBD: Endoscopic papillary large balloon dilation; AOSC: Acute obstructive suppurative cholangitis.

of ERCP for bile duct stones at a single institution. We perform 400 ERCP procedures each year. However, in this study, we only included patients treated by endoscopists who had been on ND and performed the initial endoscopic therapy for bile duct stones. This resulted in a small number of eligible subjects. However, restricting the endoscopic procedures to the treatment of bile duct stones allowed for a more precise evaluation of the influence of ND compared to previous studies that considered the effect of ND on multiple types of surgery^[8-12]. Secondly, we did not measure the exact sleep time of the endoscopists. However, night shifts and on-call duty have been found to influence circadian rhythm and worsen quality of sleep^[21,22]. A difference in bedding also influences quality of sleep^[23-26], and, therefore, ND itself is likely to influence quality of sleep. Thirdly, we were unable to compare the number of biliary duct cannulation challenges between the two groups to evaluate the direct influence of ND on the endoscopic procedure due to a lack of precise records. A further study of the number of biliary duct cannulations is desirable.

Within these limitations, we conclude that the procedure time for endoscopic therapy for bile duct stones is increased by the influence of ND. Substitution of an endoscopist after ND might be advisable to shorten the procedure.

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COMMENTS

Background

Concentration was affected by sleep deprivation. Recently, sleep deprivation or night duty (ND) was reported to influence several medical activities. However, the relationship between endoscopic therapy and sleep deprivation or ND was uncertain.

Research frontiers

Endoscopic therapy for bile duct stones requires much concentration. In Japan, many endoscopists perform endoscopic therapy after ND. This study clarified the influence of ND on endoscopic therapy for bile duct stones.

Innovations and breakthroughs

There have been no reports about the influence of ND on endoscopic therapy. In this report, ND the previous day influenced the procedure time for endoscopic therapy for bile duct stones.

Applications

This study suggested that ND the previous day influenced the procedure time of endoscopic therapy for bile duct stones. According to this result, if the first endoscopist who was on ND the previous day experiences difficulty in the endoscopic procedure, it is advisable to change endoscopists earlier.

Terminology

ERCP: endoscopic procedure that contrasts biliary duct and pancreatic duct using X-ray equipment; EST: endoscopic procedure that incises the papilla of Vater; EPBD: endoscopic procedure that dilates the biliary exit to remove the stones; EPLBD: endoscopic procedure that dilates the biliary exit using a large balloon catheter to remove large stones or many stones.

Peer-review

This study is innovative, and the conclusion is instructive and practical for endoscopic management of bile duct stones.

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Retrospective Study

Irritable bowel syndrome evaluation using computed tomography colonography

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Abstract

AIM

To evaluate the morphology of the colon in patients with irritable bowel syndrome (IBS) by using computed tomography colonography (CTC).

METHODS

Twelve patients with diarrhea type IBS (IBS-D), 13 patients with constipation type IBS (IBS-C), 12 patients with functional constipation (FC) and 14 control patients underwent colonoscopy following CTC. The lengths of the rectosigmoid colon, transverse colon and the total colon were measured. The diameters of the rectum, sigmoid colon, descending colon, transverse colon, and ascending colon were measured.

RESULTS

The mean length of the total colon was 156.5 cm in group C, 158.9 cm in group IBS-D, 172.0 cm in group IBS-C, and 188.8 cm in group FC. The total colon in group FC was significantly longer than that in group C ($P < 0.05$). The mean length of the rectosigmoid colon was 56.2 cm, 55.9 cm, 63.6 cm, and 77.4 cm (NS). The mean length of the transverse colon was 49.9 cm, 43.1 cm, 57.0 cm, and 55.0 cm. The transverse colon

in group IBS-D was significantly shorter than that in group IBS-C ($P < 0.01$) and that in group FC ($P = 0.02$). The mean diameter of the sigmoid colon was 4.0 cm, 3.3 cm, 4.2 cm, and 4.3 cm (NS). The mean diameter of the descending colon was 3.6 cm, 3.1 cm, 3.8 cm, and 4.3 cm. The descending colon diameter in group IBS-D was significantly less than that in group IBS-C ($P = 0.03$) and that in group FC ($P < 0.001$). The descending colon diameter in group FC was significantly greater than that in group C ($P = 0.04$). The mean diameter of the transverse colon was 4.4 cm, 3.3 cm, 4.2 cm, and 5.0 cm (NS).

CONCLUSION

CT colonography might contribute the clarification of subtypes of IBS.

Key words: Constipation; Irritable bowel syndrome; Computed tomography colonography

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Core tip: We report the morphology difference between diarrhea type IBS (IBS-D) and constipation type IBS (IBS-C). 12 patients with IBS-D, 13 patients with IBS-C, 12 patients with functional constipation (FC) and 14 control patients underwent colonoscopy following computed tomography colonography (CTC). The lengths and the diameters of the colon were measured. The rectosigmoid colon and transverse colon in IBS-D are shorter than that in IBS-C and FC. The sigmoid colon and descending colon in IBS-D has a diameter smaller than that in IBS-C and FC. The colonic morphology in IBS-D might be different from that in IBS-C and FC. CTC might contribute the clarification of IBS.

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INTRODUCTION

Irritable bowel syndrome (IBS), one of the most common gastrointestinal (GI) disorders in the world, is characterized by abdominal pain, discomfort, bloating, and disturbed defecation^[1,2]. It has four types: IBS with diarrhea (IBS-D), IBS with pain or discomfort and predominant constipation (IBS-C), mixed IBS (IBS-M), and unsubtyped IBS (IBS-U). IBS is very common, with a prevalence of 10%-20% in the world^[3]. In Japan, there are approximately 6%-17% of patients with IBS^[4].

Because IBS lacks characteristic imaging features

and has no diagnostic biomarkers, symptom-based criteria (Rome III) are recommended for its diagnosis^[1,2]. IBS is a prototypic functional GI disorder generally accompanied by visceral hypersensitivity, increased gut reactivity, and altered central processing in response to various stressors^[5,6]. Its features are affected by psychosocial stress, infection, gut microbiota and by the patient's genetics, gender, age, society, culture and perspective^[7-11].

Computed tomographic colonography (CTC), similar to colonoscopy but less invasive, is an examination procedure developed for detecting colonic adenomatous lesions^[12-14]. Both colonoscopy and CTC examine the full length of the colon, and their use in screening would be expected to result in colorectal cancer (CRC)-related mortality rates lower than those obtained using sigmoidoscopy or stool-based tests. For CRC and large precursor adenomas (≥ 10 mm), the sensitivity of CTC is comparable with that of colonoscopy. Not only colonic adenomatous lesions but also colonic morphology is able to be examined by using CTC. However, there is a paucity of information regarding the appropriate use of abdominal imaging in patients with IBS, and few studies have investigated typical diagnostic yields in them^[15]. Moreover, the diagnostic threshold of each IBS subtype has not been reported before.

The aim of this study was to retrospectively evaluate the GI tract morphology revealed using CTC in patients with IBS.

MATERIALS AND METHODS

Twelve patients with IBS-D (group IBS-D), 13 patients with IBS-C (group IBS-C), 13 patients with functional constipation (group FC), and 14 patients with colonic polyps and without abnormal defecation (control group C) were enrolled in this study at Saitama Medical University from May 2012 to February 2016. Patients underwent CTC soon after colonoscopy.

Preexamination preparation included a clear-liquid diet the day prior to examination and 75 mg of Laxoberon after dinner of the day prior to examination. In addition to fasting (12 h) before colonoscopy, patients underwent a standardized bowel preparation protocol using 244 g of Moviprep or 137 g of Niflec (EA Paharma Co., Japan). During the examination the patients first underwent colonoscopy with CO₂ insufflation (PCFQ260, PCFQ260AZ, or PCFH290, Olympus Medical Science Corp., Japan) using intramuscular injection of 20 mg butylscopolamine or 1 mg of glucagon. Colonoscopy was performed by three endoscopists, and as much intracolonic fluid as possible was suctioned during withdrawal of the colonoscope. Next, soon after colonoscopy, patients underwent CTC. The CTC examination entailed insertion of a small flexible rectal catheter with colonic distension produced by an automated CO₂ insufflator (20 mmHg, PROTOCO2L, Eidia Co., Japan) immediately before the scan. Single-breath-hold multidetector supine and

Table 1 Clinical characteristics of patients

	Group C	Group IBS-D	Group IBS-C	Group FC
Male/female	6/8	10/2	6/7	7/5
Mean age	64	60	61	70

IBS-D: Diarrhea type IBS; IBS-C: Constipation type IBS; FC: Functional constipation; IBS: Irritable bowel syndrome.

prone CT images were obtained using a 128-channel scanner (Siemens SOMATOM Definition Flash; Siemens Healthineers, Japan). Each image was acquired using 0.75-mm slice collimation, 1-mm reconstruction slice thickness and reconstruction increment, 120 kVp and 80 mAs after the subject had received an intramuscular injection of 20 mg butylscopolamine.

The lengths of the entire colon, rectosigmoid colon and transverse colon were measured, as the diameters of the rectum, sigmoid colon, descending colon, transverse colon and ascending colon were measured.

The study protocol was in accordance with the tenets of the revised Declaration of Helsinki (1989) and was approved by the institutional review board at our institutions. Written informed consent was obtained from all the patients.

Statistical analysis

The statistical significance of length and diameter differences was evaluated, by analysis of variance with Scheffe's method of multiple comparison, using SPSS software, version 17 (SPSS Inc., Chicago, IL). All probability values calculated in this analysis were one-sided sided, and $P < 0.05$ was considered significant.

RESULTS

Six male and 8 female patients were enrolled in group C, 10 male and 2 female patients were enrolled in group IBS-D, 6 male and 7 female patients were enrolled in group IBS-C, and 7 male and 5 female patients were enrolled in group FC. The mean age was 64 in group C, 60 in group IBS-D, 61 in group IBS-C and 70 in group FC (Table 1). Examples of CTC findings in IBS-D, IBS-C, and FC patients are shown in Figure 1.

The mean length of the total colon was 156.5 cm in group C, 158.9 cm in group IBS-D, 172.0 cm in group IBS-C, and 188.8 cm in group FC (Figure 2A). The total colon in group FC was significantly longer than that in group C ($P < 0.05$), and the total colon in group FC tended to be longer than that in group IBS-D ($P = 0.07$). The mean length of the rectosigmoid colon was 56.2 cm in group C, 55.9 cm in group IBS-D, 63.6 cm in group IBS-C, and 77.4 cm in group FC (Figure 2B). The rectosigmoid colon length in group FC tended to be greater than that in group C ($P = 0.08$) and that in group IBS-D ($P = 0.07$). The mean length of the transverse colon was 49.9 cm in group C, 43.1 cm in group IBS-D, 57.0 cm in group IBS-C, and 55.0 cm in group FC (Figure 2C). The transverse colon in group

IBS-D was significantly shorter than that in group IBS-C ($P < 0.01$) and that in group FC ($P = 0.02$).

The mean diameter of the rectum was 6.0 cm in group C, 5.4 cm in group IBS-D, 5.8 cm in group IBS-C, and 6.0 cm in group FC. There was no significant difference between the rectum diameters in any two of these groups. The mean diameter of the sigmoid colon was 4.0 cm in group C, 3.3 cm in group IBS-D, 4.2 cm in group IBS-C, and 4.3 cm in group FC (Figure 3A). The sigmoid colon diameter in group IBS-D tended to be less than that in group IBS-C ($P = 0.13$) and that in group FC ($P = 0.07$). The mean diameter of the descending colon was 3.6 cm in group C, 3.1 cm in group IBS-D, 3.8 cm in group IBS-C, and 4.3 cm in group FC (Figure 3B). The descending colon diameter in group IBS-D was significantly less than that in group IBS-C ($P = 0.03$) and that in group FC ($P < 0.001$). The descending colon diameter in group FC was significantly greater than that in group C ($P = 0.04$). The mean diameter of the transverse colon was 4.4 cm in group C, 3.3 cm in group IBS-D, 4.2 cm in group IBS-C, and 5.0 cm in group FC (Figure 3C). The transverse colon diameter in group IBS-D tended to be less than that in group FC ($P = 0.08$). The mean diameter of the ascending colon was 5.4 cm in group C, 5.8 cm in group IBS-D, 5.7 cm in group IBS-C, and 6.0 cm in group FC. None of these diameters differed significantly from any of the others.

DISCUSSION

IBS is a disease based on symptoms: abdominal pain, discomfort and abnormal defecation. IBS is not associated with serious GI diseases such as inflammatory bowel disease, infectious enterocolitis, diverticulitis, and colonic cancer. Ba enema or CTC shows no remarkable findings. Therefore, The Rome criteria were developed as a method to diagnose functional digestive disorders including IBS of FC without the need to subject the patients to invasive, expensive tests or procedures^[1]. The diagnosis of IBS is subtyped by the predominant stool pattern: IBS-D, IBS-C, IBS-M, or IBS-U. If a patient meets criteria for IBS-C or D or FC, they should not require colonoscopy or CTC to evaluate the colonic morphology or mucosa. However, several factors are related to IBS - such as psychosocial stress, infection, gut microbiota^[7-11] but it is not certain that the causes of each subtype are the same and that each subtype is the same disease. Few studies have investigated typical diagnostic yields using abdominal imagings in IBS patients^[15]. In this study the morphology of the colon was examined in IBS-D patients and IBS-C patients, FC patients and control patients. The transverse colon in IBS-D patients was significantly shorter than that in IBS-C patients and FC patients, and the length of the rectosigmoid colon in IBS-D patients tended to be less than that of the rectosigmoid colon in IBS-C patients and FC patients. The diameter of the descending colon in IBS-D patients was significantly smaller than that

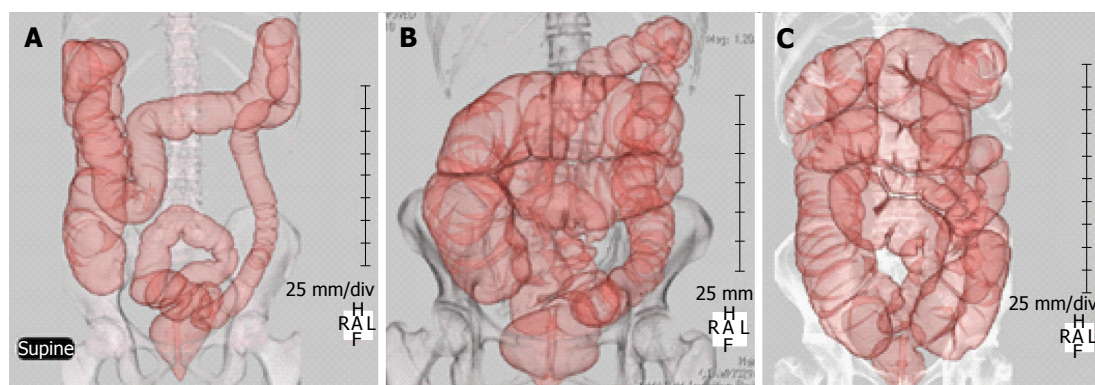


Figure 1 Computed tomography colonography. A: Typical diarrhea type IBS; B: Typical constipation type IBS; C: Typical functional constipation; IBS: Irritable bowel syndrome.

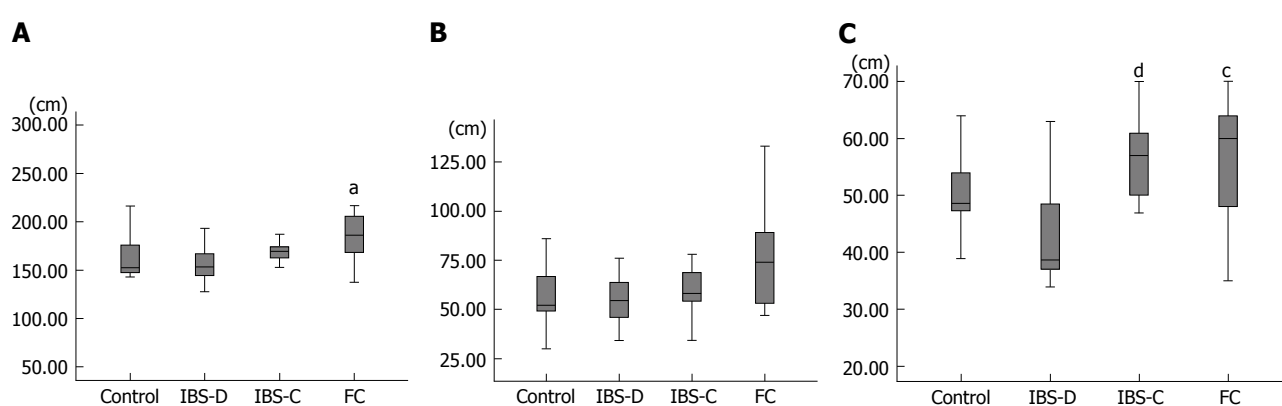


Figure 2 Length. A: total colon, ^a $P < 0.05$ vs control; B: Rectosigmoid colon; C: Transverse colon, ^c $P < 0.05$ vs IBS-D, ^d $P < 0.01$ vs IBS-D. IBS-D: Diarrhea type IBS; IBS-C: Constipation type IBS; FC: Functional constipation; IBS: Irritable bowel syndrome.

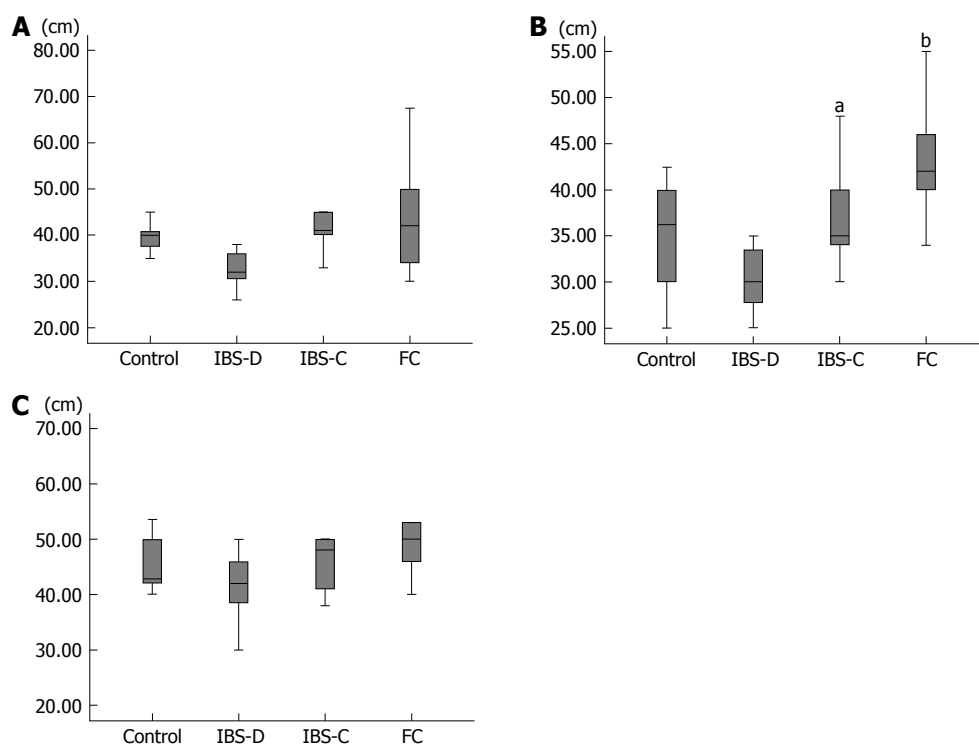


Figure 3 Diameter. A: Sigmoid colon; B: Descending colon, ^a $P < 0.05$ vs IBS-D, ^b $P < 0.001$ vs IBS-D; C: Transverse colon. IBS-D: Diarrhea type IBS; IBS-C: Constipation type IBS; FC: Functional constipation; IBS: Irritable bowel syndrome.

of the descending colon in IBS-C and FC patients. The diameter of the sigmoid colon in IBS-D patients tended to be smaller than that of the descending colon in IBS-C and FC patients. According to these data, the rectosigmoid colon and transverse colon in IBS-D patients are shorter than that in IBS-C patients and FC patients and the sigmoid colon and descending colon in IBS-D patients has a diameter smaller than that in IBS-C patients and FC patients. The colon morphology in IBS-D might be different from that in IBS-C and FC. On the other hand, neither the length nor diameter of the colon in IBS-C patients differs significantly from that of the colon in FC patients. Therefore, it is supposed that IBS-C and FC are both characterized by a longer and thicker colon.

Many IBS patients have a lowered threshold to pain or discomfort during the rectosigmoid distention^[16]. IBS patients experience as painful, rectal sensations that healthy people would regard as nonpainful: this is thought to be part of their hypersensitivity to bodily sensations. A lower tolerance of rectal distension would be expected to be associated with numerous bodily symptoms and more general psychological distress. In our data, IBS-D patients had smaller diameter in the sigmoid colon and descending colon and had a shorter rectosigmoid colon, which might mean they had a lower threshold for pain or discomfort occurring with rectosigmoid distention. However, IBS-C patients also have a lowered threshold for pain or discomfort occurring during rectosigmoid distention, and in this respect they are different from FC patients. The threshold for pain or discomfort during rectosigmoid distention in IBS-D patients might be lower than that in IBS-C patients, but data needed for comparing between IBS-D and IBS-C with regard to the threshold for pain or discomfort during rectosigmoid distention has not been reported yet.

The study by Heredia *et al.*^[17] showed that elongation of colon longitudinal muscle results in slow colonic transit in mice and presents a new mechanism of association of elongated colon and poor motility. Colonic elongation was reported as a possible underlying cause for slow colonic transit as observed with experimental stretching of the colon. Their study showed that elongation of the longitudinal muscle triggers inhibition of the colonic migrating motor complex (CMMC), resulting in slow colonic transit. Heredia *et al.*^[18] also reported in animal study that partial outlet obstruction caused an elongated impacted large bowel, slowed transit and CMMC. In human, slow colonic transit occurs in patients with chronic constipation and is known as slow transit constipation (STC)^[19]. Southwell reported that many patients with STC have an elongated transverse colon and elongated colon often occurs in patients with constipation^[20]. Yik *et al.*^[21] reported that transverse colon elongation is more common whereas sigmoid colon elongation is not more common in anorectal retention and colonic elongation may be the cause or the result of the underlying slow transit.

Mizukami *et al.*^[22-24] reported that abnormal colon morphology is common in IBS patients, and it seems to cause disorders related to defecation. Bowel morphology might be a potentially influential factor on GI symptoms. In our study, the GI morphology of IBS-D is different from that in IBS-C. IBS-D and IBS-C are classified as the same disease symptomatically (abdominal pain with abnormal bowel movement), but, pathophysiologic findings in IBS-D might be different from that in IBS-C. The morphology difference between IBS-D and IBS-C might be one of several causes of IBS. On the other hand, it is also supposed to have arisen from just the results affected by IBS symptoms.

There were several limitations in this study. Two are that it was a retrospective study and the sample size was small. We need to accumulate more clinical data in a prospective study, and a multicenter trial is necessary. Also needs is a control group without colonic polyps and abnormal bowel movements. In our study, colonic morphology in IBS-D and IBS-C were evaluated. The evaluation of colonic morphology not only in IBS-D and IBS-C but also in IBS-M and IBS-U is necessary. The threshold for pain or discomfort during rectosigmoid distention will to be measured in IBS-D patients and IBS-C patients in order to clarify the difference of pathophysiology.

In conclusion, CT colonography might contribute the clarification of subtypes of IBS according to the different morphological findings.

COMMENTS

Background

There is a paucity of information regarding the appropriate use of abdominal imaging in patients with irritable bowel syndrome (IBS). The diagnostic threshold of each IBS subtype has not been reported before.

Research frontiers

The gastrointestinal (GI) tract morphology revealed using computed tomographic colonography (CTC) in patients with IBS has not been reported.

Innovations and breakthroughs

The colonic morphology in diarrhea type IBS (IBS-D) might be different from that in constipation type IBS (IBS-C) and functional constipation (FC).

Applications

CTC might contribute the clarification of IBS.

Peer-review

The authors evaluated the GI tract morphology revealed using CTC in patients with IBS-D, IBS-C and FC. The colonic morphology in IBS-D might be different from that in IBS-C and FC. Further clinical trials in a prospective study and a multicenter trial will be necessary.

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Retrospective Study

Lymphovascular invasion in more than one-quarter of small rectal neuroendocrine tumors

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Author contributions: Kwon MJ evaluated pathologic findings and drafted the manuscript; Kwon MJ and Kang HS contributed equally to this work; Kang HS designed the study, analyzed the data and drafted the manuscript; Soh JS and Lim H revised the manuscript for important intellectual content; Kim JH and Park CK supervised the study; Park HR and Nam ES evaluated and supervised pathologic findings; all authors have read and approved the final version to be published.

Institutional review board statement: This study was conducted with the approval of the ethics committee of Hallym University Sacred Heart Hospital in Anyang, Korea, IRB No. 2016- I093.

Informed consent statement: Patients were not required to give informed consent to the study because only pathologic reevaluation and medical records were used in this retrospective study.

Conflict-of-interest statement: The authors declare no conflict of interest.

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Abstract

AIM

To identify the frequency, clinicopathological risk factors, and prognostic significance of lymphovascular invasion (LVI) in endoscopically resected small rectal neuroendocrine tumors (NETs).

METHODS

Between June 2005 and December 2015, 104 cases of endoscopically resected small (≤ 1 cm) rectal NET specimens at Hallym University Sacred Heart Hospital in Korea were retrospectively evaluated. We compared the detected rate of LVI in small rectal NET specimens by two methods: hematoxylin and eosin (H&E) and ancillary immunohistochemical staining (D2-40 and Elastica van Gieson); in addition, LVI detection rate

difference between endoscopic procedures were also evaluated. Patient characteristics, prognosis and endoscopic resection results were reviewed by medical charts.

RESULTS

We observed LVI rates of 25.0% and 27.9% through H&E and ancillary immunohistochemical staining. The concordance rate between H&E and ancillary studies was 81.7% for detection of LVI, which showed statistically strong agreement between two methods ($\kappa = 0.531$, $P < 0.001$). Two endoscopic methods were studied, including endoscopic submucosal resection with a ligation device and endoscopic submucosal dissection, and no statistically significant difference in the LVI detection rate was detected between the two (26.3% and 26.8%, $P = 0.955$). LVI was associated with large tumor size (> 5 mm, $P = 0.007$), tumor grade 2 ($P = 0.006$). Among those factors, tumor grade 2 was the only independent predictive factor for the presence of LVI (HR = 4.195, 95%CI: 1.321-12.692, $P = 0.015$). No recurrence was observed over 28.8 mo regardless of the presence of LVI.

CONCLUSION

LVI may be present in a high percentage of small rectal NETs, which may not be associated with short-term prognosis.

Key words: Rectum; Neuroendocrine tumor; Lymphatic; Immunohistochemistry; Prognosis

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Core tip: The majority of rectal neuroendocrine tumors (NETs) are small (66%-80% are ≤ 1 cm in diameter) and endoscopic resection techniques have shown successful outcomes. However, lymphovascular invasions, a well-established risk factor for lymph node metastasis, are often found at endoscopically resected specimens and there are no definite guidelines about these cases. Therefore, we investigate the frequency and prognostic significance of lymphovascular invasion (LVI) in small endoscopically resected rectal NETs. We found that LVI may be present in a high percentage of small rectal NETs by two histologic methods; hematoxylin and eosin staining and ancillary immunohistochemical staining (D2-40 and Elastica van Gieson). On the other hands, LVI was not associated with lymph node metastasis or recurrence in small rectal NETs (≤ 1 cm) during a 3 year-follow up period. Although our follow-up period was short, but I'm confident in our studies will be the cornerstone of future researches about significance of LVI in small rectal NETs.

Kwon MJ, Kang HS, Soh JS, Lim H, Kim JH, Park CK, Park HR, Nam ES. Lymphovascular invasion in more than one-quarter of small rectal neuroendocrine tumors. *World J Gastroenterol* 2016; 22(42): 9400-9410 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Rectal neuroendocrine tumors (NETs) arise from enterochromaffin endocrine cells situated within intestinal crypts of Lieberkühn^[1], comprising 25% of gastrointestinal NETs with a 5-year overall survival of 88%^[2,3]. Despite the comparatively favorable prognosis, rectal NETs are rarely aggressive and distant metastasis is of clinical concern for treating rectal NETs. The clinical or histopathologic indicators for metastasis have been demonstrated including tumor size, muscularis propria invasion, lymphovascular invasion (LVI), mitotic rate, and Ki-67 labeling index in surgically resected specimens^[4-11]. Currently, because the majority of rectal NETs are small (66%-80% are ≤ 10 mm in diameter) and found incidentally during screening colonoscopy^[12-16], endoscopic resection techniques including endoscopic submucosal resection with ligation (ESMR-L) and endoscopic submucosal dissection (ESD) are applied to treat rectal NETs. ESMR-L and ESD have shown better outcomes in terms of complete resection of rectal NETs when compared with conventional endoscopic mucosal resection^[17-21]. However, it is not known which procedure is more feasible for small rectal NETs or for which clinicopathological factors different results will be achieved. Rectal NETs ≤ 10 mm in diameter, confined to the mucosal or submucosal layer, and without LVI can be treated with endoscopic resection^[10,18,22-29]. Unexpectedly, lymph node metastasis occurs in 3% of tumors with a diameter of ≤ 10 mm^[30]. Given that LVI, as shown by the presence of tumor cells in blood vessels and/or lymphatic channels, is a high risk factor for distant or nodal metastasis and is a poor prognostic factor, LVI should be histologically assessed in specimens obtained by endoscopic resection.

Histologically, rectal NETs are composed of cells with a mixed growth pattern with trabeculae or acini of uniform cells separated by delicate and vascular stroma, which allows for easy recognition. However, marked tumor retraction from the surrounding fibrotic stroma may incorrectly give the false impression that LVI is present^[1]. Although this retraction artifact should be accurately histologically distinguished from true lymphatic or vascular invasion, identification of true LVI is not always straightforward on routine hematoxylin and eosin (H&E)-stained slides. Recently, ancillary immunohistochemical staining [D2-40, CD34, CD31, and Elastica van Gieson (EVG)] in addition to H&E histologic examination has been used to evaluate LVI in rectal NETs^[31-33]. Through these methods, the high frequency of LVI has been noted in endoscopically resected small rectal NETs^[32,33]. However, whether the increased detection rate between H&E and

ancillary studies is statistically significant has not been determined.

In the present study, we used 2 methods, H&E and ancillary immunohistochemical staining (D2-40 and EVG), to compare the detected rate of LVI in 104 endoscopically resected small rectal NET specimens and to determine the clinical impact of LVI. In addition, we evaluated differences in the LVI detection rate between endoscopic procedures and prognosis of small rectal NETs with LVI.

MATERIALS AND METHODS

Between June 2005 and December 2015, 138 patients with 139 tumors were diagnosed with rectal NET at Hallym University Sacred Heart Hospital in Anyang, Korea. Endoscopic gross tumor size ≤ 10 mm and absence of lymph node involvement or distant metastasis on the abdominal CT were the indications for endoscopic resection. The study inclusion criteria for small rectal NETs were as follows: (1) a tumor ≤ 10 mm, in diameter histologically; (2) a tumor within 15 cm of the anus; (3) no metastasis to lymph nodes or distal organs detected on abdominal computed tomography; and (4) a tumor resected in our institution for the first time. Therefore, the following cases were excluded from this analysis: 2 patients who underwent radical surgical excision with lymph node dissection owing to large tumor (3 cm and 5 cm), 4 who underwent transanal resection based on the decision of the outpatient clinic surgeon regardless of size, 7 who underwent additional transanal resection after incomplete endoscopic resection at other clinics, 12 who did not undergo additional treatment after diagnosis, 4 who were treated at other clinics, 4 who could not be evaluated for LVI owing to an insufficient specimen, and 2 with endoscopically resected tumors exceeding 1 cm (1.2 cm and 1.7 cm). As a result, 103 patients with 104 rectal NETs were included in this study; the related medical records were reviewed retrospectively. This study was conducted with the approval of the ethics committee of Hallym University Sacred Heart Hospital in Anyang, Korea. The study was carried out in accordance with the recommendations of the Declaration of Helsinki.

Methods of endoscopic resection

Three techniques were used with a single-channel scope (GIF-H260, Olympus Medical Systems Corp.) and an electrosurgical unit (ERBE VIO 300 D, ERBE Elektromedizin GmbH) after lifting the tumor with a submucosal injection of hypertonic saline solution mixed with a small amount of indigo-carmin and diluted epinephrine (1:10000). These included (1) endoscopic mucosal resection (EMR: conventional snare polypectomy); (2) ESMR-L, Figure 1A-D: Tumor was aspirated into ligator device and followed by deployment of the elastic band; Conventional snare polypectomy done below the band; and (3) endoscopic submucosal resection (ESD; Figure 1E-H): after

mucosal incision along outer border of the tumor; submucosal dissection was performed below the tumor with Dual Knife (Electrosurgical Knife ; Olympus).

Histological evaluation and immunohistochemistry

The 104 endoscopically resected cases were serially sectioned and entirely embedded for histological evaluation. H&E-stained slides from all cases were reviewed by 2 pathologists (MJK and ESN) using a multi-headed microscope. Histological evaluation including tumor size, depth of invasion, lymphatic or vascular invasion, resection margin status, mitotic count, and tumor grade was re-performed using the H&E-stained slides from the time of initial diagnosis. The immunohistochemical (Ki-67, D2-40) and histochemical (EVG) staining and re-evaluation were performed in this study. The pathological grading system of the World Health Organization 2010 criteria for tumors of the digestive system was used for classification of rectal NETs^[34]. At least 500 tumor cells were counted to determine the percentage of cells that were positive for Ki-67. Mitotic rates on H&E stain were counted in 50 high power fields (HPFs) (40 × objective, 10 × eyepiece with a field diameter of 0.55 mm and an area of 0.237 mm²; Olympus microscope BX43, Tokyo, Japan), and the mean mitotic count was calculated as the number of mitoses/10 HPFs^[35]. The tumors were classified into G1 (a mitotic count of less than 2 per 10 high-power fields and/or < 3% Ki-67) and G2 (a mitotic count of 2-20 per 10 high-power fields and/or 3%-20% Ki-67) according to the WHO 2010 classification and the North American Neuroendocrine Tumor Society guidelines^[36]. The resection margin was examined microscopically and its status determined on the basis of the general criteria for cancer involvement in a resection margin (Figure 1I-M). The completeness of resection was classified according to the extension of tumor cells into the resection margin: (1) complete (R0) resection, in which the lateral and vertical resection margins were free of tumor; (2) microscopically incomplete (R1) resection, in which the tumor extended into the lateral or vertical resection margin; and (3) macroscopically incomplete (R2) resection, in which the tumor could not be completely resected according to its endoscopic aspects. The distance between the tumor deepest margin and the endoscopic vertical resection margin was also measured and defined as the "safety resection margin" (Figure 1I and J). The involvement of tumor cells in the resection margin was also confirmed by positive synaptophysin to rule out a squeezing artifact of fibro-connective tissue.

D2-40-stained slides were assessed for lymphatic invasion. A tumor in which a lymphatic vessel showed positive staining of endothelium for D2-40 and surrounded the tumor cells was diagnosed as positive for lymphatic invasion^[37]. Venous invasion in H&E sections was defined as a tumor deposit in a space surrounded by a rim of smooth muscle and/or

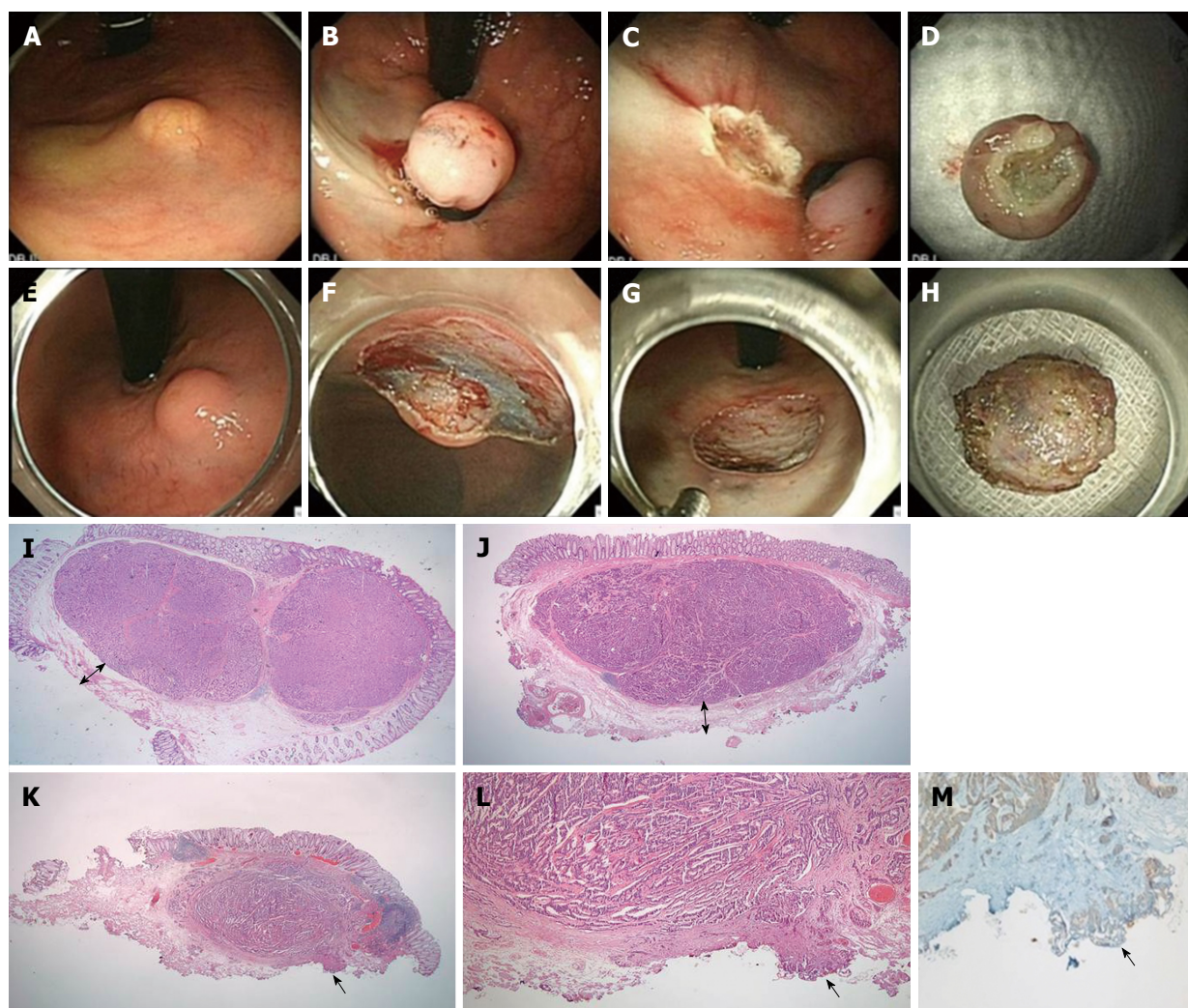


Figure 1 Endoscopic submucosal resection with a ligation device. A: NET 2 cm from anal verge; B: Aspiration of the lesion into the ligator device and deployment of the elastic band; C: Conventional snare resection below the band; D: En bloc specimen; E-H: ESD; E: NET 2 cm from the anal verge; F: Dissection with Dual Knife; G: Resection base; H: En bloc specimen; I, J: Magnified scans of H&E slides show a well-demarcated submucosal tumor with clear vertical resection margins following ESMR-L (I) and ESD (J); I-J: The vertical resection is negative (R0) and the “safety resection margin” (arrow) between the deepest margin of the tumor and the endoscopic vertical resection margin is measured; K: The magnified scan of an H&E slide of ESD shows tumor involvement in the vertical resection margin; L: The resection margin, indicated by the arrow, is involved with the neuroendocrine tumor (R1) ($\times 200$); M: The involved tumor cells are confirmed as positive for synaptophysin ($\times 200$).

containing red blood corpuscles. Venous invasion in the EVG-stained sections was defined as tumor cells observed in a vein with EVG-stained elastic lamina^[38]. Ancillary staining methods are shown in Figure 2.

Immunohistochemical staining was performed on 4- μ m-thick formalin-fixed, paraffin-embedded tumor tissue sections using the BenchMark XT automated tissue staining system (Ventana Medical Systems, Inc., Tucson, AZ, United States) according to the manufacturer's instructions, as described previously. The primary antibodies used were D2-40 (1:100; Dako, Glostrup, Denmark), Ki-67 (1:250, clone MIB-1, Dako), CD31 (1:400, JC/70A, ThermoFisher), and synaptophysin (1:2, SP11, Ventana Medical Systems). Each was used in a 40 min incubation at 37 °C; slides were then incubated with a secondary antibody (universal horseradish peroxidase (HRP) Multimer; Ventana Medical System) for 8 min at 37 °C.

The tissue sections were then incubated with a chromogendiaminobenzidine (ultraView Universal DAB Kit, Ventana Medical System) and counterstained with hematoxylin.

LVI was assessed using H&E, immunohistochemical, and histochemical stained sections (D2-40 and EVG) individually. The presence of tumor cells within vascular spaces (*i.e.*, lymphatics or small capillaries) surrounding tumors was considered LVI. Furthermore, LVI was divided into lymphatic invasion and vascular invasion depending on the presence or absence of vascular wall smooth muscle on H&E evaluation. The number of the cases positive and negative for lymphatic and vascular invasion, and LVI, was compared among the different staining procedures.

Patient follow-up

The first follow-up was done 6 mo after endoscopic

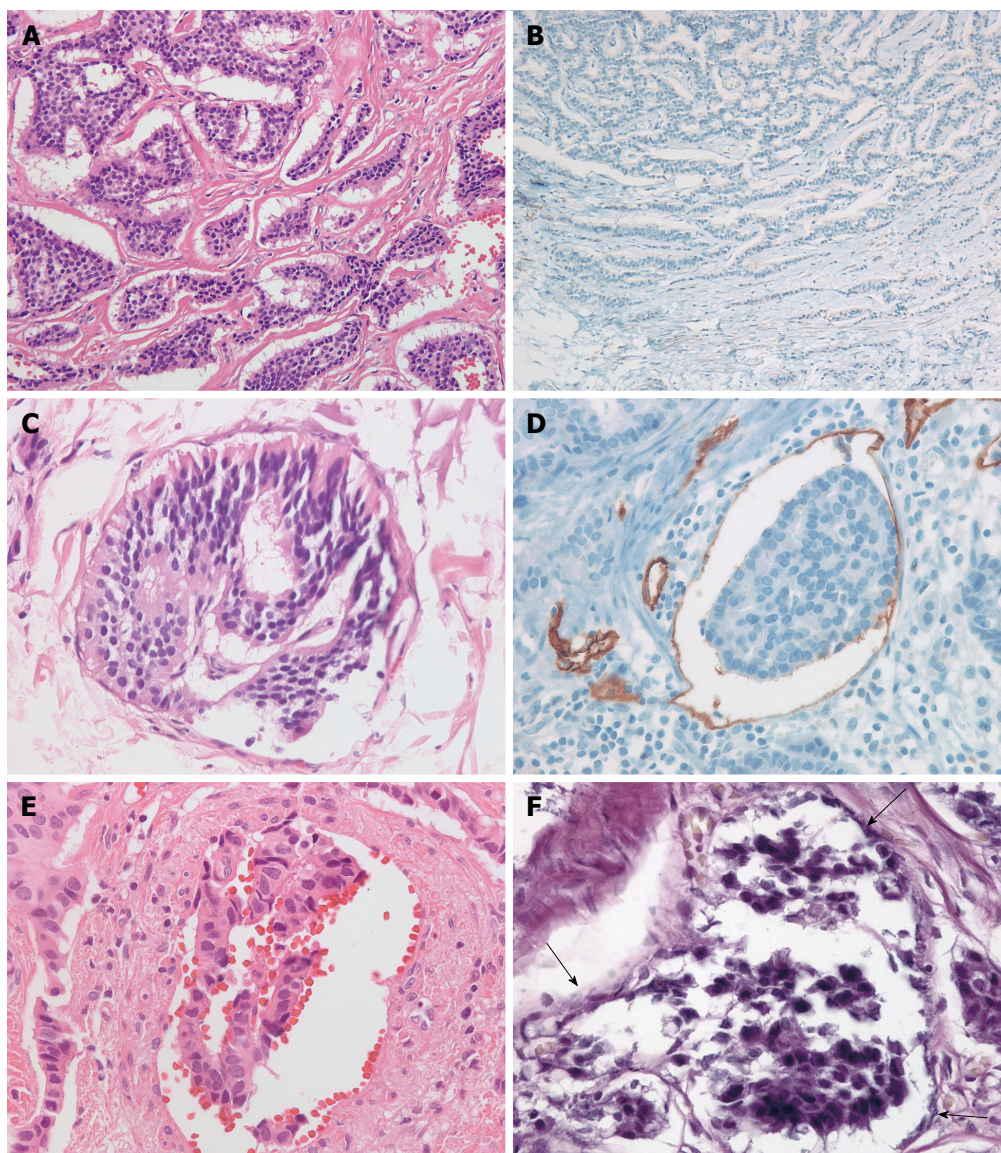


Figure 2 Ancillary staining methods. A: Rectal neuroendocrine tumors show irregular but well-demarcated islands of uniform tumor cells separated by a fibrotic stroma; B: Negative staining for D2-40 reveals retraction cleft from the surrounding fibrotic stroma; C: Lymphatic tumor invasion with thin, endothelial cell lining on H&E is identified; D: The tumor emboli in D2-40-stained lymphatic vessels reveal the recognition of lymphatic invasion; E: Vascular invasion in H&E; F: Elastica van Gieson stain reveals vascular invasion of tumor cells (arrow).

resection with colonoscopy and abdominal CT. Subsequently, endoscopy and abdominal CT were performed yearly. The follow-up duration was defined as the time from the day of endoscopic resection to the last outpatient visit day.

Data analysis and statistics

The Student's *t*-test, chi-square test and Fisher's exact test were used to analyze LVI frequency, clinicopathological factors associated with LVI, and LVI detection rate differences between endoscopic procedures. Multivariate analysis including significant predictors from the univariate analysis was performed by multiple logistic regression analysis and the overall response with a 95%CI was determined. *P* values < 0.05 were considered significant. Kaplan-Meier analysis was used for evaluation of prognosis. For the kappa value

by Kappa statistics, more than 0.5 was considered a strong association between the 2 sets. All statistical analyses were carried out using SPSS (version 18; SPSS Inc., Chicago, Illinois, United States).

RESULTS

Clinicopathological characteristics

A total of 104 cases (66 men and 37 women) with a median age of 47 years (range: 21-80 years) were included in this study. Tumors located in the rectum were an average of 8 cm away from the anal verge. Six (5.8%) patients underwent EMR, 57 (54.8%) underwent ESMR-L, and 41 (39.4%) underwent ESD. The average tumor size was 5.4 ± 2.4 mm (range, 1.2-10 mm), with 62 tumors (59.6%) measuring ≤ 5 mm and 42 tumors (40.4%) measuring 5-10 mm.

Table 1 Demographic and clinical features of patient with rectal neuroendocrine tumors

Characteristic	n = 104
Gender	
Male	67
Female	37
Age (yr), median	47 (range, 21-80)
< 60	89
≥ 60	15
Distance from anal verge, mean ± SD (cm)	8.09 ± 3.26 (range, 3-20)
Type of endoscopic resection	
EMR	6
ESMR_L	57
ESD	41
Tumor size, mean ± SD (mm)	5.4 ± 2.4 (range, 1.2-10)
≤ 5	62
> 5 and ≤ 10	42
Tumor depth	
Mucosa	3
Submucosa	101
Resection margin status	
R0	88
R1	16
Lateral (+) and deep (-)	1
Lateral (+) and deep (+)	1
Lateral (-) and deep (+)	14
Complications	
Yes	1
No	103
Follow-up	
Recurrence	0
Died	2

Three tumors (2.9%) were located at the mucosa and the other 101 (97.1%) at the submucosa. Resection margins were positive in 16 (15.4%) tumors. Procedure-related complications occurred in 1 patient who underwent ESMR-L and experienced perforation of the bowel.

Regular follow-up evaluations were performed on 68 (65.4%) patients. No patient experienced local or distant metastatic tumor recurrence after a mean follow-up of 807 d.

Three patients underwent additional surgery owing to the presence of LVI in our primary histologic reports before this study; among them, 1 patient had lymph node metastasis. This 21-year-old man's histologic evaluation showed a 5 mm tumor size, a Ki 67 index < 3%, and < 2 mitoses 10 HPFs; however, the vertical margin and lymphatic invasion were positive on the ESD specimen. There were no tumor-related deaths; 2 patients died from other causes. Kaplan-Meier analysis showed that the 5-year overall survival rate was 99%. The patient characteristics are summarized in Table 1.

Comparisons of the detection frequencies of lymphatic invasion, vascular invasion, and LVI between D2-40 and EVG stains and H&E histological evaluation

Of the 104 specimens examined by H&E, 22 (21.2%) of the tumors were considered to have lymphatic invasion. Conversely, 15 (14.4%) tumors had lymphatic

invasion detectable by D2-40. Ancillary staining including D2-40 allowed us to observe 7 lymphatic invasions (8.5%, 7/82) that were not initially detected using H&E. However, the increase of 6.8% compared with H&E was not statistically significant ($P = 0.189$).

Vascular invasion was detected in 11 (10.6%) of 104 NETs by H&E, whereas it was identified in 16 (15.4%) of 104 tumors by EVG. The detected percentage increased from 10.6% by H&E up to 15.4% by EVG. However, the difference in detection rates was not statistically significant between H&E and EVG ($P = 0.227$).

As a whole, the presence of LVI was considered positive in 26 (25.0%) of 104 tumors by H&E, and 29 (27.9%) of 104 tumors by ancillary studies. The concordance rate between H&E and ancillary studies was 81.7% for detection of LVI, which showed statistically strong agreement between two methods ($\kappa = 0.531$, $P < 0.001$). D2-40 and EVG staining enhanced LVI detection by 2.9% compared with H&E, however that difference that was not statistically significant ($P = 0.648$). LVI as assessed by H&E and immunohistochemical or histochemical procedures (D2-40 and EVG) are shown in Table 2 and Figure 3.

In 19 cases analysis for the presence of LVI using H&E did not match ancillary studies. LVI detected with H&E in 8 cases was not observed in ancillary studies. In addition, 11 cases negative for LVI using H&E were considered positive in ancillary studies. Immunostaining with CD31 and CD34 were performed on the discordant cases. Evaluation with CD31 indicated LVI was absent in the 8 cases detected with H&E, and present in the 11 cases that tested negative with H&E. However, CD34 stained in the delicate fibrovascular connective tissue of all 19 NET cases, of which non-specific staining could not be interpreted as LVI positivity.

Predictive factors for LVI based on H&E or D2-40 and EVG

Based on the comparative results between H&E and D2-40 and EVG, the results of LVI assessed by D2-40 and EVG showed statistical associations with more numbers of clinicopathological variables of NETs than H&E did. LVI assessed by D2-40 and EVG was significantly associated with tumor size, tumor grade, and mitotic count ($P = 0.007$, $P = 0.006$, and $P = 0.005$, respectively). LVI-positive cases were frequently detected in tumors that were > 5 mm, grade 2, and had a mitotic count ≥ 2. In addition, the mean Ki-67 labeling index and mean mitotic count were higher in tumors with LVI (1.54 ± 1.13 and 0.82 ± 0.88 , respectively) than in tumors without LVI (1.03 ± 0.97 and 0.32 ± 0.97 , respectively) ($P = 0.023$ and $P = 0.001$, respectively). There were no significant differences in LVI between patient's age ($P = 0.847$), gender ($P = 0.650$), tumor distance from anal verge ($P = 0.412$), or depth of tumor invasion ($P = 0.558$). The predictive parameters of LVI based on H&E or

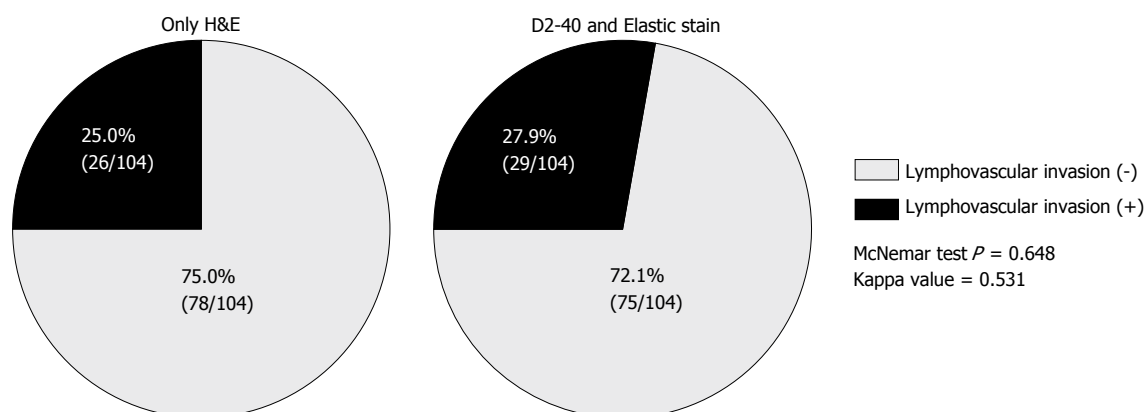


Figure 3 Comparison of pie charts of detected frequencies of lymphovascular invasion between only hematoxylin and eosin histology and acillary stains of D2-40 or Elastica van Gieson stain. H&E: Hematoxylin and eosin.

Table 2 Predictive parameters of lymphovascular invasion between hematoxylin and eosin and D2-40 and Elastica van Gieson in small rectal neuroendocrine tumors *n* (%)

	Total <i>n</i> = 104	LVI (H&E only)		<i>P</i> value	LVI (D2-40 and EVG)		<i>P</i> value
		Present <i>n</i> = 26 (25.0%)	Absent <i>n</i> = 78 (75.0%)		Present <i>n</i> = 29 (27.9%)	Absent <i>n</i> = 75 (72.1%)	
D2-40 and EVG				0.648			-
LVI (+)	29 (27.9)	18 (69.2)	11 (14.1)		-	-	
LVI (-)	75 (72.1)	8 (30.8)	67 (85.9)		-	-	
Age (yr)	48.20 ± 10.93	50.27 ± 11.28	47.51 ± 10.80	0.282	47.86 ± 11.16	48.33 ± 10.91	0.847
Sex				1.000			0.650
Male	67 (64.4)	17 (65.4)	50 (64.1)		20 (69.0)	47 (62.7)	
Female	37 (35.6)	9 (34.6)	28 (35.9)		9 (31.0)	28 (37.3)	
AV distance (cm) ¹	8.09 ± 3.25	8.35 ± 2.72	8.01 ± 3.41	0.687	7.59 ± 2.87	8.25 ± 3.37	0.412
Tumor size				0.038			0.007
≤ 5 mm	62 (59.6)	11 (42.3)	51 (65.4)		11 (37.9)	51 (68.0)	
> 5 mm	42 (40.4)	15 (57.7)	27 (34.6)		18 (62.1)	24 (32.0)	
Tumor depth				0.571			0.558
Mucosa	3 (2.9)	0 (0)	3 (3.8)		0 (0)	3 (4.0)	
Submucosa	101 (97.1)	26 (100)	75 (96.2)		29 (100)	72 (96.0)	
Tumor grade				1.000			0.006
Grade 1	95 (91.3)	24 (92.3)	71 (91.0)		20 (69.0)	68 (90.7)	
Grade 2	9 (8.7)	2 (7.7)	7 (9.0)		9 (31.0)	7 (9.3)	
Ki 67%		1.46 ± 1.01	1.08 ± 1.05	0.113	1.54 ± 1.13	1.03 ± 0.97	0.023
Ki 67 index				0.627			0.213
< 3%	98 (94.2)	24 (92.3)	74 (94.9)		26 (89.7)	72 (96.0)	
≥ 3%	6 (5.8)	2 (7.7)	4 (5.1)		3 (10.3)	3 (4.0)	
Mitotic count		0.65 ± 0.84	0.39 ± 0.69	0.125	0.82 ± 0.88	0.32 ± 0.97	0.001
Mitosis/10HPF				0.357			0.005
< 2	93 (89.4)	22 (84.6)	71 (91.0)		22 (75.9)	71 (94.7)	
≥ 2	11 (10.6)	4 (15.4)	7 (9.0)		7 (24.1)	4 (5.3)	

¹The tumor location is measured from anal verge. Bold values: *P* value < 0.05. HPF: High power field; LVI: Lymphovascular invasion; H&E: Hematoxylin and eosin stain.

D2-40 and EVG are shown in Table 2. Unlike D2-40 and EVG, analysis of LVI by H&E was only associated with tumor size (*P* = 0.038). The results of analysis of LVI using H&E were not related to tumor grade (*P* = 1.000), Ki-67 labeling index (*P* = 0.627), and mitotic count/10HPFs (*P* = 0.357).

Analysis of tumor size and grade for prediction of LVI by multivariate analysis indicated that tumor grade was the only independent predictive factor for LVI in small rectal NET patients treated with endoscopic resection (Table 3). Tumors classified as grade 2 were more likely to have LVI than grade 1 tumors (*P* = 0.015,

hazard ratio = 4.095, 95%CI: 1.321-12.692).

Correlations of LVI, tumor size, margin status, and safety margin between ESMR-L and ESD

We further investigated the possible differences in frequency of detectable LVI, tumor size, resection outcome, and safety margin between ESMR-L and ESD (Table 4). Successful complete resections (R0) by ESMR-L and ESD were achieved in 50 of 57 tumors (success rate, 87.7%) and 34 of 41 tumors (success rate, 82.9%). However, there were no statistically significant differences in the frequency of LVI, tumor

Table 3 Multivariate analyses of clinicopathological factors predictive of lymphovascular invasion in patients with rectal neuroendocrine tumors

	Lymphovascular invasion		<i>P</i> value
	HR	95% CI	
Tumor size > 5 mm	1.694	0.639-4.491	0.289
Tumor grade Grade 2	4.095	1.321-12.692	0.015

Bold values: *P* value < 0.05. HR: Hazard ratio; HPF: High power field.

Table 4 Outcomes of endoscopic resection procedures in relation to tumor size, margin status, and lymphovascular invasion *n* (%)

	ESMR-L <i>n</i> = 57	ESD <i>n</i> = 41	<i>P</i> value
LVI			0.955
Absent	42 (58.3)	30 (41.7)	
Present	15 (58.2)	11 (42.3)	
Tumor size			0.192
≤ 5 mm	38 (66.7)	22 (53.7)	
> 5 mm	19 (33.3)	19 (46.3)	
Resection outcome			0.504
Complete (R0)	50 (87.7)	34 (82.9)	
Incomplete (R1)	7 (12.3)	7 (17.1)	
Safety resection margin (μm)	725 ± 872	322 ± 348	0.002

Bold values: *P* value < 0.05. LVI: Lymphovascular invasion.

size, and resection outcome status between the 2 endoscopic resection methods (*P* = 0.955, *P* = 0.192, and *P* = 0.504, respectively).

Conversely, the vertical safety margin was significantly larger in ESMR-L than ESD (725 ± 872 μm vs 322 ± 348 μm, respectively, *P* = 0.002). The more successful safety margin was achieved in ESMR-L than in ESD.

DISCUSSION

The purpose of the present study was to investigate the frequency, risk factors and prognosis of LVI in endoscopically resected small rectal NETs ≤ 1 cm in size, and to compare the therapeutic outcome achieved with ESMR-L and ESD. We have shown that LVI was relatively common in small rectal NETs, with 27.9% exhibiting LVI. Although ancillary studies increased the detection rate of LVI, careful H&E examination was still a reliable method showing high concordance with D2-40 and EVG staining. LVI was associated with large tumor size (> 5 mm), tumor grade 2, and higher mitotic count (≥ 2). Among those factors, tumor grade 2 was the only independent predictive factor for the presence of LVI. No recurrence was observed in patients with small rectal NETs ≤ 1 cm, regardless of the presence of LVI.

Only a few studies have investigated the frequency, risk factors, and prognostic significance of pathologically proven LVI in rectal NETs ≤ 1 cm in size after endoscopic

resection^[32,33]. Prevalence of LVI in rectal NETs is 0-20% by H&E examination^[17,19,24,25,32,39]. Immunohistochemical analysis is not currently recommended for routine use to identify LVI in NETs. However, for accurate and reliable diagnosis of LVI, we applied additional staining using D2-40 and EVG to confirm the presence of LVI after H&E examination.

Staining of elastic tissue during microscopic assessment has been proposed as being a more sensitive means of revealing venous invasion within the tumor^[38]. D2-40 is the best selective immunohistochemical marker for staining lymphatic endothelium^[37]. In the present study, LVI was identified in 29 (27.9%) of 104 tumors by D2-40 and EVG, and 26 (25.0%) of 104 tumors by H&E. Although staining with D2-40 and EVG raised the detection rate of LVI by 2.9%, the difference was not statistically significant. Rather, H&E showed a high concordance rate with ancillary studies (81.7%).

There have been only two studies of D2-40 and EVG staining for identification of LVI^[32,33]. Those studies also reported a high frequency of LVI (46.7% and 22.4%) in endoscopically resected small rectal NETs^[32,33]. Taken together with our study, the frequency of LVI appears to be high in even small NETs. However, in those studies, the detection rate for LVI using H&E staining alone was much lower (1.1% and 10.2%) than the rate detected with D2-40 and EVG staining^[32,33]. The wide range of frequencies reported may be due to difficulties evaluating LVI H&E-stained sections due to retraction artifacts in the tumor. We also had 19 results (18.3%) in which the results of H&E and ancillary staining were discordant. The absence of D2-40 and EVG in 8 (42.1%) out of the 19 discordant cases was also confirmed by the absence of CD31 staining, which indicated a retraction artifact from the surrounding fibrotic stroma. Although the immunohistochemical and/or special staining used in our study was not demonstrated as a statistically significant indicator for the identification of LVI, the ancillary studies may be of help to differentiate retraction artifacts. The high concordance rate in our study between H&E and ancillary studies may be because two pathologists carefully re-evaluated H&E-stained slides from all cases and discussed the findings of LVI using a multi-headed microscope. The previous studies did not describe the number of pathologist participating in slide review^[32,33].

The metastatic potential and aggressive behavior of a rectal NET are generally proportional to tumor size^[30]. A close relationship has been noted between tumor size or LVI and risk of metastasis even in small rectal NETs. LVI-positive tumors have significantly larger tumor size (median 5 mm) than those without LVI (median 4 mm)^[32]. The metastasis rate of early stage rectal NETs (10 mm or less in size) was 9.7% (58/595)^[2]. Three tumors (25%) out of 12 with lymph node metastasis were less than 10 mm^[5]. In our study, LVI was frequently detected in tumor size > 5 mm in univariate analysis. However, the multivariate analysis failed to demonstrate the correlation between tumor

size and LVI.

LVI was also associated with tumor grade 2 and increased mitotic count (≥ 2). Tumor grade 2 was the only independent predictive factor for the presence of LVI. The majority of small rectal NETs are NET G1 (97.6%-100%)^[8,25,32,33,39,40]. Few studies have demonstrated a correlation between LVI and grade 2 in small rectal NETs, although NETs G1 and G2 exhibit significant differences in patient survival. The present study included 16 NETs classified as G2 (15%). Interestingly, a significant association between grade 2 and LVI was only found in the LVI results assessed by D2-40 and EVG but not by H&E staining. Thus, immunohistochemically confirmed LVI may more precisely reflect on clinicopathological features of such tumors.

Strategies for treating small rectal NETs ≤ 1 cm in size with LVI remain controversial, and clear-cut indications for local resection and additional surgery have not been established. Patients with rectal NETs without metastasis have a good prognosis if they undergo endoscopic resection; the 3-year survival rate is 100%^[23]. In the present study, an excellent prognosis was found in the small rectal NETs. There was no recurrence or metastasis in patients with LVI during follow-up periods of 28.8 mo in our study. Similarly, no metastasis or recurrence has been reported in the small rectal NETs with LVI but without additional surgery over the 5 years median follow-up period^[32,33]. Furthermore, recurrences have not been observed following the removal of tumors 20 mm in size and positive for LVI, but not in any tumors < 20 mm, even if they were positive for LVI during a 10-year period^[39]. In contrast, a delayed localized recurrence has been unexpectedly reported 23 years after endoscopic resection of 4 mm sized rectal G1 NET^[41], and the size of a lymph node metastasis has remained unchanged during 7 years of follow-up^[42], suggesting that the metastatic lymph node growth rate may be extremely low in some cases. However, there are no definite guidelines for regular follow-up of LVI-positive small rectal NETs^[10,27]. While small rectal NETs seem to have a favorable short-term prognosis, the long-term prognosis may be difficult to determine.

Complete resection of rectal NETs is difficult to achieve with conventional endoscopic resection techniques because these tumors often extend into the submucosa. We found that ESMR-L ($725 \pm 872 \mu\text{m}$) showed a larger safety resection margin than ESD ($725 \pm 872 \mu\text{m}$ vs $322 \pm 348 \mu\text{m}$) despite similar rates of complete resection between two methods (ESMR-L 87.7% and ESD 82.9%). It may be that ESMR-L gets more submucosal tissue below NETs because submucosal aspiration is done by negative pressure. The short procedure time of ESMR-L may result in a smaller coagulation effect in the submucosa than ESD^[21]. We found that there was no statistical difference in LVI detection rates between two endoscopic methods. The subsequent surgical resection with lymph node

dissection for small rectal NETs with LVI after endoscopic resection has no worldwide accepted consensus. This study showed excellent outcomes of endoscopic resection with LVI. After endoscopic resection is completely achieved through ESMR-L and ESD, close follow-up should be pursued in cases with LVI^[23].

The approximately 3-year follow-up period may be a limitation of our study. Nevertheless, some significant findings emerged from our results. The present study demonstrated that LVI in small rectal NETs may be high, and that this may not be associated with lymph node metastasis or recurrence in small rectal NETs (≤ 1 cm) during a 3 year-follow up period. Application of ancillary studies may help differentiate retraction artifacts from true LVI, which may contribute to a close association with clinicopathological characteristics of rectal NETs.

Small rectal NETs have a favorable prognosis and successful outcomes following endoscopic resection. However, a low but real risk of metastasis remains, as in our results, and there are several cases of recurrence during long-term observation. Therefore, careful histologic examination for LVI and prospective studies with long-term follow up are needed to determine the natural course of small, endoscopically resected rectal NETs.

COMMENTS

Background

Rectal neuroendocrine tumors (NETs) arise from enterochromaffin endocrine cells and are found incidentally during sigmoidoscopy or colonoscopy. On endoscopy, they typically appear as sessile, subepithelial tumors covered with yellow, discolored epithelium. Rectal NETs ≤ 10 mm in diameter, within the mucosal or submucosal layer, can be treated with endoscopic resection and have a good prognosis. However, lymphovascular invasion (LVI), a well-established risk factor for lymph node metastasis, is often found in endoscopically resected specimens, and there are no definite guidelines about these cases. Therefore, the authors investigated the frequency and prognostic significance of LVI in small, endoscopically resected rectal NETs.

Research frontiers

Immunohistochemical analysis is not currently recommended for routine use to identify LVI in NETs. However, for accurate and reliable diagnosis of LVI, the authors undertook additional immunohistochemical staining using D2-40 and Elastica van Gieson staining to confirm the presence of LVI.

Innovations and breakthroughs

The authors observed LVI rates of 25% and 27.9%, higher than previously reported, through hematoxylin and eosin (H&E) and additional immunohistochemical staining. On the other hand, LVI was not associated with lymph node metastasis or recurrence in small rectal NETs (≤ 1 cm) during a 3 year-follow up period.

Applications

After endoscopic resection of rectal NETs, even in small tumors (≤ 10 mm), careful histologic examination for LVI is needed. Furthermore, long-term prospective studies are required to determine the natural course of endoscopically resected rectal NETs.

Peer-review

In this article, the authors analyzed the frequency of LVI in endoscopically resected small rectal NETs by precise methods and compared these results

with conventional H&E staining. By these methods, they found that the frequency of LVI was higher than the previously reported ratio. Although they could not determine the relationship between LVI and clinical outcome, such as survival and recurrence, this study provides very important insights for future study.

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Retrospective Study

Potential model for differential diagnosis between Crohn's disease and primary intestinal lymphoma

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Abstract

AIM

To evaluate the usefulness of different parameters to differentiate Crohn's disease (CD) from primary intestinal lymphoma (PIL).

METHODS

The medical records of 85 patients with CD and 56 patients with PIL were reviewed retrospectively. Demographic, clinical, laboratory, endoscopic, and computed tomographic enterography (CTE) parameters were collected. The univariate value of each parameter was analyzed. A differentiation model was established by pooling all the valuable parameters. Diagnostic efficacy was analyzed, and a receiver operating characteristic (ROC) curve was plotted.

RESULTS

The demographic and clinical parameters that showed significant values for differentiating CD from PIL included age of onset, symptom duration, presence of diarrhea, abdominal mass, and perianal lesions ($P < 0.05$). Elevated lactate dehydrogenase and serum $\beta 2$ -microglobulin levels suggested a PIL diagnosis ($P < 0.05$). The endoscopic parameters that showed significant values for differentiating CD from PIL included multiple-site lesions, longitudinal ulcer, irregular ulcer,

and intraluminal proliferative mass ($P < 0.05$). The CTE parameters that were useful in the identification of the two conditions included involvement of ≤ 3 segments, circular thickening of the bowel wall, wall thickness > 8 mm, aneurysmal dilation, stricture with proximal dilation, "comb sign", mass showing the "sandwich sign", and intussusceptions ($P < 0.05$). The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of the differentiation model were 91.8%, 96.4%, 93.6%, 97.5%, and 88.5%, respectively. The cutoff value was 0.5. The area under the ROC curve was 0.989.

CONCLUSION

The differentiation model that integrated the various parameters together may yield a high diagnostic efficacy in the differential diagnosis between CD and PIL.

Key words: Primary intestinal lymphoma; Crohn's disease; Differential diagnosis; Endoscopy; CT enterography

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Core tip: Crohn's disease and primary intestinal lymphoma (PIL) have overlapping clinical manifestations. Misdiagnosis of PIL would lead to disastrous outcomes in patients. Consequently, the differential diagnosis between these two conditions has perplexed clinical practitioners for decades. In this article, we evaluated the usefulness of different parameters, including clinical manifestations, laboratory tests, endoscopic features, and computed tomographic enterographic characteristics for differentiating these two conditions and established an objective differentiation model that would yield a high diagnostic efficacy in order to avoid misdiagnosis of PIL. This is a first study which focuses on the differential diagnosis of these two diseases.

Zhang TY, Lin Y, Fan R, Hu SR, Cheng MM, Zhang MC, Hong LW, Zhou XL, Wang ZT, Zhong J. Potential model for differential diagnosis between Crohn's disease and primary intestinal lymphoma. *World J Gastroenterol* 2016; 22(42): 9411-9418 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9411.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9411>

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory granulomatous disorder that can affect any segment of the gastrointestinal (GI) tract, especially the terminal ileum and ileocecal region. The incidence of CD in China is increasing in recent years^[1]. However, the incidence of noncaseating granuloma as a gold standard criterion for the diagnosis of CD is quite low in terms of pathological findings, especially those from biopsy^[2]. Moreover,

the clinical manifestations of CD and other GI diseases in terms of symptoms, and endoscopic and radiological findings overlap, making the diagnosis of CD an even more complicated issue^[3].

The estimated incidence of extranodal lymphomas in the GI tract is approximately 5%-20%^[4]. On the other hand, primary GI lymphomas only account for 1%-4% of all GI cancers^[5]. In the past decade, the incidence of primary GI lymphomas has been increasing worldwide^[6]. The so-called primary intestinal lymphoma (PIL) includes about 30% of primary GI lymphomas that occur in the small intestine, ileocecal region, and colorectum. Derived from submucosal lymphoid tissue, PILs were mostly classified as a subtype of non-Hodgkin's lymphoma. The clinical features of PIL lack specificity, and definitive diagnosis often relies on histological confirmation. However, in clinical practice, qualified specimens are sometimes difficult to acquire through endoscopy due to the small size and superficial nature of PILs. Furthermore, for some lesions confined to the deep small intestine, accurate localization and diagnosis without surgery are challenging^[7]. Consequently, the preoperative diagnostic rate of PIL is low (30.5% as estimated)^[8].

Therefore, it is important to establish a correct differential diagnosis between CD and PIL. One concern is that PIL has a malignant potential to some extent, and a delayed diagnosis may lead to lethal outcomes. However, these two conditions have overlapping characteristics in terms of symptoms, and laboratory, endoscopic, and radiological findings. An inconvenient truth is that the risk of misdiagnosis could not be neglected in some intricate cases^[9,10].

In the past decade, the advent of new technology such as double-balloon enteroscopy (DBE) and CTE provided an unprecedented opportunity for us to have better knowledge and visualization of small bowel lesions, and the bowel wall and extraluminal. DBE and CTE, when properly performed and accurately interpreted, are helpful in the diagnosis and management of small bowel disease and act as a good complement to other examinations^[11-13]. Undoubtedly, they would play an important role in the preoperative differential diagnosis between CD and PIL.

In recent years, we have done much work in the differential diagnosis between CD and PIL. In this study, we retrospectively enrolled patients with CD and PIL treated in our hospital and evaluated the diagnostic values of clinical, laboratory, endoscopic, and CTE parameters.

MATERIALS AND METHODS

Patients enrolled

A retrospective study of a single center was designed. The medical records of inpatients with CD and PIL treated from August 1, 2005, to March 31, 2016 in Ruijin Hospital, Shanghai, China were reviewed. None of

the cases was complicated with both CD and intestinal lymphoma or intestinal lymphoma in preexisting CD. Patients with CD were included in our final cohort if they met the following criteria: (1) newly diagnosed with CD with lower GI tract involvement; (2) aged >18 years; (3) with a definite diagnosis for at least 1 year; (4) had a Montreal classification of L1, L2, or L3 (with an involvement of the ileum, colon, or both)^[14]; (5) underwent endoscopy and CTE at least once during hospitalization; and (6) had an exclusion of other concomitant GI diseases. Diagnosis of PIL was based on Dawson standards as follows^[15]: (1) no enlargement of the peripheral or mediastinal lymph nodes; (2) normal white blood cell count; (3) predominance of alimentary tract lesions with only regional lymph node involvement; and (4) no involvement of the liver and spleen. Patients with PIL were included in our final cohort if they met the following criteria: (1) newly diagnosed as having PIL; (2) aged >18 years; (3) had a definitive histological diagnosis through endoscopy or surgery; (4) underwent endoscopy and CTE for at least once during hospitalization; and (5) had an exclusion of other concomitant GI diseases.

Clinical evaluation

All the data of the enrolled patients with CD and PIL were reviewed. Parameters regarding demographic information, clinical manifestations, laboratory data, endoscopic feature, and CTE characteristics were collected prior to treatment.

The demographic parameters included sex, age of onset, height, and weight. Clinical manifestations were symptom duration, abdominal pain, diarrhea, abdominal distension, nausea, hematochezia, fever, weight loss, abdominal mass, perianal lesions, history of GI surgery, perforation, and extraintestinal manifestations. The laboratory data documented were as follows: hemoglobin (Hb) level, hematocrit (Hct) level, platelet (Plt) count, albumin (Alb) level, elevated erythrocyte sedimentation rate (ESR), elevated C-reactive protein (CRP) level, elevated lactate dehydrogenase (LDH) level, and elevated serum β_2 -microglobulin (β_2 -MG) level.

DBE was performed if the lesion was confined to the small intestine, while colonoscopy was performed for patients with only colonic involvement. Endoscopic features included multiple-site lesions, pseudo-polyp formation, aphthoid ulcer, longitudinal ulcer, irregular ulcer, intraluminal proliferative mass, bowel stricture, and anorectal involvement.

All of our enrolled patients had undergone CTE at least once and was evaluated by an experienced radiologist independently. CTE characteristics mainly included involvement of ≤ 3 segments, circular thickness of the bowel wall, wall thickness of > 8 mm, target sign, enhancement after a contrast-enhanced scan, aneurysmal dilation, stricture with proximal dilation, abscess, phlegmon, ascites, "comb sign",

enlargement of the abdominal lymph nodes, enhanced density of the peri-intestinal fat, mass showing the "sandwich sign", and intussusceptions.

Statistical analysis

SPSS 19.0 was used for the data analyses and screening for potential valuable parameters for differential diagnosis between CD and PIL. Continuous variables were expressed as mean \pm SD, and a comparison was performed by using the Student *t* test if the data had a normal distribution. Median values (upper and lower quartiles) were calculated, and the Wilcoxon rank sum test was used to analyze the data that did not have a normal distribution. Binary categorical variables were expressed as frequency and percentage values, while comparisons were made using the Chi-square or Fisher's exact test. A probability (*P*) value of < 0.05 was considered to be statistically significant. Then, continuous variables were converted to binary categorical variables based on the Youden index. All of the parameters with significant differences in differentiating diagnosis were graded, with CD1 and PIL-1. A differentiation model was created by adding all of the scores of the valuable parameters. The total score was calculated, and a receiver operating characteristic (ROC) curve was plotted. The cutoff value was obtained from the Youden index. Sensitivity, specificity, accuracy, PPV, and NPV were calculated to evaluate the diagnostic efficacy of the model.

RESULTS

Demographic, clinical, and laboratory features of the patients with CD and those with PIL

The demographic, clinical, and laboratory features of CD and PIL are listed in Table 1. No significant difference was found with respect to the patients' sex, height, and weight. The CD patients were significantly younger than the PIL patients in terms of age of onset (32.0 ± 9.9 years vs 52.8 ± 16.3 years, $P < 0.05$). The CD patients had a longer symptom duration before definite diagnosis than the PIL patients [median time, 16 (11-19) mo vs 2 (1-6) mo, $P < 0.05$]. Based on the Youden index, age of onset of < 40 years was graded 1, while symptom duration of < 12.5 mo was graded 1. For clinical manifestations, the incidence of diarrhea and perianal lesions in CD was significantly higher than that in PIL ($P < 0.05$). In contrast, the incidence of an abdominal mass in PIL was significantly higher than that in CD ($P < 0.05$). For other clinical parameters, including abdominal pain, abdominal distension, nausea, hematochezia, fever, weight loss, history of GI surgery, perforation, and extraintestinal manifestations, no significant difference was found between these two conditions. As shown by the laboratory data, the mean levels of hemoglobin, hematocrit, platelet, and albumin had no significant

Table 1 Demographic, clinical and laboratory parameters of Crohn's disease and primary intestinal lymphoma patients *n* (%)

Parameters	CD <i>n</i> = 85	PIL <i>n</i> = 56	<i>P</i> value	Score
Gender (male/female)	48/37	32/24	0.937	N/A
Age of onset	32.0 ± 9.9	52.8 ± 16.3	< 0.001	1
Height (cm)	166.1 ± 6.6	164.6 ± 7.3	0.189	N/A
Weight (kg)	59.3 ± 9.4	57.7 ± 10.2	0.346	N/A
Symptom duration (mo)	16 (11-19)	2 (1-6)	< 0.001	-1
Abdominal pain	64 (75.3)	39 (69.6)	0.459	N/A
Diarrhea	60 (70.6)	15 (26.8)	< 0.001	1
Abdominal distension	29 (34.1)	21 (37.5)	0.681	N/A
Nausea	12 (14.1)	13 (23.2)	0.166	N/A
Hematochezia	22 (25.9)	11 (19.6)	0.392	N/A
Fever	13 (15.3)	15 (26.8)	0.094	N/A
Weight loss	42 (49.4)	32 (57.1)	0.368	N/A
Abdominal mass	5 (5.9)	9 (16.1)	0.048	-1
Perianal lesions	37 (43.5)	2 (3.6)	< 0.001	1
History of GI surgery	12 (14.1)	4 (7.1)	0.189	N/A
Perforation	4 (4.7)	5 (8.9)	0.515	N/A
Extraintestinal manifestation	6 (7.1)	2 (3.6)	0.614	N/A
Hemoglobin(g/L)	106.9 ± 17.8	109.7 ± 22.4	0.408	N/A
Hematocrit	35.1 ± 3.9	33.9 ± 5.7	0.127	N/A
Platelet ($1 \times 10^9/L$)	263.7 ± 96.2	261.2 ± 116.5	0.890	N/A
Albumin (g/L)	29.7 ± 6.1	31.3 ± 7.0	0.151	N/A
Elevated ESR	53 (62.4)	28 (50.0)	0.147	N/A
Elevated CRP	59 (69.4)	31 (55.4)	0.089	N/A
Elevated lactate dehydrogenase	2 (2.4)	8 (14.3)	0.018	-1
Elevated serum β_2 -microglobulin	0 (0.0)	12 (21.4)	< 0.001	-1

CD: Crohn's disease; PIL: Primary intestinal lymphoma.

differences between CD and PIL. Furthermore, the levels of serum inflammatory markers such as ESR and CRP tended to elevate in more CD patients than PIL patients, but the differences were not significant. LDH level was found to be elevated in the PIL patients (8/56), much higher than that in the CD patients (2/85; $P < 0.05$). Moreover, we found that 21.4% (12/56) of the patients with PIL had an elevated β_2 -MG level, while none of the CD patients had elevated serum β_2 -MG levels.

Endoscopic features of the patients with CD and those with PIL

The endoscopic parameters of CD and PIL are summarized in Table 2. Lesions of CD tended to involve multiple sites compared with those of PIL ($P < 0.05$). The morphology of ulcers under endoscopy differed between CD and PIL patients. Longitudinal ulcers (Figure 1D) were more apparent in the CD patients ($P < 0.05$), whereas irregular ulcers (Figure 2F) were more common in the PIL patients ($P < 0.05$). We also found that intraluminal proliferative mass (Figure 2E) was more frequent in the PIL patients than in the

Table 2 Endoscopic parameters of Crohn's disease and primary intestinal lymphoma patients *n* (%)

Parameters	CD <i>n</i> = 85	PIL <i>n</i> = 56	<i>P</i> value	Score
Multiple-site lesions	73 (85.9)	19 (33.9)	< 0.001	1
Pseudo-polyp formation	26 (30.6)	10 (17.9)	0.090	N/A
Aphthoid ulcer	37 (43.5)	21 (37.5)	0.477	N/A
Longitudinal ulcer	69 (81.2)	5 (8.9)	< 0.001	1
Irregular ulcer	31 (36.5)	32 (57.1)	0.016	-1
Intraluminal proliferative mass	11 (12.9)	31 (55.4)	< 0.001	-1
Bowel stricture	27 (31.8)	15 (26.8)	0.527	N/A
Anorectal involvement	13 (15.3)	4 (7.1)	0.146	N/A

CD: Crohn's disease; PIL: Primary intestinal lymphoma.

Table 3 Computed tomography enterography parameters of Crohn's disease and primary intestinal lymphoma patients *n* (%)

Parameters	CD <i>n</i> = 85	PIL <i>n</i> = 56	<i>P</i> value	Score
Involvement of ≤ 3 segments	31 (36.5)	45 (80.4)	< 0.001	-1
Circular thickening of bowel wall	33 (38.8)	35 (62.5)	0.006	-1
Wall thickness of > 8 mm	21 (24.7)	45 (80.4)	< 0.001	-1
Aneurysmal dilation	5 (5.9)	27 (48.2)	< 0.001	-1
Target sign	25 (29.4)	12 (21.4)	0.292	N/A
Enhancement after a contrast-enhanced scan	67 (78.8)	37 (66.1)	0.092	N/A
Stricture with proximal dilation	19 (22.4)	4 (7.1)	0.017	1
Abscess	7 (8.2)	2 (3.6)	0.449	N/A
Phlegmon	8 (9.4)	2 (3.6)	0.324	N/A
Ascites	5 (5.9)	7 (12.5)	0.174	N/A
"Comb sign"	61 (71.8)	18 (32.1)	< 0.001	1
Enlargement of the abdominal lymph nodes	42 (49.4)	36 (64.3)	0.082	N/A
Enhanced density of the peri-intestinal fat	30 (35.3)	26 (46.4)	0.186	N/A
Mass showing the "sandwich sign"	2 (2.4)	9 (16.1)	< 0.001	-1
Intussusceptions	0 (0.0)	3 (5.4)	0.031	-1

CD: Crohn's disease; PIL: Primary intestinal lymphoma.

CD patients ($P < 0.05$). Other parameters, including pseudo-polyp formation (Figure 1E), aphthoid ulcer, bowel stricture, and anorectal involvement (Figure 1F), showed no significant differences between the two diseases.

CTE features of the patients with CD and those with PIL

For the CTE parameters listed in Table 3, we found that involvement of ≤ 3 segments, circular thickening of the bowel wall (Figure 2D), wall thickness > 8 mm,

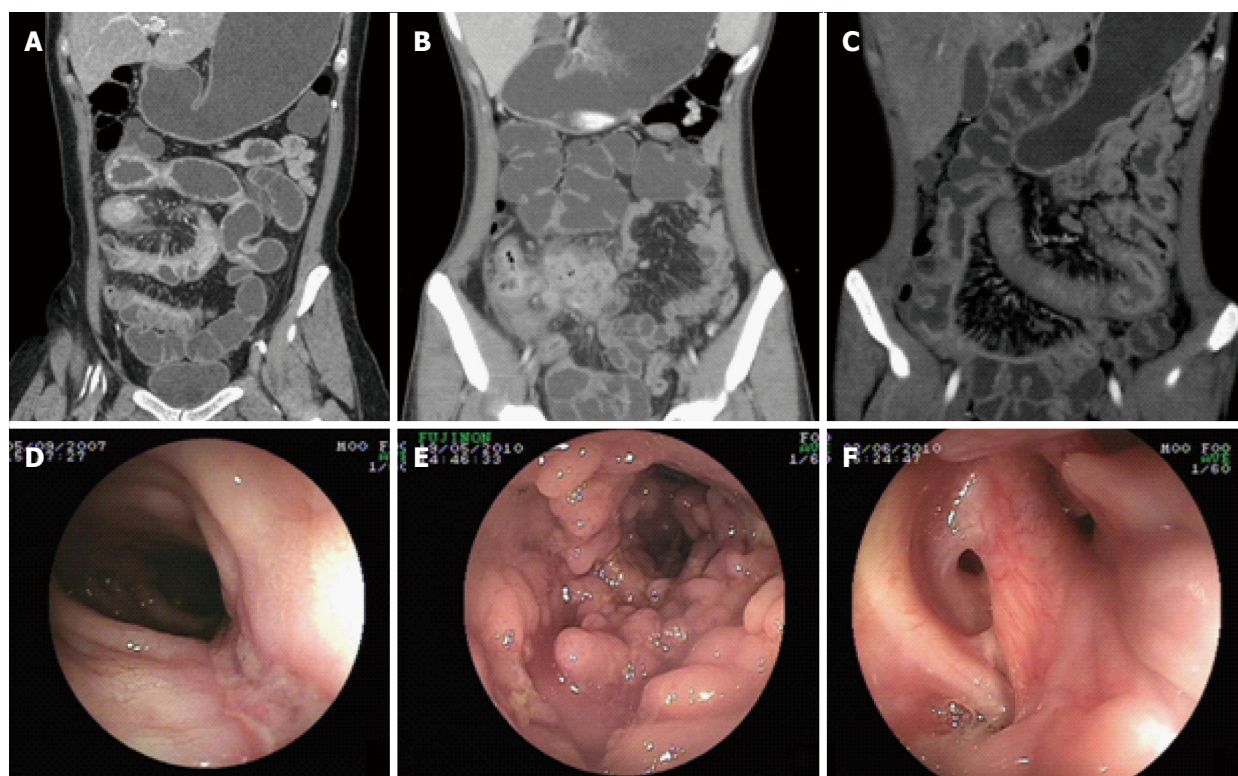


Figure 1 Endoscopic and computed tomographic enterography features in Crohn's disease. A: Coronary reconstructed CTE reflected stricture with proximal dilation and comb sign in ileum; B: Phlegmon in distal ileum; C: Stretching and densifying of distal mesenteric artery so-called comb sign in ileum; D: DBE found longitudinal ulcer in distal ileum; E: Colonoscopy detected Pseudo-polyps formation in ascending colon; F: Perianal involvement in CD.

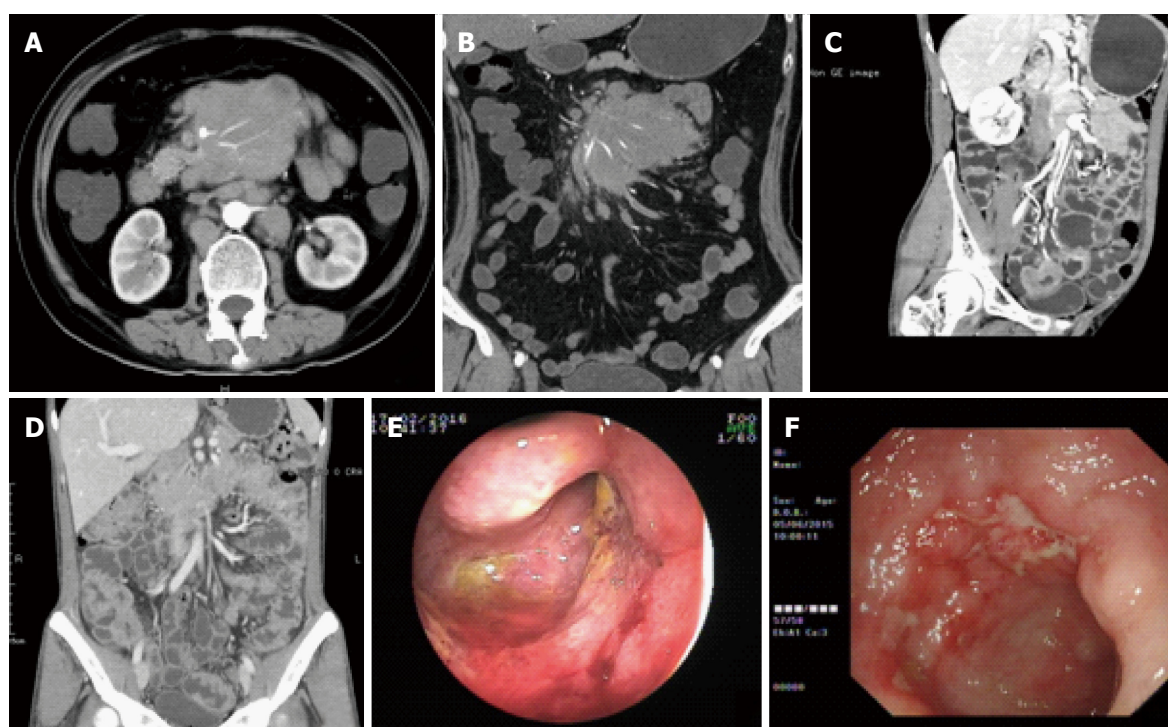


Figure 2 Endoscopic and computed tomographic enterography features in primary intestinal lymphoma. A and B: Axial and coronal reconstructed CTE sections showed mass of sandwich sign in mesentery area; C: Coronary reconstructed CTE reflected aneurysmal dilation in pelvic intestine; D: Coronary reconstructed CTE displayed circular thickening of bowel wall without stricture in ileocecal region; E: DBE showed intraluminal proliferative mass in proximal ileum; F: Colonoscopy revealed irregular ulcer in ileocecal region.

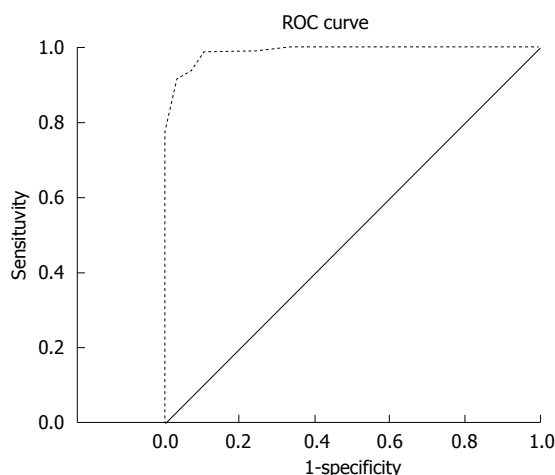


Figure 3 Receiver operating characteristic curve of differentiating model between Crohn's disease and primary intestinal lymphoma (area under the ROC curve = 0.989). ROC: Receiver operating characteristic.

and aneurysmal dilation (Figure 2C) were significantly more prevalent in the PIL patients than in the CD patients ($P < 0.05$). Stricture with proximal dilation (Figure 1A) in CD was more common compared with that in PIL. In terms of parenteral manifestations, the "comb sign" (stretching and densifying of the distal mesenteric artery; Figure 1C) indicated a diagnosis of CD ($P < 0.05$). On the other hand, mass showing the "sandwich sign" (enhanced vessel and nonenhanced fat inside the mesenteric mass; Figure 2A and B) and intussusceptions were more frequently observed in PIL than in CD ($P < 0.05$), suggesting that these parameters were indicative of a PIL diagnosis. No significant difference was found between these two groups in terms of target sign, abscess enhancement after a contrast-enhanced scan, phlegmon (Figure 1B), ascites, enlargement of abdominal lymph nodes, and enhanced density of peri-intestinal fat.

Total score of the differential parameters, diagnostic efficacy of the differentiation model, and ROC curve in the patients with CD and those with PIL

The total score was calculated by pooling all of the valuable differential parameters together. A differentiating diagnostic model was established, with high sensitivity (91.8%), specificity (96.4%), accuracy (93.6%), PPV (97.5%), and NPV (88.5%). A ROC curve was plotted (Figure 3). Based on the Youden index, a diagnostic point of 0.5 was obtained ($P > 0.5$, predictable diagnosis of CD; $P < 0.5$, diagnosis of PIL) and the area under the ROC curve was 0.989.

DISCUSSION

The incidence of CD, which was formerly considered as a Western disease, was estimated to have increased by threefold during the past two decades in China, making it a major health concern in the nation^[16,17].

Differential diagnosis between CD and PIL is challenging because of the overlapping manifestations of the two conditions^[18]. A correct and timely differential diagnosis is of great importance for the contradictory medications and sometimes lethal prognosis of PIL^[19]. In addition, correct differential diagnosis through routine clinical examinations without surgery should be a goal worth pursuing by clinicians. To the best of our knowledge, no diagnostic algorithm has been established between CD and PIL that collated numerous parameters. In the present study, we analyzed the differential diagnostic values of demographic, clinical, endoscopic, and CTE data of CD and PIL patients. Then, using valuable parameters, we investigated a differentiation model for a more objective and easier facilitation of differential diagnosis between CD and PIL.

From among various demographic and clinical factors, in this study we found that five factors, including age of onset, symptom duration, diarrhea, abdominal mass, and perianal lesions, were most helpful in the differential diagnosis of CD and PIL. Among these factors, age of onset of < 40 years, diarrhea, and perianal lesions favored a diagnosis of CD, whereas symptom duration of < 12.5 mo and abdominal mass favored a diagnosis of PIL. For the other demographic and clinical parameters, no significant differences were found between the two groups, which further confirm that CD and PIL exhibit overlapping manifestations and symptoms. The results of our study were similar to those of Zou *et al.*^[20] and Wang *et al.*^[21].

Our study showed that CD and PIL patients displayed no significant difference in routine laboratory test results, including hemoglobin level, hematocrit level, platelet count, albumin level, elevated ESR, and elevated CRP level. On the contrary, elevated LDH and serum β_2 -MG levels exhibited significant differences between CD and PIL. These may be attributed to the hematologic malignant nature of PIL. The two above-mentioned laboratory parameters only require a blood sample from the patient, thus highlighting their convenience, replicability, minimal invasiveness, and high specificity in the diagnosis of PIL. Thus, in the process of differentiating CD from PIL, measurement of serum LDH and β_2 -MG levels should be a routine laboratory test.

Endoscopy is the first choice of clinical practitioners for detecting bowel lesions and evaluating therapeutic response. In this study, we found that CD tended to have multiple-site involvement compared with PIL. Moreover, the morphology of bowel ulcers differs between CD and PIL patients. The ulcers of CD patients were longitudinal and stretched across several intestinal folds. In contrast, the morphology of PIL ulcers was characterized by irregular ulcers. In addition, we found that intraluminal proliferative mass was more common in PIL than in CD, probably because of its malignant nature.

CTE is an emerging noninvasive technology for the diagnosis and evaluation of small bowel lesions. It

offers an unparalleled tool to detect bowel wall lesions and extra-enteric complications, which is a necessary complement to endoscopy. Our study illustrated that inside the lumen, the presence of involvement of ≤ 3 segments, circular thickening of the bowel wall, wall thickness of > 8 mm, and aneurysmal dilation indicated a probable diagnosis of PIL. These are the characteristic imaging findings of PIL, which may be attributed to the proliferation of lymphoma cells without damage of normal intestinal wall cells. As a result, in spite of diffuse thickening of the bowel wall, the incidences of stricture and obstruction are relatively low. On the other hand, proliferation of lymphoma cells brings invasion to the nervous plexus of the bowel wall, leading to a decreased muscular tension of the bowel wall and aneurysmal dilation^[22]. Compared with that in PIL, the chronic intestinal inflammation in CD would end up with fibrosis. Consequently, stricture with proximal dilation would occur. Regarding extra-enteric manifestations, our study showed that the "comb sign" was more likely found in the CD patients, while the mass showing the sandwich sign and intussusceptions was more likely found in the PIL patients. Our findings further proved that PIL had characteristic features on CTE which provides a new prospective that extra-luminal manifestations should not be neglected in differentiating CD from PIL.

Although a series of differentiating parameters had been proposed, none of them had high sensitivity and specificity at the same time. For this reason, differentiating between these two conditions through a single parameter is difficult. Thus, we established a diagnostic model that combines all of the valuable parameters. Through later analysis, we proved that the sensitivity, specificity, accuracy, PPV, and NPV of our model were 91.8%, 96.4%, 93.6%, 97.5%, and 88.5%, respectively. Based on the Youden index, the cutoff value was 0.5 and the area under the curve was 0.989. These prove that our differentiation model produced a high diagnostic efficacy. In addition, it is easy for clinicians to apply in clinical practice. The differentiation model could help avoid a misdiagnosis between CD and PIL.

Positive positron emission tomography/computed tomography (PET/CT) plays an important role in the diagnosis and therapeutic evaluation of lymphomas^[23]. However, we did not take PET/CT parameters into consideration in our differentiation model. Our concern was that the CD patients were already at an increased risk of malignancies^[24]. Besides, PET/CT is too expensive. Thus, it is not suitable for all CD patients to undergo PET/CT examination. We recommend that only intricate cases with a grade of nearly 0.5 in our differentiation model should proceed to PET/CT examination. If a more accurate diagnosis can not be made, surgery should be earnestly considered.

This study has some limitations. First, this study is retrospective; thus, diseases other than CD and PIL were not included in the analysis. Consequently,

clinicians could use our model to avoid misdiagnosing PIL as CD when they had excluded other diseases, which may hamper its application. In the future, a prospective study should be conducted to discriminate bowel ulcers. Second, the number of patients enrolled was limited, and our differentiation model should be further proved. We are looking forward to conducting a multicenter collaboration to enlarge the sample size and modify or further prove our differentiation model.

In conclusion, CD and PIL have overlapping features, which continuously perplex clinicians. However, some valuable parameters can be used to differentiate these two conditions, including clinical manifestations, laboratory test results, endoscopic features, and CTE characteristics. Our differential model integrated various parameters to yield a high diagnostic efficacy, which should be further proved.

COMMENTS

Background

Crohn's disease (CD) and primary intestinal lymphoma (PIL) have overlapping clinical manifestations which sometimes make differential diagnosis difficult. Misdiagnosis of PIL could cause disastrous outcomes in patients. For lesions in the deep small intestine, qualified tissues for accurate diagnosis is difficult to acquire without surgery.

Research frontiers

Some studies focused on the differential diagnosis of small bowel ulcers. However, currently, none of these studies focused on the differential diagnosis between CD and PIL without histology. Some valuable parameters have been proposed, but no scoring system has been established for differential diagnosis.

Innovations and breakthroughs

In this study, the authors evaluated the usefulness of various parameters, including clinical manifestations, laboratory test results, endoscopic features, and computed tomographic enterographic characteristics, for differentiating CD from PIL. A scoring system was established. These will help clinical practitioners to avoid misdiagnosis of these two conditions.

Applications

This scoring system would provide a more objective and convenient tool for the differential diagnosis between CD and PIL.

Peer-review

It is an interesting retrospective study on the clinical, laboratory, endoscopic, and CTE features of CD and PIL. The authors defined some important parameters that are important in the differential diagnosis of these two entities.

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Retrospective Study

Full-thickness myotomy is associated with higher rate of postoperative gastroesophageal reflux disease

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Abstract

AIM

To compare long-term occurrence of gastroesophageal reflux disease (GERD) between two different types of peroral endoscopic myotomy (POEM) for achalasia.

METHODS

We included all patients with achalasia who underwent POEM at our hospital from August 2011 to October 2012 and had complete GERD evaluation with ≥ 3 years of follow-up. They were divided into circular or full-thickness myotomy groups according to the depth of myotomy. Demographics, Eckardt score, manometry results, 24-h pH monitoring, and GERD symptoms were recorded and compared between the two groups.

RESULTS

We studied 56 patients (32 circular myotomy and 24 full-thickness myotomy) with complete GERD evaluation. There was no significant difference between the two groups in terms of treatment success (defined as Eckardt score ≤ 3), postoperative Eckardt score, mean basal lower esophageal sphincter pressure, and 4-s integrated relaxation pressure (4sIRP). Postoperative abnormal esophageal acid exposure was found in 25 patients (44.6%). A total of 13 patients (23.2%) had GERD symptoms and 12 had esophagitis (21.4%). Clinically relevant GERD (abnormal esophageal acid exposure associated with GERD symptoms and/or esophagitis) was diagnosed in 13 patients (23.2%).

Multivariate analysis revealed that full-thickness myotomy and low level of postoperative 4sIRP were predictive factors for clinically relevant GERD.

CONCLUSION

Efficacy and manometry are comparable between achalasia patients treated with circular or full-thickness myotomy. But patients with full-thickness myotomy and low postoperative 4sIRP have more GERD.

Key words: Achalasia; Gastroesophageal reflux disease; Peroral endoscopic myotomy; Circular myotomy; Full-thickness myotomy

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Core tip: Gastroesophageal reflux disease (GERD) is a major concern following peroral endoscopic myotomy for achalasia. Although short-term follow-up did not show an increased rate of GERD in patients who received full-thickness myotomy compared with circular myotomy, the long-term difference is still unknown. We found that full-thickness myotomy is associated with a higher rate of clinically relevant GERD.

Wang XH, Tan YY, Zhu HY, Li CJ, Liu DL. Full-thickness myotomy is associated with higher rate of postoperative gastroesophageal reflux disease. *World J Gastroenterol* 2016; 22(42): 9419-9426 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9419.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9419>

INTRODUCTION

Achalasia is a rare primary esophageal dysmotility disorder characterized by failed relaxation of the lower esophageal sphincter (LES) and absence of peristalsis of the distal esophagus^[1]. All therapeutic options focus on reducing the pressure gradient across the LES, which can result in potential gastroesophageal reflux disease (GERD). Therefore, an antireflux procedure is usually added to surgical myotomy, and it has been proven useful in reducing the rate of postoperative GERD^[2,3]. Peroral endoscopic myotomy (POEM) is an endoscopic, minimally invasive treatment for achalasia in which myotomy is performed within a submucosal tunnel approached from the esophageal lumen, and this procedure can relieve symptoms and improve quality of life^[4,5]. In contrast to Heller myotomy, no antireflux procedure is included in POEM, and the reported rates of postoperative reflux have varied widely, from 0% to 53%^[6-9]. To reduce GERD, circular myotomy without disturbing the longitudinal muscle is usually recommended by most researchers^[6,9,10]. Some researchers have performed full-thickness myotomy, and several clinical studies have demonstrated that full-thickness myotomy can accelerate recovery of

esophageal emptying and reduce operating time, while not increasing the rate of GERD^[11,12]. However, these studies had only short-term follow-up, and whether full-thickness myotomy increases the rate of GERD in the long term is still unknown.

The aims of the present study were to compare the long-term occurrence of GERD in achalasia patients treated with circular or full-thickness myotomy during POEM, and to identify the predictive factors of postoperative GERD.

MATERIALS AND METHODS

Patients

This was a single-center retrospective study conducted in China. The study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University, Changsha, China, and all the participants gave signed informed consent. One hundred and ten patients with achalasia underwent POEM at our hospital between August 2011 and October 2012. In the beginning, simple circular myotomy was performed for all patients with achalasia and there were 32 patients included, but we found that some patients did not receive satisfactory outcome. So, we began to perform full-thickness myotomy beginning in March 2012, and there were 24 patients enrolled in total. Each patient was called back for an assessment of GERD at least 3 years after POEM. Only patients who underwent a complete assessment of GERD, including symptom evaluation (including Eckardt score^[13] and GerdQ score^[14]), esophagogastroduodenoscopy (EGD), high-resolution manometry (HRM) and esophageal pH monitoring were included in the study. The patients' demographics and clinical history, perioperative data, myotomy type, and follow-up data were retrospectively collected and recorded.

POEM

POEM was performed as previously described under general anesthesia *via* intratracheal intubation, with the patient in the left lateral position^[13]: (1) a submucosal injection into the posterior esophageal wall (5 o'clock position) was administered at 8-10 cm above the esophagogastric junction (EGJ); (2) a 2-3-cm longitudinal mucosal incision was made to create a tunnel entry; (3) a submucosal tunnel was created, passing over the EGJ, and approximately 3 cm into the proximal stomach; (4) myotomy was started from 2 to 3 cm below the tunnel entry. Some patients received circular myotomy alone and others received full-thickness myotomy at approximately 3 cm above or below the EGJ; and (5) several metal clips were applied to close the mucosal entry. Figures 1 and 2 describe the procedure of circular and full-thickness myotomy. Patients were maintained as nil by mouth for 24 h with a liquid diet for 3 d, and returned gradually to a normal diet within 2 wk. Intravenous proton pump inhibitor (PPI) was used for 3 d.

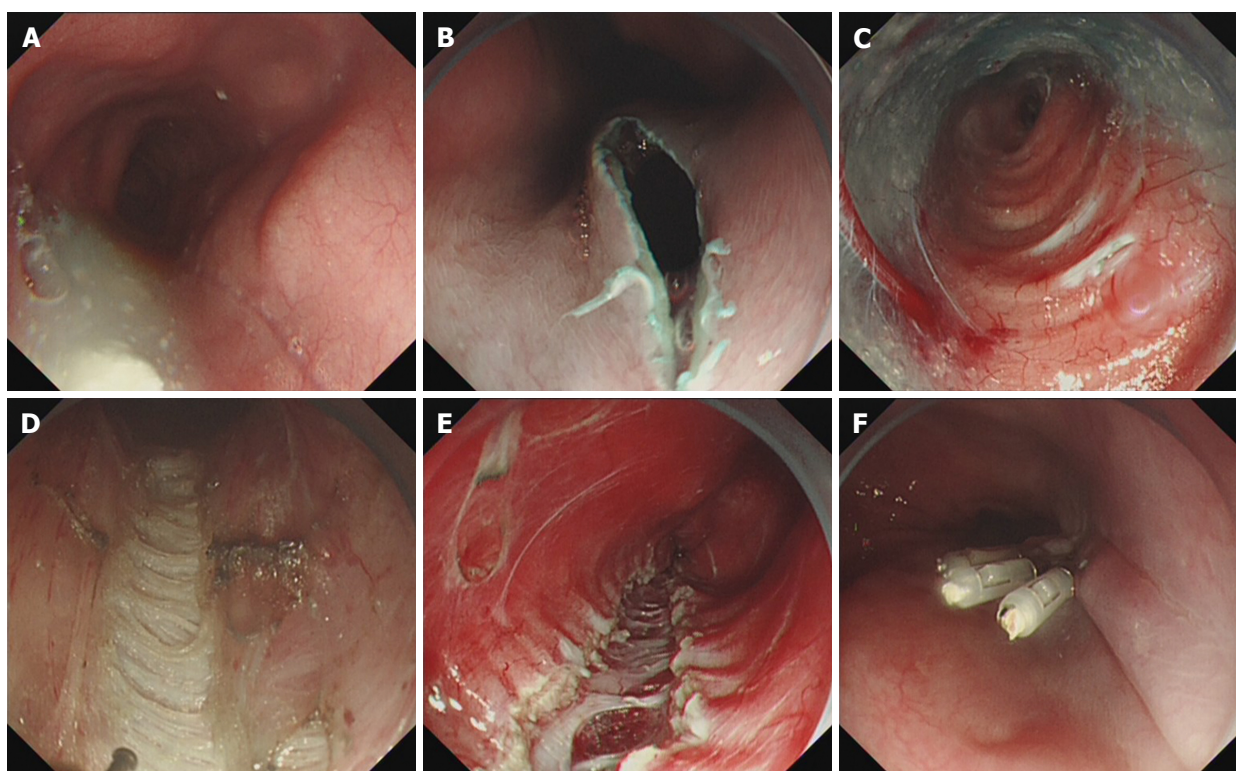


Figure 1 Case illustration of peroral endoscopic circular myotomy. A: Endoscopy showing dilated esophagus; B: Longitudinal mucosal incision was made to create a tunnel entry; C: Submucosal tunnel; D: Endoscopic image of the circular muscle; E: Circular myotomy; F: Tunnel entry was closed with several clips.

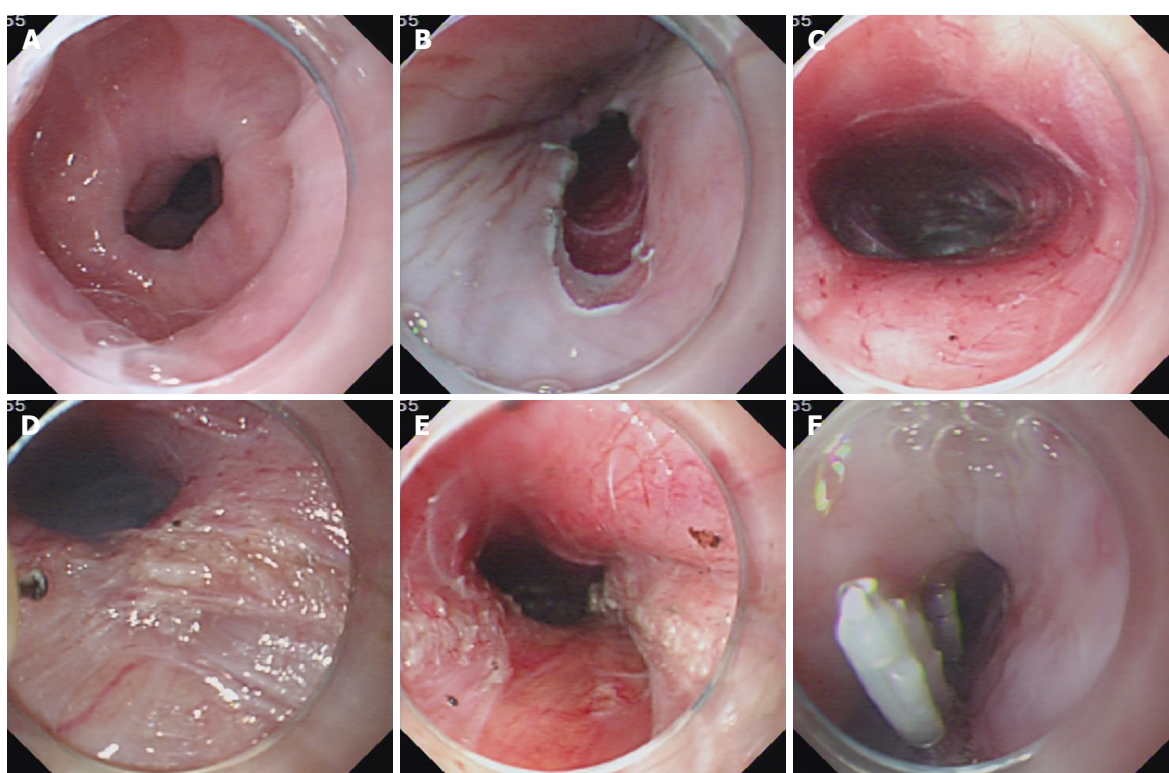


Figure 2 Case illustration of peroral endoscopic full-thickness myotomy. A: Endoscopy showing dilated esophagus; B: Longitudinal mucosal incision was made to create a tunnel entry; C: Submucosal tunnel; D: Circular myotomy was initially performed; E: Full-thickness myotomy was performed; F: Tunnel entry was closed with several clips.

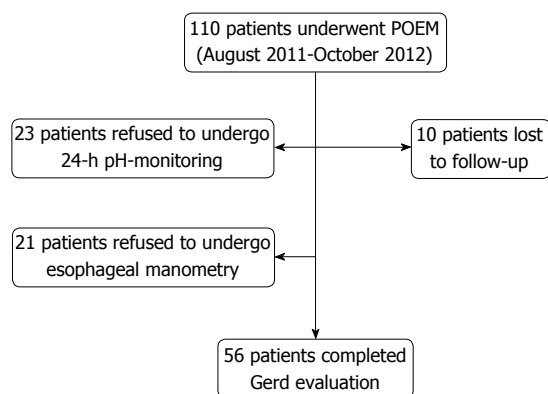


Figure 3 Patient selection and enrollment. POEM: Peroral endoscopic myotomy; GERD: Gastroesophageal reflux disease.

HRM

HRM was done using a solid-state manometric assembly with 36 pressure sensors spaced at 1-cm intervals (Manoscan ESO; Given Imaging, Yoqneam, Israel). The HRM assembly was placed transnasally and the manometric catheter was positioned to record from the hypopharynx to the stomach with approximately five intragastric sensors. The manometric protocol included a 5-min period to assess basal LES pressure (LESP), 10 water swallows of 5 mL each, and one water swallow each of 1 mL (dry), 10 mL and 20 mL. Basal LESP and average 4-s integrated relaxation pressure (4sIRP) were recorded.

Esophageal pH monitoring

Twenty-four-hour esophageal pH monitoring (Digitrapper pH-Z Monitoring; Given Imaging) was carried out before EGD. The pH-monitoring catheter with two sensors was introduced transnasally into the esophagus. One of the two sensors was pushed through the EGJ into the stomach; the other sensor was kept approximately 5 cm proximal to the EGJ. Data were recorded for 24 h. Measurements were always carried out after cessation of acid-suppressive medication for at least 7 d. In the analysis of pH signals, episodes with pH < 4 caused by stasis-associated acidification of esophageal contents were discarded.

Study outcome

Primary outcome was clinically relevant GERD defined as abnormal esophageal acid exposure associated with GERD symptoms and/or esophagitis^[15]. Secondary outcomes were Eckardt score and manometry results. Abnormal esophageal acid exposure was defined by percentage total reflux time (%TRT; esophageal pH < 4) > 5%. GerdQ score was used to assess for symptoms suggestive of GERD, and a total GerdQ score > 7 was considered indicative of significant GERD symptoms^[14]. Reflux esophagitis was classified according to the Los Angeles Classification^[16].

Statistical analysis

SPSS version 21.0 was used for data analysis.

Continuous variables were presented as mean \pm standard deviation and analyzed using Student's *t*-test. Qualitative data were presented as frequencies and calculated using the χ^2 test or Fisher's exact test. Univariate and multivariate analyses were used to identify predictive factors associated with clinically relevant GERD. Variables analyzed included preoperative data [sex, age, disease course, previous treatment, esophageal shape (sigmoid vs non-sigmoid), Eckardt score, LESP, 4sIRP, and achalasia type according to the Chicago Classification], perioperative data (myotomy depth), and postoperative factors LESP, 4sIRP, Eckardt score]. A two-tailed *P* value < 0.05 was considered as statistically significant in all cases.

RESULTS

Efficacy of POEM

Fifty-four of the original 110 cases were not included in the present study. We enrolled 56 patients (mean age: 42 years, range: 14–71 years; female/male: 30/26) with completed assessment of GERD (Figure 3). Thirty-two patients received circular myotomy and 24 received full-thickness myotomy. All of the patients underwent POEM, and treatment success (defined as Eckardt score ≤ 3) was achieved in all 56 patients during a mean follow-up of 39.3 mo. Mean Eckardt score decreased significantly from 6.5 ± 1.4 (baseline value) to 0.4 ± 0.7 ($P < 0.001$). Postoperative mean basal LESP and 4sIRP were significantly lower compared to the preoperative values (39.0 ± 6.6 and 28.6 ± 5.1 mmHg vs 14.4 ± 3.4 and 10.5 ± 2.6 mmHg, respectively, $P < 0.01$). There was no significant difference between circular and full-thickness myotomy in terms of treatment success, pre- and postoperative esophageal manometry and Eckardt score (Table 1).

Clinically relevant GERD after POEM

Postoperative abnormal esophageal acid exposure was attested in 25 patients (44.6%). A total of 13 patients (23.2%) had GERD symptoms and 12 had esophagitis (21.4%). Overall, clinically relevant GERD (abnormal esophageal acid exposure, associated with GERD symptoms and/or esophagitis) was diagnosed in 13 patients (23.2%). Among the 12 cases of esophagitis, five were Los Angeles Grade A and seven were Grade B (Figure 4). Patients with GERD symptoms had complete clinical remission under standard PPI therapy (*i.e.*, pantoprazole 40 mg q.d. or lansoprazole 30 mg q.d.). In cases of esophagitis, double-dose PPI (*i.e.*, esomeprazole 40 mg b.i.d. or lansoprazole 30 mg b.i.d.) was prescribed for 6 wk. A follow-up EGD was carried out 6 wk after diagnosis and PPI therapy. Esophagitis healed completely in all patients, and no cases of Barrett's esophagus were diagnosed.

Predictive factors for clinically relevant GERD

Univariate analysis revealed that full-thickness myotomy,

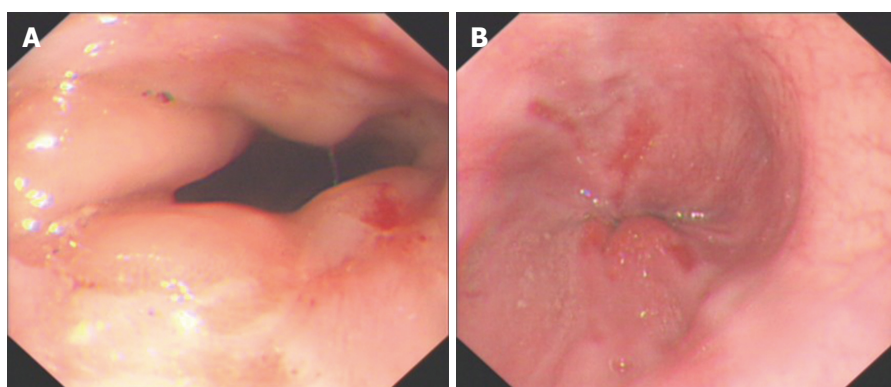


Figure 4 Endoscopic images of esophagitis after peroral endoscopic myotomy. A: Los Angeles Grade A esophagitis; B: Los Angeles Grade B esophagitis.

Table 1 Comparison of characteristics between the two groups

	Circular myotomy (n = 32)	Full-thickness myotomy (n = 24)	P value
Sex, M/F	13/19	11/13	0.315
Age (yr)	41.5 ± 10.8	44.5 ± 14.5	0.375
Disease course (yr)	5.3 ± 7.0	6.6 ± 8.4	0.535
Esophagus type, S/non-S	1/31	3/21	0.303
Previous therapy, Yes/No	6/26	5/19	0.846
Achalasia type, I / II / III	5/24/3	4/18/2	0.987
Pre- Eckardt score	6.4 ± 1.3	6.5 ± 1.6	0.784
POEM LESP, mmHg	39.5 ± 7.1	38.3 ± 6.0	0.502
4sIRP, mmHg	28.4 ± 5.0	28.7 ± 5.3	0.849
Follow-up, mo	39.8 ± 4.2	38.6 ± 1.8	0.201
Post- Eckardt score	0.47 ± 0.67	0.38 ± 0.65	0.602
POEM LESP (mmHg)	14.6 ± 3.7	14.0 ± 3.0	0.500
4sIRP (mmHg)	10.6 ± 2.8	10.2 ± 2.2	0.545
GERD symptoms	15.6% (5/32)	33.3% (8/24)	0.12
pH test +	40.6% (13/32)	50% (12/24)	0.485
Esophagitis (%)	15.6% (5/32)	29.2% (7/24)	0.222
Clinically relevant GERD	12.5% (4/32)	37.5% (9/24)	0.028

S/non-S: Sigmoid-type/non-sigmoid-type; GERD: Gastroesophageal reflux disease; pH test +: Abnormal esophageal acid exposure.

lower postoperative LESP and 4sIRP were predictive factors for clinically relevant GERD. Multivariate analysis further indicated that full-thickness myotomy and lower postoperative 4sIRP were predictive factors for clinically relevant GERD (Table 2). Patients who underwent full-thickness myotomy had a higher rate of clinically relevant GERD (circular vs full-thickness myotomy, 4/32 vs 9/24, $P < 0.05$). Patients who had clinically relevant GERD had a lower level of post-POEM 4sIRP than those without clinically relevant GERD (8.6 ± 1.6 mmHg vs 11.0 ± 2.7 mmHg, $P < 0.05$).

DISCUSSION

We demonstrated that the treatment efficacy and manometry outcomes were comparable between circular and full-thickness myotomy during a minimum follow-up of 3 years. However, patients with full-thickness myotomy may have a higher rate of clinically relevant GERD. To the best of our knowledge, this is the first study to compare efficacy and postoperative GERD between circular and full-thickness myotomy

with long-term follow-up.

Each treatment of achalasia aims at reducing basal and swallow-induced pressure of LES to relieve symptoms, facilitate esophageal emptying and prevent development of mega-esophagus^[1]. However, iatrogenic GERD caused by decreased LESP is a common concern after endoscopic or surgical treatment of achalasia. GERD can cause symptoms such as heartburn and reflux, which impair quality of life of affected patients and increase their economic burden. Some GERD patients may develop esophagitis with the possible, even if rare, evolution of peptic stricture, Barrett's esophagus and even esophageal adenocarcinoma^[17,18]. Thus, preventing postoperative GERD is important for patients who receive endoscopic or surgical treatment of achalasia. In Heller myotomy, which is still the standard method for treating achalasia, it is accepted that partial fundoplication is mandatory and effective for reducing the incidence of postoperative GERD^[2,3]. POEM is an endoscopic treatment for achalasia in which myotomy is performed within a submucosal tunnel approached from the esophageal lumen, and because it includes no

Table 2 Multivariate analyses for risk factors of clinically relevant gastroesophageal reflux disease

Item	B	SE	Sig	OR	95%CI of OR
Constant	8.810	3.261	0.007	0.000	
Full-thickness myotomy	1.835	0.806	0.023	6.262	1.289, 30.413
Postoperative 4sIRP	0.769	0.299	6.628	2.158	1.202, 3.877

4sIRP: 4-s integrated relaxation pressure; OR: Odds ratio.

antireflux procedure, it has the potential to cause GERD.

The reported rates of prevalence of GERD after POEM vary from 0% to 53%^[6-9]. Some of this variability may relate to differences in subjective and objective measurements of GERD. Rarely, GERD was systematically evaluated on the entire POEM population with EGD, HRM and pH monitoring. Familiari *et al.*^[15] enrolled 103 patients, and found that 50.5% had abnormal esophageal acid exposure, 18.4% had heartburn, 20.4% had esophagitis, and 29.1% had clinically relevant GERD. In the present study, postoperative esophageal acid exposure was found in 44.6% of the patients, 23.2% had GERD symptoms, 21.4% had esophagitis, and 23.2% had clinically relevant GERD, which was consistent with Familiari's study^[15]. Jones *et al.*^[14] found that 58% (15/26) of the achalasia patients had abnormal pH test results after POEM, and subjective symptoms were not correlated with pH test results. Our study also found that not all the patients with abnormal pH test results (25/56) had GERD symptoms (13/56).

In POEM, circular myotomy with preservation of the longitudinal muscle is usually recommended to decrease the potential risk of postoperative GERD, as the longitudinal muscle plays an important role against GERD. However, complete myotomy involving both circular and longitudinal layers is a prerequisite for sufficient and long-term reduction of LESP and is the basis for excellent results of conventional surgical myotomy^[19,20]. Thus, some researchers perform full-thickness myotomy considering that it theoretically results in better long-term efficacy. von Renteln *et al.*^[12] enrolled 16 patients (9 circular myotomy and 7 full-thickness myotomy) with 3 mo follow-up, and found that there was no significant difference in efficacy and complications between the two groups, but full-thickness myotomy may be superior in rapid esophagogastric emptying. Li *et al.*^[11] enrolled 234 patients (131 circular myotomy and 103 full-thickness myotomy) with a follow-up of 6-10 mo, and showed that there was no difference in short-term outcomes between the two groups. However, full-thickness myotomy decreased the procedure time without increasing procedure-related and clinical reflux complications. Whether full-thickness myotomy leads to better long-term efficacy and/or increased rate of

GERD remains unclear.

In the present study, 32 patients received circular myotomy and 24 received full-thickness myotomy with a minimum follow-up of 3 years. We demonstrated that the treatment efficacy and manometry outcomes were comparable between the two groups, but patients with full-thickness myotomy had a higher rate of clinically relevant GERD. This result is not in favor of full-thickness myotomy, but a randomized prospective study with long-term follow-up is warranted to confirm our conclusion and to analyze cost-effectiveness.

Familiari *et al.*^[15] found that patients with heartburn had a lower postoperative 4sIRP compared to patients without symptoms (7.6 ± 3.8 mmHg vs 10.01 ± 4.4 mmHg, $P < 0.05$). We had consistent results in our study: low postoperative 4sIRP was a predictive factor of clinically relevant GERD after POEM, and the level of 4sIRP was lower in patients with than without clinically relevant GERD. Average 4sIRP > 15 mmHg is considered indicative of impaired LES relaxation and characteristic of achalasia, and 4sIRP < 15 mmHg is regarded as indicative of treatment success. However, both Familiari's and our studies suggested that postoperative 4sIRP was not "the lower, the better". Further studies may evaluate the appropriate level of postoperative 4sIRP to balance the treatment efficacy and risk of postoperative GERD.

The present study had several limitations. First, this was a retrospective study, and only some of our patients who underwent complete assessment of GERD were included. Some of the patients were lost to follow-up or refused to undergo pH testing or HRM. This bias affected the chances of calculating the real prevalence of GERD after POEM, as those who had symptoms were more likely to undergo the assessment. Second, GERD evaluation was done only for those with a minimum follow-up of 3 years, thus we do not know how postoperative GERD changes with time. GERD is regarded as a late complication after POEM, and its incidence increases with time^[7]. Thus, GERD evaluation should also be carried out at 5 years, 10 years or even longer after POEM. Third, depth of myotomy was the only perioperative data that we measured. Other data such as myotomy length (all patients underwent a standard myotomy length) and orientation of myotomy (all patients underwent posterior myotomy) were not included in the study. As a consequence, it is not possible to define whether these parameters are associated with postoperative GERD.

In conclusion, our study shows that the treatment efficacy and manometry outcomes were comparable between the circular and full-thickness myotomy groups. However, patients with full-thickness myotomy and low postoperative 4sIRP may have a higher rate of clinically relevant GERD. Randomized, large-scale studies are warranted to confirm our conclusions.

COMMENTS

Background

All therapeutic options for achalasia focus on reducing the pressure gradient across the lower esophageal sphincter (LES), and an antireflux procedure is generally recommended in surgery to reduce the risk of potential gastroesophageal reflux disease (GERD). In contrast to Heller myotomy, no antireflux procedure is included in peroral endoscopic myotomy (POEM), especially full-thickness myotomy, and the rate of postoperative reflux varies from 0% to 53%.

Research frontiers

Several clinical studies have compared the safety and efficacy of circular and full-thickness myotomy, and have demonstrated that full-thickness myotomy can accelerate recovery of esophageal emptying and reduce operating time while not increasing the rate of GERD. However, these studies had short-term follow-up, and whether full-thickness myotomy increases the rate of GERD in the long term is still unknown.

Innovations and breakthroughs

Whether the risk of postoperative GERD in full-thickness myotomy is higher than in circular myotomy is still unclear. This study compared the long-term occurrence of GERD between circular and full-thickness myotomy during POEM for achalasia, and identified the predictive factors. This is the first study to compare the efficacy and postoperative GERD between circular and full-thickness myotomy with long-term follow-up.

Applications

In the present study, the authors have demonstrated that achalasia patients who received full-thickness myotomy during POEM had a higher rate of postoperative GERD without obvious advantages of better efficacy than circular myotomy, which suggest that circular myotomy is optimal during POEM for achalasia.

Terminology

Full-thickness myotomy is a type of POEM that includes completeness of myotomy involving both circular and longitudinal muscle fibers to preserve long-term reduction of LES pressure. GERD is a chronic condition with mucosal damage caused by stomach acid coming up from the stomach into the esophagus. Occasional reflux causes heartburn, but chronic reflux leads to reflux esophagitis, GERD, and sometimes Barrett's esophagus.

Peer-review

In this study, the authors analyzed the result of POEM and the incidence of GERD in a group of 56 patients who underwent POEM for esophageal achalasia and received a full assessment for GERD 3 years after the procedure. The authors have presented interesting new insights on iatrogenic GERD rates for different types of POEM procedures for a respectable follow-up period.

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Observational Study

Occult hepatitis B virus infection is not associated with disease progression of chronic hepatitis C virus infection

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Abstract

AIM

To clarify the prevalence of occult hepatitis B virus (HBV) infection (OBI) and the association between OBI and liver disease progression, defined as development of liver cirrhosis or hepatocellular carcinoma (HCC), worsening of Child-Pugh class, or mortality in cases of chronic hepatitis C virus (HCV) infection.

METHODS

This prospective cohort study enrolled 174 patients with chronic HCV infection (chronic hepatitis, $n = 83$; cirrhosis, $n = 47$; HCC, $n = 44$), and evaluated disease progression during a mean follow-up of 38.7 mo. OBI was defined as HBV DNA positivity in 2 or more

different viral genomic regions by nested polymerase chain reaction using 4 sets of primers in the S, C, P and X open reading frame of the HBV genome.

RESULTS

The overall OBI prevalence in chronic HCV patients at enrollment was 18.4%, with 16.9%, 25.5% and 13.6% in the chronic hepatitis C, liver cirrhosis and HCC groups, respectively ($P = 0.845$). During follow-up, 52 patients showed disease progression, which was independently associated with aspartate aminotransferase > 40 IU/L, Child-Pugh score and sustained virologic response (SVR), but not with OBI positivity. In 136 patients who were not in the SVR state during the study period, OBI positivity was associated with neither disease progression, nor HCC development.

CONCLUSION

The prevalence of OBI in chronic HCV patients was 18.4%, and OBI was not associated with disease progression in South Koreans.

Key words: Hepatitis B virus; Hepatitis C virus; Disease control; Oncogenesis

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Core tip: Whether occult hepatitis B virus (HBV) infection affects the outcomes of chronic hepatitis C virus infection is controversial. This prospective observational study aimed to clarify the association between occult HBV infection and liver disease progression defined as development of liver cirrhosis, worsening of Child-Pugh class, hepatocellular carcinoma or mortality in patients with chronic hepatitis C infection in South Korea.

Cho JH, Lee SS, Choi YS, Jeon YJ, Chung JW, Baeg JY, Si WK, Jang ES, Kim JW, Jeong SH. Occult hepatitis B virus infection is not associated with disease progression of chronic hepatitis C virus infection. *World J Gastroenterol* 2016; 22(42): 9427-9436 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9427.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9427>

INTRODUCTION

Prevalence of hepatitis C virus (HCV) infection involves about 3% of the global population, or approximately 170 million people^[1]. Chronic HCV infection can lead to liver cirrhosis or hepatocellular carcinoma (HCC) in 20%-30% of cases^[2-4]. The HCV life cycle occurs exclusively in the cell cytoplasm, and the HCV RNA genome does not integrate into the host genome^[5,6]. Therefore, the pathogenic mechanisms of HCV include

complex interaction of HCV proteins and host proteins, inducing chronic inflammation, inhibition of apoptosis, stimulation of cell proliferation and fibrosis, leading to genetic alteration of hepatocytes, liver cirrhosis and ultimately HCC^[7]. In contrast, hepatitis B virus (HBV) has a direct oncogenic effect by integration of HBV DNA into the host genome, causing insertional mutagenesis, in addition to the indirect effects by HBV protein-host interaction.

HCV and HBV have similar transmission routes, and coinfection with HCV and HBV can increase the risk of HCC, compared to HCV mono-infection^[8]. Even if serum hepatitis B surface antigen (HBsAg) is negative, HBV DNA can exist in the liver or blood of people in an occult HBV infection (OBI) state. The pathogenic role of OBI in the development of cirrhosis or HCC among patients with chronic HCV infection is still extremely controversial^[9-12]. Moreover, the prevalence of OBI in chronic HCV-infected patients shows a wide range of variation in different global regions^[13,14]. There are no data on the prevalence or effect of OBI in chronic HCV infection in South Korea, an HBV endemic region.

This study aimed to clarify the prevalence of OBI in the blood of patients with chronic HCV infection, and to estimate the association between OBI and liver disease progression defined as development of liver cirrhosis, decompensation (worsening of Child-Pugh class), HCC or mortality among the subjects by prospective observation. We also investigated the HBV genotype on the HBsAg-coding open reading frame of HBV gene (S-ORF) in OBI-positive patients.

MATERIALS AND METHODS

Subjects

This prospective cohort study included 174 consecutively enrolled patients with HBsAg-negative, chronic HCV infection in Seoul National University Bundang Hospital between November 2005 and May 2014. Chronic HCV infection was defined as HCV RNA positivity > 6 mo with HBsAg negativity. Among them, 83 patients were given diagnoses of chronic hepatitis C, 47 patients of liver cirrhosis, and 44 patients of HCC. The diagnostic criteria for liver cirrhosis included the presence of portal hypertension manifested as splenomegaly, thrombocytopenia $< 100000/\text{mm}^3$, ascites, varices, or hepatic encephalopathy and imaging findings compatible with liver cirrhosis. The diagnosis of HCC was based on histological examination or typical radiographic image findings consisting of arterial enhancement and venous wash-out of hepatic nodules on contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI)^[15]. HBsAg-positive patients or human immunodeficiency virus coinfecting patients were excluded. Informed consent was obtained from all patients, and the study protocol was approved by the Institutional Review Board of the hospital.

Table 1 Baseline characteristics of 174 patients with chronic hepatitis C virus infection

Variable	Total (n = 174)	Chronic hepatitis (n = 83)	Liver cirrhosis (n = 47)	HCC (n = 44)
Mean age, yr ^{b,c}	66.5 ± 9.9	65.6 ± 9.8	65.3 ± 10.1	69.7 ± 9.3
Male sex	105 (60.3)	54 (65.1)	24 (51.1)	27 (61.4)
Body mass index (kg/m ²)	23.3 ± 3.0	23.6 ± 3.0	23.0 ± 3.2	23.0 ± 2.8
Ex or current smoker (n = 173)	51 (29.5)	26 (31.3)	10 (21.7)	15 (34.1)
Alcohol intake (social or heavy) (n = 173)	80 (46.2)	42 (50.6)	17 (37.0)	21 (47.8)
Anti-HBc (n = 100)	75 (75.0)	35 (71.4)	18 (85.7)	22 (73.3)
Hemoglobin (g/dL) ^c	13.5 ± 1.8	14.1 ± 1.5	13.5 ± 1.9	12.4 ± 1.9
Platelet (× 10 ³ /μL) ^{a,b}	151.3 ± 87.4	188.0 ± 55.7	117.5 ± 120.2	118.3 ± 66.2
Albumin (g/dL) ^{a,b}	4.0 ± 0.5	4.2 ± 0.2	3.8 ± 0.5	3.7 ± 0.5
Total bilirubin (mg/dL) ^a	1.0 ± 0.6	1.0 ± 1.7	1.2 ± 0.6	1.1 ± 0.8
ALP (IU/L) ^{a,b}	100.3 ± 47.3	89.2 ± 30.9	102.0 ± 37.0	119.3 ± 71.4
AST (IU/L) ^{a,b}	75.8 ± 103.3	76.0 ± 141.9	72.6 ± 47.8	78.8 ± 45.3
ALT (IU/L)	77.2 ± 138.5	89.7 ± 193.2	68.0 ± 59.3	63.3 ± 41.0
Creatinine (mg/dL) ^a	0.9 ± 0.4	1.0 ± 0.5	0.8 ± 0.2	0.9 ± 0.2
Prothrombin time (INR) ^{a,b}	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
AFP > 20 ng/mL (n = 171) ^{a,b}	30 (17.5)	5 (6.2)	9 (19.6)	16 (36.4)
Child-Pugh class (A/B/C) ^{a,b}				
A	160 (92.0)	83 (100)	40 (85.1)	37 (84.1)
B	12 (6.9)	0	5 (10.6)	7 (15.9)
C	2 (1.1)	0	2 (4.3)	0
MELD score (n = 167) ^{a,b}	8.5 ± 2.3	8.0 ± 2.3	9.3 ± 2.2	8.9 ± 2.1
HCV genotype (1/2) (n = 135)	60/75 (44.4%/55.6%)	32/35 (47.8%/52.2%)	16/26 (38.1%/61.9%)	12/14 (46.2%/53.8%)
Antiviral treatment				
No antiviral treatment	102 (58.7)	45 (54.2)	25 (53.2)	32 (72.7)
Treatment without SVR	34 (19.5)	14 (16.9)	11 (23.4)	9 (20.5)
Treatment with SVR ^{b,c}	38 (21.8)	24 (28.9)	11 (23.4)	3 (6.8)

Data are presented as mean ± SD or number (%). ^aP value < 0.05 between patients with chronic hepatitis and liver cirrhosis; ^bP value < 0.05 between patients with chronic hepatitis and HCC; ^cP value < 0.05 between patients with liver cirrhosis and HCC. ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; INR: International normalized ratio; SVR: Sustained virologic response.

Data collection and patient follow-up

At enrollment, data on the demographics, socioeconomic status, alcohol consumption, and smoking habits were obtained using a standard questionnaire. In addition, laboratory tests, ultrasonography or CT examination of the liver was performed for all patients at baseline. Those collected data were entered into the electronic case report form at the Korean HCV cohort study group homepage (available from URL: <http://www.hcvcohort.or.kr/>).

All patients were monitored for clinical status and given laboratory tests and imaging examinations, including ultrasonography, CT or MRI, every 3–12 mo. A total of 72 patients were treated with Pegylated interferon alpha (peg-IFN α) and ribavirin for 24–48 wk according to HCV genotype before the study enrollment (n = 24) and during the study period (n = 48). Survival and mortality, including cause of death, were confirmed using examination of the final medical records, telephone calls to participants or their family members, and death certificate data obtained from the Korean Statistical Information Service^[16]. The disease progression was defined as: (1) occurrence of liver cirrhosis, HCC, decompensation (worsening of Child-Pugh class), or mortality in patients with chronic hepatitis; (2) occurrence of HCC, decompensation, or mortality in patients with cirrhosis; and (3) occurrence

of mortality in patients with HCC. Time to disease progression was defined as the interval between the date of enrollment and the date of occurrence of HCC, liver cirrhosis, worsening of Child Pugh class, death, last observation, or September 30, 2015.

Blood collection and HBV DNA detection

Serum or plasma samples were obtained from 174 patients and stored at -70 °C. HBV DNA was extracted from 400 μL of serum or plasma using a QIAamp DNA Blood Mini Kit, (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. DNA was eluted from the spin column in 50 μL of elution buffer. Nested polymerase chain reaction (PCR) was conducted with AccuPower HotStart PCR PreMix (Catalog No. k-5051; Bioneer Inc., Seoul, Republic of Korea) using 4 sets of primers to detect S, C, P and X regions of the HBV genome (Supplementary Table 1). According to the *Taormina Expert Meeting Statements*^[17], the presence of OBI was defined as proved positivity in 2 or more different viral genomic regions by nested PCR. AM6 plasmid purchased from the Korea Cell Line Bank (positive control) and serum obtained from normal healthy persons or distilled water (negative control) was used. The first round of PCR was performed in a final volume of 20 μL at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s,

52–65 °C for 30 s (52 °C for S, 58 °C for C, 58 °C for P, 65 °C for X) and 72 °C for 30 s, and a final extension step of 72 °C for 5 min. Using 5 µL of the first PCR product, the second round PCR was performed under the same conditions as the first round of PCR except for the annealing step temperature (58 °C for S, 52 °C for C, 52 °C for P, 65 °C for X). By nested PCR with sets of 10-fold serially diluted AM6 templates, the detection limit was estimated as 54.5 copies/µL of AM6 plasmid.

HBV DNA sequence analyses and genotyping

For 5 OBI-positive samples, the entire S region (the preS1, preS2 and S region) of the HBV genome was amplified by nested PCR using different sets of primers. The PCR products were purified and sequenced to identify the HBV genotype. The condition for the first and second round of PCR was 95 °C for 5 min, followed by 30 cycles of 95 °C 60 s, 52 °C 45 s, and 72 °C 90 s with a final extension of 72 °C for 5 min. The PCR products were analyzed by electrophoresis on 1% agarose gels, which were stained with Gel Red, and visualized on a UV transilluminator. The PCR products were sequenced by a commercial sequencing company (Bioneer Inc.). HBV DNA sequences were aligned using Clustal W (<http://www.clustal.org>), and phylogenetic trees were constructed using the neighbor-joining method. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 re-samplings. MEGA version 6.0.6 was used for phylogenetic tree construction and mutation analysis. We determined HBV genotypes by phylogenetic analysis based on 14 reference strains obtained from GenBank (accession numbers AY641558.1, AF286594.1, AY247032.1, AY641559.1, D16667.1, D50519.1, AF305422.1, M57663.2, X70185.1, AB100695.1, D00329.1, AB074755.1, X02496.1, AB554024.1).

Statistical analysis

Categorical variables were compared with the chi-square test or 2-tailed Fisher's exact test, and continuous variables were compared with the Mann-Whitney test or Kruskal-Wallis test. The cumulative probabilities of disease progression and HCC were analyzed using the Kaplan-Meier method. Predictors associated with disease progression and HCC were determined by the Cox proportional regression model. Risk was expressed by hazard ratio and 95%CI. A *P* value of < 0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using PASW software (version 22; IBM SPSS Statistics for Windows, Armonk, NY, United States).

RESULTS

Patient characteristics

The baseline characteristics of the 174 study patients with chronic HCV infection are summarized in Table 1,

showing the mean age to be 66.5 years, male sex for 60.3%, and past or current alcohol users of 46.2%. They included 83 patients with chronic hepatitis, 47 patients with liver cirrhosis and 44 patients with HCC. The HCV genotype 1 and 2 were detected in 60 patients (44.4%) and 75 patients (55.6%), respectively. Antiviral therapy with peg-IFN α and ribavirin combination regimen was undertaken in 72 patients before enrollment or during follow-up, and overall sustained virologic response (SVR) rate (defined as undetectable serum HCV RNA at 24 wk after the end of the treatment) was 52.8%. The anti-hepatitis B core immunoglobulin G test was performed in 100 patients, for which 75 patients showed positive results.

Prevalence of OBI and clinical factors related to OBI positivity

The positive detection rate of plasma or serum HBV DNA was 18.4% (32 among 174 total patients) defined as at least 2 positive results on nested PCR among 4 different sets covering 4 ORFs of HBV genomes. The positive rate was not different among 3 different diagnostic categories at enrollment: 14 of 83 (16.9%) in chronic hepatitis C, 12 of 47 (25.5%) in liver cirrhosis, and 6 of 44 (13.6%) in HCC (Figure 1). Therefore, the prevalence of OBI did not parallel the severity of liver disease at study enrollment.

To investigate the clinical factors that might be associated with OBI positivity, we compared various variables including age, sex, body mass index, laboratory results, model for end-stage liver disease score, Child-Pugh score and HCV genotypes between the patients with and without OBI. However, there were no significant differences between OBI-positive and OBI-negative patients, as shown in Table 2.

Effect of OBI positivity on disease progression and HCC development

During the mean follow-up duration of 37.4 mo, 52 patients showed composite disease progression: 12 patients developed liver cirrhosis from chronic hepatitis; 13 patients developed decompensated cirrhosis (worsening of Child-Pugh class); 14 patients developed HCC; and 13 patients died. As shown in Table 3, OBI positivity was not a significant factor associated with disease progression in either univariate or multivariate analysis. However, AST > 40 IU/L, increased Child-Pugh score at enrollment, and SVR were significantly associated with disease progression in multivariate analysis. Moreover, there was no significant difference in development of disease progression between the patients with or without OBI (Figure 2A).

Of the 130 patients without HCC at enrollment, there was no significant difference in HCC development between the patients with or without OBI (Figure 2B). On multivariate analysis, Child-Pugh score was an independent factor for HCC development (Table 4).

After exclusion of 34 patients who achieved SVR

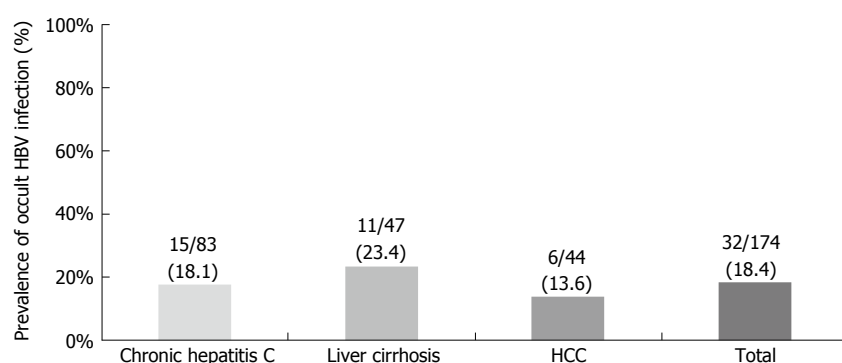


Figure 1 At study enrollment, the prevalence of occult hepatitis B virus infection in blood of patients with chronic hepatitis C virus infection. The prevalence of HBV DNA in blood among 174 patients was 32 (18.4%) by nested PCR; 15 of 83 (18.1%) were positive in the chronic hepatitis C group, 11 of 47 (23.4%) in the liver cirrhosis group, and 6 of 44 (13.6%) in the HCC group. The difference was not significant. HCC: Hepatocellular carcinoma.

Table 2 Comparison of clinical factors between patients with and without occult hepatitis B virus infection

Variable	OBI (+) (n = 32)	OBI (-) (n = 142)	P value
Mean age, yr	67.1 ± 9.6	66.4 ± 9.9	0.952
Male sex	19 (59.4)	86 (60.6)	0.527
Body mass index (kg/m ²)	23.9 ± 3.2	23.2 ± 3.0	0.180
Anti-HBc (n = 100)	6 (66.7)	69 (75.8)	0.399
Anti-HBs (n = 157)	14 (48.3)	72 (56.3)	0.283
Hemoglobin (g/mL)	13.3 ± 1.3	13.5 ± 1.9	0.364
Platelet (× 10 ³ /mL)	150.5 ± 79.5	151.5 ± 89.3	0.978
Albumin (g/dL)	3.9 ± 0.5	4.0 ± 0.4	0.593
Total bilirubin (mg/dL)	1.1 ± 0.8	1.0 ± 0.5	0.118
AST (IU/L)	62.7 ± 40.3	78.8 ± 112.6	0.546
ALT (IU/L)	54.7 ± 43.5	82.2 ± 151.6	0.148
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.4	0.779
PT-INR	1.0 ± 0.1	1.0 ± 0.1	0.459
MELD score	8.3 ± 1.9	8.6 ± 2.4	0.744
HCV genotype (1/2)	12/15 (44.4%/55.6%)	48/60 (44.4%/55.6%)	0.587
AFP > 20 ng/mL	6 (19.4)	23 (16.5)	0.441
Child-Pugh class			0.436
A	28 (87.5)	132 (93.0)	
B	4 (12.5)	8 (5.6)	
C	0	2 (1.3)	
Antiviral treatment			
No antiviral treatment	21 (65.6)	81 (57.0)	0.430
Treatment without SVR	5 (19.2)	29 (26.4)	0.616
Treatment with SVR	6 (18.8)	32 (22.5)	0.814
Disease progression	7 (21.9)	45 (31.7)	0.392
Development of HCC	3 (11.5)	11 (10.6)	1.000
Follow-up period (mo)	42.5 ± 34.7	36.3 ± 26.9	0.555

Data are presented as mean ± SD or number (%). OBI: Occult hepatitis B virus infection; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; PT-INR: Prothrombin time-international normalized ratio; SVR: Sustained virologic response.

during the study period, OBI was not a significant factor associated with either disease progression, nor HCC development (Supplementary Figure 1).

OBI positivity according to serological markers of HBV

Anti-HBs and anti-HBc were both evaluated in 87 patients. OBI positivity was 2.9% (1/34) in anti-HBc (+) and anti-HBs (+) patients, 9.4% (3/32) in anti-HBc (+) and anti-HBs (-) patients, 11.1% in anti-HBc (-) and anti-HBs (+) patients, and 15.4% in anti-HBc (-) and anti-HBs (-) patients. Therefore, positivity for anti-HBc

alone did not represent OBI positivity.

HBV DNA genotype of HBV strains in OBI-positive patients

In the 32 OBI-positive patients, 5 samples were available for full S-ORF sequence analysis including preS1, preS2 and S gene. The phylogenetic analysis showed that all 5 samples were HBV genotype C2, which was same as the HBV genotype reported in almost all patients with chronic HBV infection in South Korea.

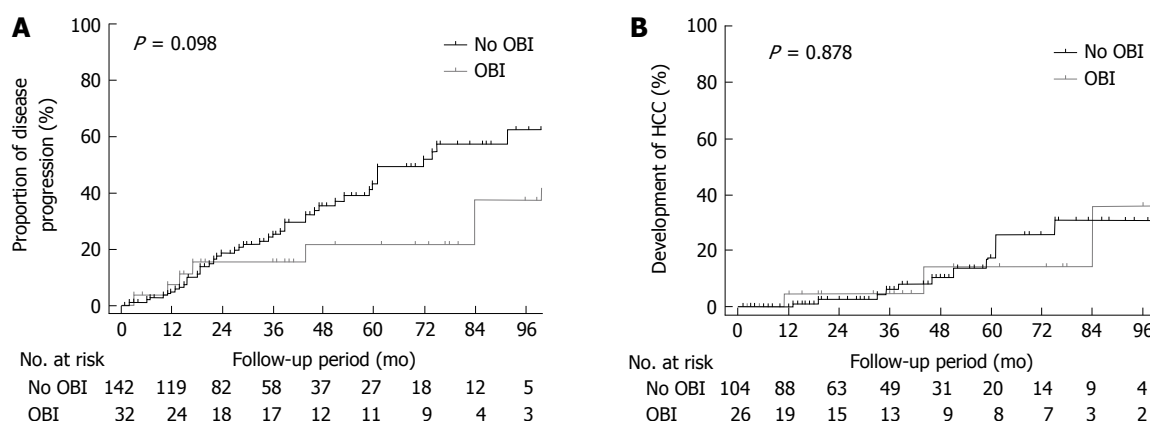


Figure 2 Effect of occult hepatitis B virus infection positivity on disease progression and hepatocellular carcinoma development. A: Cumulative incidence of disease progression defined as development of liver cirrhosis or HCC, worsening of Child Pugh class (A→B or C), or mortality according to OBI; B: Cumulative incidence of development of HCC according to OBI. HCC: Hepatocellular carcinoma; OBI: Occult hepatitis B virus infection.

Table 3 Clinical factors associated with disease progression, defined as development of liver cirrhosis, decompensation, hepatocellular carcinoma, or mortality ($n = 174$)

Variable	HR	P value	Adjusted HR	P value
OBI	0.494 (0.210-1.164)	0.107	0.510 (0.208-1.251)	0.141
Age (per year)	1.020 (0.990-1.050)	0.194		
Male sex	0.935 (0.534-1.636)	0.814		
Anti-HBc positivity	2.291 (0.878-5.976)	0.090		
AST > 40 IU/L	3.730 (1.740-7.996)	0.001	3.419 (1.117-10.463)	0.031
ALT > 40 IU/L	2.454 (1.273-4.730)	0.007	0.737 (0.297-1.826)	0.510
GGT > 70 IU/L	2.736 (1.571-4.765)	< 0.001		
AFP > 20 ng/mL	2.247 (1.238-4.079)	0.008	1.370 (0.721-2.600)	0.336
Child-Pugh score (per unit)	2.136 (1.628-2.802)	< 0.001	1.716 (1.230-2.394)	0.001
MELD score (per unit)	1.111 (1.009-1.224)	0.032	0.987 (0.860-1.134)	0.856
SVR	0.263 (0.104-0.663)	0.005	0.317 (0.121-0.828)	0.019
HCV genotype 1	1.465 (0.780-2.753)	0.231		

Disease progression defined as: (1) occurrence of liver cirrhosis, hepatocellular carcinoma (HCC), decompensation, or mortality in patients with chronic hepatitis; (2) occurrence of HCC, decompensation, or mortality in patients with cirrhosis; and (3) occurrence of mortality in patients with HCC. HR: Hazard ratio; OBI: Occult hepatitis B virus infection; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; MELD: Model for end-stage liver disease; SVR: Sustained virologic response; HCV: Hepatitis C virus.

DISCUSSION

In this study, the prevalence of OBI was 18.4% in the blood of HBsAg-negative patients with chronic HCV infection in South Korea by nested PCR, in whom OBI prevalence was not higher in the HCC group (13.6%) than in the chronic hepatitis group (18.1%) or liver cirrhosis group (23.4%). Moreover, neither positive OBI nor presence of anti-HBc was significantly associated with disease progression or HCC development on multivariate analysis. The HBV genotype detected in all 5 OBI-positive patients was genotype C2, which was the same as almost all of the detected genotypes in chronic hepatitis B patients in Korea.

The prevalence of OBI in the liver tissue or serum of anti-HCV-positive patients has been reported variously, both in prospective and retrospective studies. The prevalence of OBI detected in liver tissue was reported as 38.8% in 326 Italian patients with

chronic HCV infection^[18], 57% in 100 Portuguese patients^[19], and 15.0% in 167 Japanese patients as determined by nested PCR^[13], and 50% in 44 patients with HCV-related advanced cirrhosis in the United States as determined by real-time PCR^[20].

The prevalence of OBI in serum/plasma in HCV-related liver disease patients was 5.6% (8/141)^[21], 7.8% (11/140)^[22], and 5.2% (9/173) as determined by real-time PCR in Japan^[23], but it was 43.6% (204/468) in one Japanese study that using nested PCR with only one set of primers covering the X region^[24]. The OBI prevalence in chronic HCV patients was 45.7% (42/92) in Morocco^[25] and 20% (18/50) in Iran^[26], while none of 100 Portuguese patients showed serum OBI^[19]. A retrospective study in Taiwan showed that serum OBI prevalence as determined by nested PCR using 3 sets of primers in patients with chronic HCV infection was 14.8% (31 of 210), which did not differ from that of healthy controls (15%, 15/100), and the prevalence of

Table 4 Clinical factors associated with development of hepatocellular carcinoma

Variable	HR	P value	Adjusted HR	P value
OBI	0.904 (0.251-3.264)	0.878	0.860 (0.209-3.535)	0.835
Age (per year)	1.016 (0.962-1.073)	0.571		
Male sex	1.531 (0.536-4.374)	0.427		
AST > 40 IU/L	6.120 (1.351-27.729)	0.019	3.383 (0.664-17.226)	0.142
ALT > 40 IU/L	3.573 (0.976-13.075)	0.054		
Creatinine (per unit)	0.051 (0.003-0.892)	0.042	0.075 (0.003-2.200)	0.133
AFP > 20 ng/mL	3.381 (1.129-10.121)	0.029	1.706 (0.512-5.678)	0.384
PT-INR (per unit)	10.081 (1.036-77.802)	0.027		
Child-Pugh score (per unit)	3.065 (1.741-5.399)	< 0.001	2.818 (1.547-5.135)	0.001
MELD score (per unit)	1.057 (0.862-1.296)	0.597		
SVR	0.289 (0.064-1.302)	0.106		
HCV genotype 1	2.049 (0.644-6.517)	0.224		

HR: Hazard ratio; OBI: Occult hepatitis B virus infection; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; PT-INR: Prothrombin time-international normalized ratio; MELD: Model for end-stage liver disease; SVR: Sustained virologic response; HCV: Hepatitis C virus.

OBI did not parallel the severity of liver disease (14.5% in chronic hepatitis, 8% in liver cirrhosis, and 22% in HCC)^[27]. In the present study, the prevalence of OBI in plasma was 18.4% (32 of 174), which did not increase with the severity of liver disease, showing similar results to the Taiwan study consisting of the subjects and detection methods similar to this study.

The variously reported OBI prevalence in patients with chronic HCV infection may be related to the different study subjects, different materials used (liver tissue vs serum), or differences in PCR technology such as nested PCR or real-time PCR using different numbers of primer sets. Our method followed the *Taormina Expert Meeting Statements*^[17], in which the presence of OBI was defined as proved positivity in 2 or more different viral genomic regions by nested PCR and included adequate positive and negative controls. Previous data suggested that the prevalences of OBI detected in liver tissue were higher than those in blood, though reports of head-to-head comparisons of both methodologies are limited^[19,28]. Integration of the HBV genome into the host genome is a plausible explanation. In addition, risk factors of HCV infection may affect the prevalence of OBI. As shown in the above United States study, a history of intravenous drug abuse (IVDU) was found in 66% of the subjects, as opposed to 1% of our study population. Because HBV and HCV can share the parenteral transmission route, repeated exposure to HBV or HCV during IVDU may result in relatively high frequency of OBI in those subjects rather than those who were infected through perinatal infection as in Korea.

This study showed that OBI prevalence did not increase according to the severity of liver disease ($n = 174$), and during a mean prospective follow-up of 38 mo, OBI was not associated with either HCC development or the overall disease progression. However, SVR, Child-Pugh score and AST level were independent factors associated with the disease

progression. Even in the patients who did not receive treatment or did not achieve SVR ($n = 136$), OBI positivity was not associated with either HCC development or disease progression.

The role of OBI in disease progression or development of HCC in patients with chronic HCV infection is still a matter of considerable controversy. Some studies have reported that OBI may contribute to the development of HCC or cirrhosis in chronic HCV-infected patients^[9,10,18,21,23,24,29]. In contrast, other reports have shown that OBI is not an important factor in the progression to HCC or cirrhosis^[11,12,14,27,28,30,31]. Most previous studies were cross-sectional studies^[12,16,18,24], and a meta-analysis including both prospective and retrospective studies reported that OBI contributed to the development of HCC^[32]. A prospective study in Italy showed that among 94 patients who were tested for liver OBI and followed for a median 11 years, HCC developed more often in the OBI-positive group (13/37) than in the OBI-negative group (5/57), and OBI-positive patients had shorter cumulative survival rate than OBI-negative patients. Though this study suggests that OBI may lead to HCC development and lower survival, only 94 among a total 326 original study group were followed (follow-up missing rate of 71.2%) and 79 out of 94 patients underwent antiviral treatment with an SVR rate of 33% (26/79). Considering that SVR is a strong independent factor for HCC development or survival, more studies are needed to clarify the significance of OBI in HCV-related liver disease progression.

The possible mechanisms of OBI involvement in hepatocarcinogenesis were HBV-induced accelerated inflammation, HBV genome integration in the host DNA, or promoting effect of HBV proteins in malignant transformation. In a woodchuck model, persistently low level of viremia in liver tissue in the absence of woodchuck HBsAg can lead to liver injury and HCC development^[33]. However, there has been no clear

evidence supporting the hepatocarcinogenic role of OBI in HCV-related liver disease. Two recently reported independent *in vitro* studies showed that both HBV and HCV can replicate in the same hepatocyte cells without evidence of viral interference. Therefore, HCV and HBV may interfere with each other by indirect effects of host-viral interactions or host immune response.

Moreover, the epidemiology of HCV or HBV is different according to geographic regions or population. For example, the most common mode of transmission of HBV in Korean people has been perinatal transmission, while HCV infection occurs in later life. In contrast, IVDUs in an HBV non-endemic area may be infected by both viruses simultaneously or repeatedly. Those differences in epidemiology may lead to differences in immunological response to OBI in HCV-infected patients in different regions. In this study, a phylogenetic analysis showed that 5 strains from the occult HBV-infected subjects were all genotype C2, which was detected in nearly 100% of chronic hepatitis B patients in Korea. Because of sample availability, only 5 samples among the 32 patients with OBI were evaluated. Several mechanisms have been considered for occult infections by HBV, such as low HBV DNA and HBsAg levels, mutations in HBV DNA sequence, viral DNA integration in the host genome, infection of peripheral blood mononuclear cells, production of immune complexes containing HBV, altered host immune response, and interference of HCV^[34-37].

The present study had several limitations, including being a single center study with relatively small sample size, no availability of liver tissue and no results on quantitative evaluation of HBV DNA.

In conclusion, this study demonstrates that the prevalence of OBI in blood in patients with chronic HCV infection in South Korea was 18.4%, with no significant correlation between OBI positivity and disease progression or HCC. Controversy still exists regarding the role of OBI; therefore, well-designed prospective multicenter studies and experimental studies are warranted.

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COMMENTS

Background

Whether the occult hepatitis B virus (HBV) infection (OBI) affects the outcomes

of chronic hepatitis C virus (HCV) infection is controversial. This study aimed to clarify the prevalence of OBI and the association between OBI and liver disease progression in cases of chronic HCV infection.

Research frontiers

The prevalence of OBI in chronic HCV-infected patients has been reported variously in different global regions. The pathogenic role of OBI in the disease progression or development of hepatocellular carcinoma (HCC) in patients with chronic HCV infection is still a matter of considerable controversy. There are no data on the prevalence or effect of OBI in chronic HCV infection in Korea, an HBV endemic region.

Innovations and breakthroughs

The positive detection rate of plasma or serum HBV DNA was 18.4% (32 among 174 patients), defined as at least 2 positive results on nested polymerase chain reaction (PCR) using 4 sets of primers in the S, C, P and X open reading frame of the HBV genome. However, OBI positivity was not a significant factor associated with disease progression. In addition, there was no significant difference in HCC development between the patients with or without OBI.

Applications

This study demonstrates that the prevalence of OBI in patients with chronic HCV infection in Korea was 18.4%, with no significant correlation between OBI positivity and disease progression or HCC. Controversy still exists regarding the role of OBI; therefore, well-designed prospective multicenter studies and experimental studies are warranted.

Terminology

According to the *Taormina Expert Meeting Statements*, the presence of OBI was defined as proved positivity in 2 or more different viral genomic regions by nested PCR using 4 sets of primers to detect the S, C, P and X regions of the HBV genome. The disease progression was defined as: (1) occurrence of liver cirrhosis, HCC, decompensation (worsening of Child-Pugh class), or mortality in patients with chronic hepatitis; (2) occurrence of HCC, decompensation, or mortality in patients with cirrhosis; and (3) occurrence of mortality in patients with HCC.

Peer-review

This observational study represents an unbiased and well-articulated report on the usefulness of OBI measurement as a prognostic factor for liver disease progression.

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Observational Study

Prevalence of depression and anxiety in patients with chronic digestive system diseases: A multicenter epidemiological study

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Abstract

AIM

To investigate the prevalence of depression and anxiety in patients with chronic digestive system diseases.

METHODS

A total of 1736 patients with chronic digestive system

diseases were included in this cross-sectional study, including 871 outpatients and 865 in-patients. A self-designed General Information for Patients of the Department of Gastroenterology of General Hospitals questionnaire was used to collect each patient's general information, which included demographic data (including age, sex, marital status, and education) and disease characteristics (including major diseases, disease duration, principal symptoms, chronic pain, sleep disorder, and limited daily activities).

RESULTS

The overall detection rate was 31.11% (540/1736) for depression symptoms alone, 27.02% (469/1736) for anxiety symptoms alone, 20.68% (359/1736) for both depression and anxiety symptoms, and 37.44% (650/1736) for either depression or anxiety symptoms. Subjects aged 70 years or above had the highest detection rate of depression (44.06%) and anxiety symptoms (33.33%). χ^2 trend test showed: the higher the body mass index (BMI), the lower the detection rate of depression and anxiety symptoms ($\chi^2_{\text{trend}} = 13.697$, $P < 0.001$; $\chi^2_{\text{trend}} = 9.082$, $P = 0.003$); the more severe the limited daily activities, the higher the detection rate of depression and anxiety symptoms ($\chi^2_{\text{trend}} = 130.455$, $P < 0.001$, $\chi^2_{\text{trend}} = 108.528$, $P < 0.001$); and the poorer the sleep quality, the higher the detection rate of depression and anxiety symptoms ($\chi^2_{\text{trend}} = 85.759$, $P < 0.001$; $\chi^2_{\text{trend}} = 51.969$, $P < 0.001$). Patients with digestive system tumors had the highest detection rate of depression (57.55%) and anxiety (55.19%), followed by patients with liver cirrhosis (41.35% and 48.08%). Depression and anxiety symptoms were also high in subjects with comorbid hypertension and coronary heart disease.

CONCLUSION

Depression and anxiety occur in patients with tumors, liver cirrhosis, functional dyspepsia, and chronic viral hepatitis. Elderly, divorced/widowed, poor sleep quality, and lower BMI are associated with higher risk of depression and anxiety.

Key words: Depression; Anxiety; Chronic digestive system diseases; Psychiatric illnesses

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Core tip: Depressive and anxiety disorders are common psychiatric illnesses. Depression and anxiety can not only lower the quality of life but also affect the therapeutic effects on somatic diseases. Research has shown that detection rates of depression and anxiety symptoms are high in patients with chronic digestive system diseases, especially in patients with digestive system tumors, liver cirrhosis, functional dyspepsia, and chronic viral hepatitis. Elderly patients, divorced/widowed patients, patients with a low degree of education, limited daily activities, poor sleep quality, or a lower body mass index are at higher risk for

depression and anxiety symptoms.

Zhang AZ, Wang QC, Huang KM, Huang JG, Zhou CH, Sun FQ, Wang SW, Wu FT. Prevalence of depression and anxiety in patients with chronic digestive system diseases: A multicenter epidemiological study. *World J Gastroenterol* 2016; 22(42): 9437-9444 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9437.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9437>

INTRODUCTION

Depressive and anxiety disorders are common psychiatric illnesses. According to statistics from the World Health Organization, there were more than 350 million people suffering from depressive disorders worldwide in 2012, suggesting that depressive disorders have become an important source of the global burden of diseases^[1]. The digestive system is vulnerable to the influence of emotional factors, because its function is regulated mainly by the vegetative nervous system and the endocrine system, and the center of both systems has the same anatomical location as the subcortical integration center of the emotional center^[2].

Previous studies have shown that depression and anxiety are risk factors for diseases of the digestive system^[3-5]. Digestive system disease patients with depressive and anxiety disorders often have more serious somatic symptoms, longer time to disease recovery and worse prognosis, and therefore tend to consume more medical resources^[6-8]. On the other hand, several studies have shown that the prevalence of depressive and anxiety disorders in patients with digestive system diseases is often high^[9-11], and these patients tend to visit the gastroenterology department of general hospitals because of more prominent digestive system symptoms, mild depressive or anxiety symptoms, or the concern of being labelled as "mentally ill patients"^[12].

Although digestive system diseases are closely related to anxiety, depressive and other mood disorders, studies have shown that the symptoms of mood disorders, such as depression and anxiety, in the vast majority of patients with digestive system diseases cannot be identified by gastroenterologists. As a result, 40%-90% of patients with depressive and anxiety disorders cannot acquire corresponding medical and health services and treatment^[13-15].

In view of the above, understanding and evaluating the prevalence of depressive and anxiety disorders in patients with digestive system diseases is of great significance. Although such studies have been previously conducted in China, many of them are confined to a kind of disease, or the subjects are either outpatients or in-patients. Therefore, the results obtained cannot fully reflect the overall prevalence

of depressive and anxiety disorders in patients with digestive system diseases.

The present study included both outpatients and in-patients with chronic diseases treated at the departments of gastroenterology in eight general hospitals in Shandong Province and evaluated the prevalence of depressive and anxiety disorders in these patients. The results obtained will provide a basis for identifying patients with chronic digestive system diseases at higher risk of anxiety and depression, and this will help determine targeted interventions and improve the prognosis and quality of life of these patients.

MATERIALS AND METHODS

Subjects

Both outpatients and in-patients with chronic diseases treated at the departments of gastroenterology of eight general hospitals in Shandong Province were included. Inclusion criteria were: (1) patients with clearly diagnosed chronic digestive system diseases, aged 18 years or above; (2) patients who were willing to participate in the study; and (3) patients who were conscious, had no mental retardation, and can complete the questionnaire independently. Exclusion criterion was patients who cannot complete the study due to severe mental or somatic disorders. Patients who could not complete all the 14 items of the Hospital Anxiety and Depression (HAD) scale were also excluded from the study. Based on these criteria, a total of 1736 patients were finally included in the study, including 871 outpatients and 865 in-patients.

Methods

This was a cross-sectional study. A self-designed General Information for Patients of the Department of Gastroenterology of General Hospitals questionnaire was used to collect each patient's general information. The questionnaire was completed by well-trained physicians using face-to-face interview after informed consent was obtained from patients. General information included demographic data (including age, sex, marital status, and education) and disease characteristics (including major diseases, disease duration, principal symptoms, chronic pain, sleep disorder, and limited daily activities). Chronic pain was defined as pain that had lasted 3 mo or longer^[16]. The symptoms of depression and anxiety were identified using the HAD scale, and the scale was completed by the subjects themselves. The degree of symptoms of depression and anxiety was assessed using the Rating Scale for Mental Health (revised version), and a score of ≥ 9 was considered to indicate positive symptoms.

Statistical analysis

After checking the completed questionnaires, Epidata3.0 was used to establish a database. SPSS17.0 software

was used to perform statistical analyses. The detection rates of depression and anxiety symptoms between different populations were compared using the χ^2 test at a significance level of 0.05.

RESULTS

General information

A total of 1736 subjects were included, of whom 937 (53.97%) were male and 799 (46.03%) were female, with a median age of 52.00 years (range, 18-90 years). Among them, 87.73% (1523/1736) were married and 26.15% (454/1736) had attended college or earned a bachelor's degree (Table 1).

The diseases diagnosed in subjects mainly included chronic gastritis (807; 46.49%), functional dyspepsia (227; 13.08%), digestive system tumors (212; 12.21%), chronic peptic ulcer (187; 10.77%), inflammatory bowel disease (181; 10.43%), gastroesophageal reflux disease (141; 8.12%), liver cirrhosis (104; 5.99%), irritable bowel syndrome (96; 5.53%), and chronic viral hepatitis (59; 3.40%) (Table 2). The median duration of diseases was 8.71 years (range, 1.20-44.00 years).

Overall detection rates of depression and anxiety symptoms

As shown in Table 3, the overall detection rate was 31.11% (540/1736) for depression symptoms alone, 27.02% (469/1736) for anxiety symptoms alone, 20.68% (359/1736) for both depression and anxiety symptoms, and 37.44% (650/1736) for either depression or anxiety symptoms. In outpatients, the detection rate was 28.01% (244/871) for depression symptoms alone, 22.96% (200/871) for anxiety symptoms alone, 18.48% (161/871) for both depression and anxiety symptoms, and 32.49% (283/871) for either depression or anxiety symptoms. In in-patients, the detection rate was 34.22% (296/865) for depression symptoms alone, 31.10% (269/865) for anxiety symptoms alone, 22.89% (198/865) for both depression and anxiety symptoms, and 42.23% (367/865) for either depression or anxiety symptoms.

Detection rates of depression and anxiety symptoms in subjects with different demographic characteristics

As shown in Table 1, the detection rate of depression symptoms did not differ significantly between men and women ($\chi^2 = 1.681$, $P = 0.195$). Subjects aged 70 years or above had the highest detection rate of depression symptoms (44.06%), followed by subjects aged 50-59.99 years (36.00%). With regard to marital status, the detection rate of depression symptoms was highest in divorced/widowed subjects (41.35%) and lowest in unmarried subjects (17.43%). With regard to education level, the detection rate of depression symptoms was highest in subjects with primary school education or below (45.60%) and lowest in subjects

Table 1 Detection rates of depression and anxiety symptoms in subjects with different demographic characteristics

Variable	n (%)	Depression symptoms			Anxiety symptoms		
		n (%)	χ^2	P value	n (%)	χ^2	P value
Sex			1.681	0.195		0.779	0.377
Male	937 (53.97)	279 (29.78)			245 (26.15)		
Female	799 (46.03)	332 (32.67)			224 (28.04)		
Age, yr			48.522	< 0.001		24.254	< 0.001
18-30	164 (9.45)	38 (23.17)			31 (18.90)		
30-40	234 (13.48)	57 (24.36)			50 (21.37)		
40-50	363 (20.91)	81 (22.31)			83 (22.87)		
50-60	375 (21.60)	135 (36.00)			123 (32.80)		
60-70	339 (19.53)	114 (33.63)			95 (28.02)		
> 70	261 (15.03)	115 (44.06)			87 (33.33)		
Marital status			14.656	0.001		8.490	0.014
Married	1523 (87.73)	478 (31.39)			416 (27.31)		
Divorced/widowed	104 (5.99)	43 (41.35)			35 (33.65)		
Unmarried	109 (6.28)	19 (17.43)			18 (16.51)		
Education			55.293	< 0.001		13.184	0.010
Primary school or below	386 (22.24)	176 (45.60)			128 (33.16)		
Junior high school	440 (25.35)	137 (31.14)			120 (27.27)		
Senior high school or polytechnic school	424 (24.42)	108 (25.47)			105 (24.76)		
College or bachelor's degree	454 (26.15)	114 (25.11)			112 (24.67)		
Master's degree or above	32 (1.84)	5 (15.63)			4 (12.50)		
Body mass index, kg/m ²			16.273	< 0.001		9.092	0.011
< 18.5	88 (5.07)	42 (47.19)			31 (34.83)		
18.5-23.99	947 (54.55)	307 (32.42)			274 (28.93)		
≥ 24	697 (40.15)	191 (27.29)			164 (23.43)		
Chronic pain			6.426	0.011		1.540	0.215
Yes	262 (15.09)	99 (37.79)			79 (30.15)		
No	1474 (84.91)	441 (29.92)			390 (26.46)		
Daily activities			132.199	< 0.001		108.699	< 0.001
Non-limited	1457 (83.93)	373 (25.60)			325 (22.31)		
Limited but independent	253 (14.57)	147 (58.10)			125 (49.41)		
Limited but dependent	26 (1.50)	20 (76.92)			19 (73.08)		
Sleep quality			87.741	< 0.001		53.651	< 0.001
Good	539 (31.05)	104 (19.29)			99 (18.37)		
Fair	898 (51.73)	285 (31.74)			245 (27.28)		
Poor or very poor	299 (17.23)	151 (50.50)			125 (41.81)		

Table 2 Detection rates of depression and anxiety symptoms in subjects with different diseases n (%)

Disease	Total	Depression symptoms	Anxiety symptoms
Peptic ulcer	187 (10.77)	50 (26.74)	42 (22.50)
Digestive system tumors	212 (12.21)	122 (57.55)	117 (55.19)
Chronic gastritis	807 (46.49)	221 (27.39)	177 (21.93)
Functional dyspepsia	227 (13.08)	78 (34.36)	58 (25.55)
Inflammatory bowel disease	181 (10.43)	58 (32.04)	46 (25.41)
Irritable bowel syndrome	96 (5.53)	28 (29.17)	24 (25.00)
Chronic viral hepatitis	59 (3.40)	20 (33.90)	16 (27.12)
Liver cirrhosis	104 (5.99)	43 (41.35)	50 (48.08)
Gastroesophageal reflux disease	141 (8.12)	34 (24.11)	29 (20.57)

with graduate level or above (15.63%). The detection rate of depression symptoms was higher in patients with chronic pain than in those without ($\chi^2 = 6.426$, $P = 0.011$). χ^2 trend test showed: the higher the body mass index (BMI), the lower the detection rate of depression symptoms ($\chi^2_{\text{trend}} = 13.697$, $P < 0.001$); the more severe the limited daily activities, the higher the detection rate of depression symptoms ($\chi^2_{\text{trend}} = 130.455$, $P < 0.001$); and the poorer the sleep quality, the higher the detection rate of depression symptoms

($\chi^2_{\text{trend}} = 85.759$, $P < 0.001$).

The detection rate of anxiety symptoms did not differ significantly between men and women ($\chi^2 = 0.779$, $P = 0.377$). Subjects aged 70 years or above had the highest detection rate of anxiety symptoms (33.33%), followed by subjects aged 50-59.99 years (32.80%). With regard to marital status, the detection rate of anxiety symptoms was highest in divorced/widowed subjects (33.65%) and lowest in unmarried subjects (16.51%). With regard to education level,

Table 3 Overall detection rates of depression and anxiety symptoms *n* (%)

Patients	<i>n</i>	Depression symptoms	Anxiety symptoms	Depression and anxiety symptoms	Depression or anxiety symptoms
Outpatients	871	244 (28.01)	200 (22.96)	161 (18.48)	283 (32.49)
In-patients	865	296 (34.22)	269 (31.10)	198 (22.89)	367 (42.43)
Overall	1736	540 (31.11)	469 (27.02)	359 (20.68)	650 (37.44)

Table 4 Detection rates of depression and anxiety symptoms in subjects with different comorbidities

Comorbidity	<i>n</i> (%)	Depression symptoms			Anxiety symptoms		
		<i>n</i> (%)	χ^2	<i>P</i> value	<i>n</i> (%)	χ^2	<i>P</i> value
Hypertension			6.402	0.011		5.734	0.017
No	1342 (77.30)	397 (29.58)			344 (25.63)		
Yes	394 (22.70)	143 (36.29)			125 (31.73)		
Diabetes			7.646	0.006		7.424	0.006
No	1585/91.30	478/30.16			414/26.12		
Yes	151/8.70	62/41.06			55/36.42		
Coronary heart disease			6.060	0.014		3.396	0.065
No	1603/92.34	486/30.32			424/26.45		
Yes	133/7.66	54/40.60			45/33.83		
Cerebrovascular disease			0.766	0.382		0.120	0.729
No	1627/93.72	502/30.85			438/26.92		
Yes	109/6.28	38/34.86			31/28.44		

the detection rate of anxiety symptoms was highest in subjects with primary school education or below (33.16%) and lowest in subjects with graduate level or above (12.50%) (Table 1). The detection rate of anxiety symptoms did not differ significantly between patients with chronic pain and those without ($\chi^2 = 1.540$, $P = 0.215$). χ^2 trend test showed that: the higher the BMI, the lower the detection rate of anxiety symptoms ($\chi^2_{\text{trend}} = 9.082$, $P = 0.003$); the more severe the limited daily activities, the higher the detection rate of depression symptoms ($\chi^2_{\text{trend}} = 108.528$, $P < 0.001$); and the poorer the sleep quality, the higher the detection rate of depression symptoms ($\chi^2_{\text{trend}} = 51.969$, $P < 0.001$).

Detection rates of depression and anxiety symptoms in subjects with different diseases

Patients with digestive system tumors had the highest detection rate of depression (57.55%), followed by patients with liver cirrhosis (41.35%), patients with functional dyspepsia (34.36%), and patients with chronic viral hepatitis (33.90%). Patients with digestive system tumors had the highest detection rate of anxiety (55.19%), followed by patients with liver cirrhosis (48.08%), patients with chronic viral hepatitis (27.12%), and patients with functional dyspepsia (25.55%) (Table 2).

Detection rates of depression and anxiety symptoms in subjects with different comorbidities

The detection rates of depression and anxiety symptoms were significantly higher in subjects with comorbid hypertension than in those without (36.29% vs 29.58%, 31.73% vs 25.63%, $P < 0.05$) and in

subjects with comorbid diabetes than in those without (41.06% vs 30.16%, 36.42% vs 26.12%, $P < 0.05$). The detection rate of depression symptoms was significantly higher in subjects with comorbid coronary heart disease than in those without (40.60% vs 30.32%, $P = 0.014$) but the detection rate of anxiety symptoms did not differ significantly between subjects with comorbid coronary heart disease and those without ($P > 0.05$) (Table 4).

DISCUSSION

This cross-sectional study investigated the prevalence of symptoms of depression and anxiety in both outpatients and in-patients with chronic diseases treated at the departments of gastroenterology of general hospitals. Given that the sample size was large, the subjects were collected from eight general hospitals in Shandong Province, and multiple common digestive system diseases were involved, the results obtained are of great clinical value.

Detection rates of depression and anxiety symptoms in patients with chronic diseases treated at the departments of gastroenterology of general hospitals

This study showed that the detection rate of depression or anxiety symptoms in patients with chronic diseases treated at the departments of gastroenterology of general hospitals was 37.44% of all subjects (42.43% of in-patients and 32.49% of outpatients), which was lower than that (53.1%) reported by Li *et al*^[11] who investigated 1995 outpatients at the departments of gastroenterology of 15 general hospitals in five regions, and that (58.3%) reported by Jiang *et al*^[17]

who investigated 517 outpatients at the departments of gastroenterology of three general hospitals in Beijing. This difference may be caused by the fact that the cutoff value for HAD score used in the previous two studies was ≥ 8 (as opposed to ≥ 9 used in the present study).

The detection rate of depression symptoms in patients with chronic diseases treated at the departments of gastroenterology of general hospitals was 31.11%, which was lower than that (37%) reported by a previous study (the LIDO study^[18]) that included 18489 patients in primary care in six countries. Such discrepancy may be due to different subjects and screening tools used. The detection rate of depression symptoms in outpatients was 28.01%, which was lower than that (35.1%) reported by Mei *et al.*^[19] who investigated 1428 outpatients at the department of gastroenterology, and the detection rate of depression symptoms of in-patients was 34.22%, which was lower than that (40.8%) reported by Li *et al.*^[20] who investigated 133 in-patients at the department of gastroenterology. These differences may be due to different screening criteria used between studies.

The detection rate of anxiety symptoms in patients with chronic diseases treated at the departments of gastroenterology of general hospitals was 27.02% in all subjects and 22.96% in outpatients, which were higher than those (5.8% and 31.10%) reported by Mei *et al.*^[19] who investigated 1428 outpatients at the department of gastroenterology, but lower than that (50.4%) reported by Li *et al.*^[21] who investigated 133 in-patients at the department of gastroenterology.

The differences in these findings suggest that the differences in evaluation tools and endpoints may cause different results. Since the present study utilized the HAD scale, which is the most widely used tool worldwide, and included 1736 patients from eight tertiary hospitals located in different regions of Shandong Province, it can well reflect the actual situation of patients at departments of gastroenterology in Shandong Province.

Distribution of chronic digestive system disease patients with depression and anxiety symptoms

With regard to the type of diseases, patients with digestive system tumors had the highest detection rates of depression and anxiety symptoms, followed by patients with liver cirrhosis. This may be because these two types of diseases are more severe and cause greater psychological and economic burden for patients.

The detection rates of depression and anxiety symptoms in patients with functional dyspepsia were 34.36% and 25.55%, respectively, which were comparable to those reported by Li *et al.*^[21]. Our results also showed that the detection rates of depression and anxiety symptoms were higher in patients with hypertension or diabetes than in those

without. Lloyd *et al.*^[22] found that diabetes, especially diabetes with multiple complications, can increase the incidence of depression. Sun *et al.*^[23] discovered that BMI, glycosylated hemoglobin level, and use of insulin can affect the mood of diabetes patients and result in higher levels of depression and anxiety in diabetes patients than in normal people. Jiang *et al.*^[24] found that the incidence and severity of depression were higher in patients with hypertension than in those without.

Although many previous studies indicated that women were more likely to develop depressive and anxiety disorders than men^[25-27], the present study revealed no significant differences in the detection rates of depression and anxiety symptoms between men and women, which is consistent with the finding of Zhang *et al.*^[28] who investigated 2877 outpatients from 50 general hospitals in Beijing.

The present study also showed that the detection rates of depression and anxiety symptoms were higher in patients aged 50 years or above than in younger patients, suggesting that patients aged > 50 years may be a high risk population for depression and anxiety, which is consistent with the findings of many previous studies that elderly patients have a higher prevalence of depressive and anxiety disorders than younger patients^[24,29]. Compared to unmarried or married subjects, divorced/widowed subjects had higher detection rates of depression and anxiety symptoms. This may be because divorced/widowed subjects received less family and social support. With regard to education level, patients with a low level of education had higher detection rates of depression and anxiety symptoms, which is consistent with the finding of previous studies^[19]. This may be because patients with a high level of education have better awareness of disease status and self-adjustment.

This study also showed that higher severity of limited daily activities was associated with higher detection rates of depression and anxiety symptoms, which is consistent with the findings of Ji *et al.*^[30]. The underlying reasons are that patients with limited daily activities have a lower degree of independence, are often reliant on their families, and tend to develop feelings of guilt, self-abasement, self-blame and frustration, which can result in the occurrence of depression and anxiety.

We also found that patients with a lower BMI had higher detection rates of depression and anxiety symptoms. Janssen *et al.*^[31] also discovered that depression and anxiety symptoms were more common in patients with a lower BMI. Since patients with a lower BMI tend to have poorer digestion and absorption, the related somatic symptoms may be more serious. In addition, patients with poor sleep quality had higher detection rates of depression and anxiety symptoms, which is consistent with the finding of Hong *et al.*^[32] that sleep disorders are significantly associated with depression and anxiety.

In summary, the detection rates of depression and

anxiety symptoms are high in patients with chronic digestive system diseases, especially in patients with digestive system tumors, liver cirrhosis, functional dyspepsia, and chronic viral hepatitis. Elderly patients, divorced/widowed patients, patients with a low degree of education, limited daily activities, poor sleep quality, or a lower BMI are at higher risk for depression and anxiety symptoms. Depression and anxiety can not only lower the quality of life but also affect the therapeutic effects on somatic diseases. Comprehensive measures, including psychological counseling, healthy eating and sleeping habits, active exercise, and anti-depressive therapy, can improve quality of life and therapeutic efficacy, and are safe.

However, there is an ongoing clinical problem: although many studies have found that the incidence of depression and anxiety is high in patients of departments of gastroenterology, the diagnosis and treatment rates are low. The extent of the impact of these factors requires further study. Gastroenterologists are encouraged to pay more attention to the psychological status of patients in their daily clinical activities, and hospitals should strengthen the training of non-psychiatric physicians to improve their diagnostic and therapeutic skills for depression and anxiety in order to promote early recognition of these psychological conditions. In addition, there remains a need for further study on how to develop more effective comprehensive treatments, such as psychological counseling, life style guidance, and drug intervention, in gastroenterology departments in order to improve therapeutic efficacy, shorten therapeutic duration, and reduce therapeutic costs.

COMMENTS

Background

Depressive and anxiety disorders are common psychiatric illnesses. Digestive system disease patients with depressive and anxiety disorders often have more serious somatic symptoms, longer time to disease recovery and worse prognosis, and therefore tend to consume more medical resources. Understanding and evaluating the prevalence of depressive and anxiety disorders in patients with digestive system diseases is of great significance.

Research frontiers

In China, depressive and anxiety disorders are common psychiatric illnesses. However, there are very few English language studies in the literature concerning the diagnosis of depressive and anxiety disorders. This research was conducted to investigate the overall prevalence of depressive and anxiety disorders in patients with digestive system diseases in China.

Innovations and breakthrough

This cross-sectional study investigated the prevalence of symptoms of depression and anxiety in both outpatients and in-patients with chronic diseases treated at departments of gastroenterology of general hospitals.

Applications

The research provides a basis for identifying patients with chronic digestive system diseases at higher risk of anxiety and depression, and this will help determine targeted interventions and improve the prognosis and quality of life

of these patients.

Peer-review

In this manuscript, the authors investigated the prevalence of depression and anxiety in patients with chronic digestive system diseases. They provide a basis for identifying patients with chronic digestive system diseases at higher risk of anxiety and depression, and this will help determine targeted interventions and improve the prognosis and quality of life of these patients.

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Complete response with sorafenib and transcatheter arterial chemoembolization in unresectable hepatocellular carcinoma

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Abstract

Patients with advanced hepatocellular carcinoma (HCC) showing portal vein tumor thrombosis (PVTT) have an extremely poor prognosis. According to treatment guidelines, the only option for HCC patients with PVTT is sorafenib chemotherapy. However, in Asia, various treatments have been attempted and possible prolongation of overall survival has been repeatedly reported. We herein report the first case of a patient with an initially unresectable advanced HCC with PVTT who underwent curative hepatectomy after sorafenib and transcatheter arterial chemoembolization (TACE) showing complete histological response. Two months after induction with sorafenib, a significant decrease in serum alpha-fetoprotein level was observed and computed tomography imaging showed a significant decrease in tumor size. Because of remaining PVTT, TACE and curative resection were performed. The combination of sorafenib and TACE may be an effective treatment for HCC patients with PVTT.

Key words: Hepatocellular carcinoma; Sorafenib; Complete response; Portal vein tumor thrombosis; Transcatheter arterial chemoembolization

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Core tip: Patients with advanced hepatocellular carcinoma (HCC) showing portal vein tumor thrombosis (PVTT) have an extremely poor prognosis. The only proposed treatment option for HCC patients with PVTT is sorafenib chemotherapy. However, in Asia, various treatments have been attempted and possible prolongation of overall survival has been repeatedly reported. Here we report the first case of a patient with an initially unresectable advanced HCC and PVTT who underwent curative hepatectomy after sorafenib and transcatheter arterial chemoembolization (TACE) showing complete histological response. The combination of sorafenib and TACE may be an effective treatment for HCC patients with PVTT.

Takano M, Kokudo T, Miyazaki Y, Kageyama Y, Takahashi A, Amikura K, Sakamoto H. Complete response with sorafenib and transcatheter arterial chemoembolization in unresectable hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(42): 9445-9450 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9445.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9445>

INTRODUCTION

Patients with advanced hepatocellular carcinoma (HCC) showing portal vein tumor thrombosis (PVTT) have an extremely poor prognosis^[1,2]. The median survival of untreated HCC with PVTT has been reported to be 2.7-6 mo^[2,3]. According to the American Association for the Study of the Liver Disease/Barcelona Clinic for Liver Cancer Staging System and treatment guidelines, the only proposed treatment option for HCC patients with PVTT is sorafenib chemotherapy^[4]. In Asia, various treatments including hepatectomy and transcatheter arterial chemoembolization (TACE) have been attempted and possible prolongation of overall survival (OS) has been repeatedly reported^[5]. We herein report the first case of a patient with an initially unresectable advanced HCC with PVTT who underwent curative hepatectomy after sorafenib and TACE, showing complete histological response.

CASE REPORT

A 67-year-old man was diagnosed with HCC through abdominal ultrasound during examination for elevated liver enzymes by his family doctor and was referred to our hospital. The patient had no history of alcohol abuse, hepatitis B or C infection. Serum α -fetoprotein (AFP) level and protein induced by vitamin K absence or antagonist-II level were 1736 ng/mL (normal range: < 10 ng/mL) and 15388 mAU/mL (normal range: < 40 mAU/mL), respectively. Contrast-enhanced computed tomography (CT) scan revealed the presence of an 8.7 cm \times 6 cm tumor in the right paramedian sector,

showing early enhancement in the arterial phase and wash-out in the late phase together with PVTT limited to the first-order branch and invading the right portal vein (Figure 1). Right hepatectomy was considered to be necessary for curative resection. Although the patient's liver function was Child-Pugh A, the patient's indocyanine green retention rate at 15 min was 21% (normal range; < 10%) and right hepatectomy was considered to be intolerable according to our institutional criteria^[6]. Therefore, sorafenib was orally administered twice daily at a dose of 800 mg. During sorafenib treatment, the patient had no adverse event. Two months later, a significant decrease in serum AFP level was observed (195 ng/mL) (Figure 2). The CT scan showed a significant decrease in tumor size (3 cm); however, PVTT remained in the right portal vein (Figure 3). Four months later, serum AFP level decreased to within normal range (4.5 mg/mL), and 14 months later, CT scan revealed the residual PVTT in right portal vein (Figure 4). Portography revealed filling defect in S8 and digital subtraction arteriography showed irregular shaped tumor stain. Thus TACE was performed with 30 mg of miripulatin, 3 mL of lipiodol and gelatin sponge particle (Figure 5), followed by right paramedian sectionectomy. During the operation, neither the main tumor nor the PVTT was identified through intraoperative ultrasound. The operation time was 318 min and the estimated blood loss was 762 mL. The patient's postoperative course was uneventful, and he was discharged from hospital on postoperative day 11. Pathological examination revealed complete necrosis without viable tumor cells both in the scar of PVTT and the main tumor. To date, no recurrence has been observed after 12 mo of follow-up.

DISCUSSION

Sorafenib has been reported to prolong survival in patients with unresectable or advanced HCC; however, complete response (CR) was not achieved in these reports^[4,7,8]. In Asian countries, including Japan, liver resection and TACE have been reported to improve the prognosis of patients with HCC with PVTT. In the presence of PVTT, TACE is theoretically contraindicated in Western countries because of the potential risk of hepatic insufficiency that results from ischemia following TACE. However, recent studies demonstrate that TACE can be safely performed in the presence of adequate collateral circulation around the occluded portal vein^[9,10]. A median OS period after treatment with TACE was reported to be 5.6-16.5 mo in patients with HCC accompanied by PVTT^[11-13]. A median OS period after treatment with surgery was reported to be 6-19.9 mo^[14-16]. Furthermore, Minagawa *et al.*^[16] reported a high survival rate in these patients with the combination of TACE followed by hepatic resection. In the very recent paper, liver resection is associated with prolongation of overall survival of HCC with PVTT^[13].

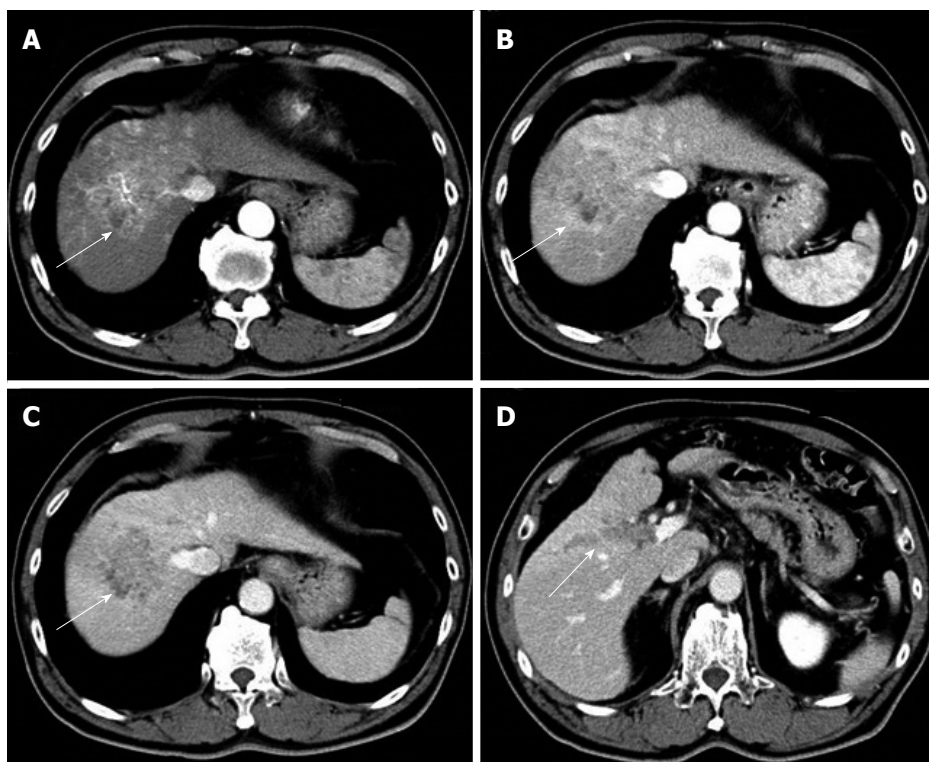


Figure 1 Contrast-enhanced computed tomography before sorafenib introduction. Heterogeneous hypervascularized tumor (A-C, arrow) in the right paramedian sector, showing early enhancement in the arterial phase and wash-out in the late phase together with portal vein tumor thrombosis (D, arrow) limited to the first-order branch and invading the right portal vein.

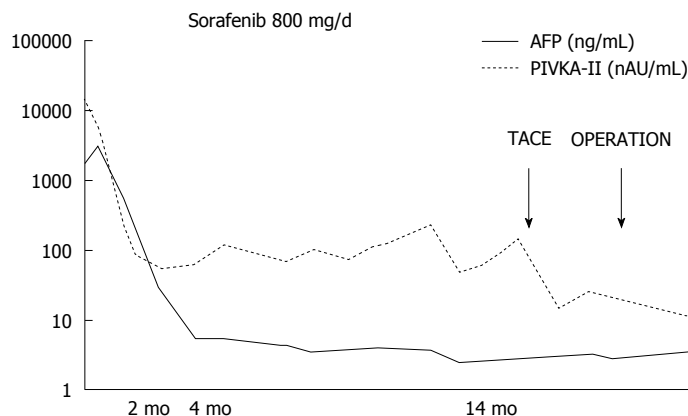


Figure 2 Clinical course as assessed by tumor markers and therapeutic events. AFP: α -fetoprotein; PIVKA-II: Vitamin K absence or antagonist-II; TACE: Transcatheter arterial chemoembolization.

Thus, TACE and surgery are the common choices of the treatment for HCC in Japan. Anticoagulants (e.g., low molecular weight heparin, warfarin and oral anticoagulant) has been reported to be effective for portal vein thrombosis^[17]. However, evidence is limited concerning the effect of anticoagulants other than anti-cancer treatment for PVTT. After treatment of HCC with PVTT with sorafenib, an OS period of 6.2-12.3 mo has been reported^[13,18]. Although CR after sorafenib treatment is rare, to the best of our knowledge, 10 cases of HCC patients with PVTT who achieved CR after treatments including sorafenib have been reported

(Table 1)^[19-27]. Four of the 10 cases underwent hepatectomy and had confirmed histological CR. Five of the 10 cases only underwent sorafenib treatment, and the other cases had other combined treatment modalities. The median time to normalized level of serum AFP was 4.5 mo (range, 2.75-6.5 mo). The median time to CR is 8 mo (range, 6-16.5 mo). All cases including ours, showed disappearance of the main tumor and PVTT. We herein report the first case of histologically confirmed CR of HCC with PVTT after sorafenib and TACE. Combination of sorafenib and TACE may be an effective treatment for HCC patients with PVTT.

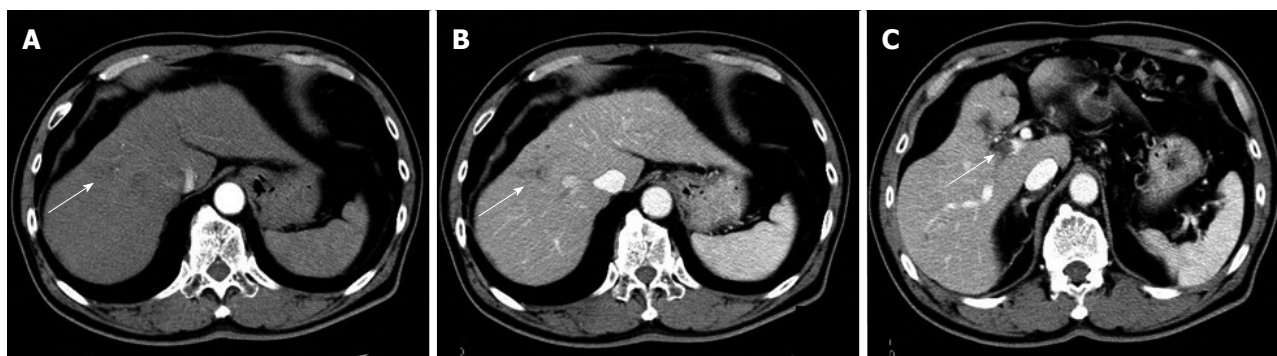


Figure 3 Two months after sorafenib induction. Computed tomography showed the significantly decrease in size (3 cm) and hypervascularization (A and B, arrow), and portal vein tumor thrombosis remained in the second-order branch of portal vein (C, arrow).

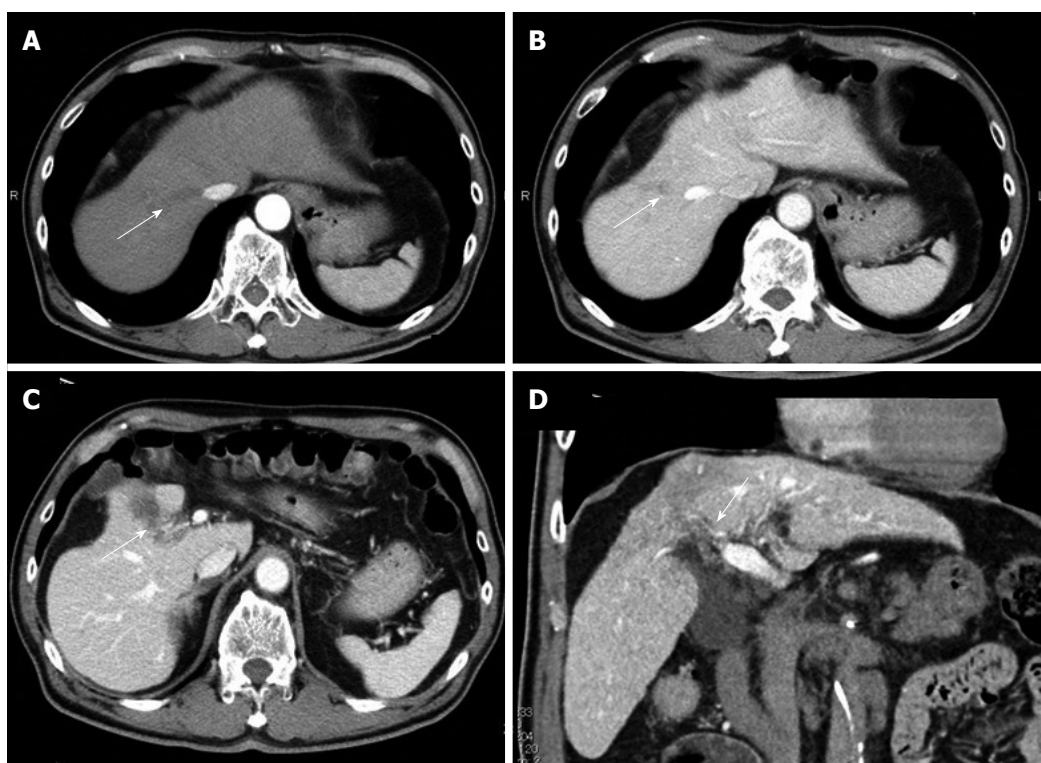


Figure 4 Fourteen months after sorafenib induction. Computed tomography revealed the tumor disappearance (A and B, arrow) and the residual portal vein tumor thrombosis (C and D, arrow) in the right anterior portal vein.



Figure 5 Angiography 14 mo after sorafenib induction. A: Portography revealed filling defect in S8 (arrow); B: Digital subtraction arteriography showed irregular shaped tumor stain (arrow); C: Transcatheter arterial chemoembolization was performed with 30 mg of miripulatin, 3 mL of lipiodol and gelatin sponge particle.

Table 1 Cases of hepatocellular carcinoma patients with portal vein tumor thrombosis who achieved complete response in the literature

Age	Sex	Etiology	Extrahepatic metastasis	Pre-AFP	Post-AFP	Sorafenib dose	Duration (mo)	Time to CR (mo)	Resection	Other therapy	Follow-up period (mo)
54	Male	Hepatitis C	Lung	52347	30.2	800-400	5	5	+	EBRT	14
83	Male	None	Lung	41948	W.N.L.	800-400-200-100	34	8	-	TACE RFA	34
59	Male	Hemochromatosis	Little omentum LN	866	W.N.L.	800	6	6	+	HAI None	16
57	Male	Hepatitis B	None	17000	W.N.L.	800-400	12	12	+	None	12
74	Male	Hepatitis C	ND	3300	W.N.L.	400	8	8	-	None	24
84	ND	Hepatitis C	None	353	W.N.L.	800	12	6	-	None	12
69	Male	Hepatitis C	None	n.d.	ND	800-400-200	62	23	-	None	62
74	Male	None	ND	33058	2	800-400-200	19	19	-	None	19
68	Male	Hepatitis C	Dissemination	4773	45.7	800-400	28	24	+	None	40
48	Male	Hepatitis C	None	135835	W.N.L.	800	9	4	-	None	31
67	Male	None	None	3385	W.N.L.	800	14	14	+	TACE	26

AFP: α -fetoprotein; CR: Complete response; LN: Lymph node; W.N.L.: Within normal limit; EBRT: External beam radiotherapy; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; HAI: Hepatic arterial infusion chemotherapy; ND: Not described.

COMMENTS

Case characteristics

A 67-year-old man had no symptom.

Clinical diagnosis

On physical examination, he had a palpable mass in right upper quadrant of the abdomen.

Differential diagnosis

Hepatocellular carcinoma, metastatic liver tumor, intrahepatic cholangiocarcinoma, malignant lymphoma and liver hemangioma.

Laboratory diagnosis

The patient have elevated hematological value for alkaline phosphatase (316 IU/L), Glutamic-oxaloacetic transaminase (80 IU/L), glutamic pyruvic transaminase (89 IU/L), γ -glutamyltranspeptidase (338 IU/L), α -fetoprotein (1736.3 ng/mL), protein induced by vitamin K absence or antagonist-II (15388 mAU/mL).

Imaging diagnosis

Contrast-enhanced computed tomography scan revealed the presence of an 8.7 cm \times 6 cm tumor in the right paramedian sector, showing early enhancement in the arterial phase and wash-out in the late phase together with portal vein tumor thrombosis limited to the first-order branch and invading the right portal vein.

Pathological diagnosis

Histological examination after sorafenib chemotherapy and transcatheter arterial chemoembolization showed complete necrosis without viable tumor cells both in the scar of portal vein tumor thrombosis and the main tumor.

Treatment

The patient received a sorafenib chemotherapy and transcatheter arterial chemoembolization.

Related reports

Sorafenib chemotherapy is associated with prolongation of overall survival of advanced hepatocellular carcinoma (HCC), compared with best supportive care. However, complete response after sorafenib treatment with or without other treatments is very rare.

Term explanation

Portal vein tumor thrombosis is a form of venous thrombosis affecting the hepatic portal vein, caused by tumor invasion.

Experiences and lessons

This case report presents a new choice of treatment for advanced hepatocellular carcinoma accompanying with portal vein tumor thrombosis. Combination of sorafenib and transcatheter arterial chemoembolization may be an effective treatment for HCC patients with portal vein tumor thrombosis.

Peer-review

The authors have described a case of advanced hepatocellular carcinoma with portal vein thrombosis that showed complete response after sorafenib and transcatheter arterial chemoembolization. The article provides another choice of treatment for advanced hepatocellular carcinoma.

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