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ABOUT COVER

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MINIREVIEWS

Research advances of vasoactive intestinal peptide in the pathogenesis of ulcerative colitis by regulating interleukin-10 expression in regulatory B cells

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Abstract

Ulcerative colitis (UC) is a chronic relapsed intestinal disease with an increasing incidence around the world. The pathophysiology of UC remains unclear. However, the role of the interaction between the enteric nervous system and the immune system in the pathogenesis of UC has been the focus of attention and has become a research hotspot. Vasoactive intestinal peptide (VIP) is a kind of endogenous neuropeptide with regulatory activity on intestinal immunity. It has been shown to regulate immune disorders in animal and human experiments and has become an effective anti-inflammatory and immune modulator that affects the innate immune system and adaptive immune system. Regulatory B cells (Bregs) are a new group of B cells that negatively regulate the immunity and have received extensive attention in immune circles. Bregs can regulate immune tolerance by producing interleukin (IL)-10, IL-35, and transforming growth factor-β, suppressing autoimmune diseases or excessive inflammatory responses. The secretion of IL-10 by Bregs induces the development of T helper (Th) 0 and Th2 cells. It also induces Th2 cytokines and inhibits Th1 cytokines, thereby inhibiting Th1 cells and the Th1/Th2 balance. With further clarity on the mechanism of the regulation of IL-10 expression by VIP in Bregs in colitis patients, we believe that

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Bregs can provide a novel strategy for the clinical treatment of UC. Thus, we aim to review the current literature on this evolving topic.

Key Words: Vasoactive intestinal peptide; Ulcerative colitis; Interleukin-10; Bregs; Pathogenesis

Core Tip: The pathophysiology of ulcerative colitis remains unclear. Vasoactive intestinal peptide (VIP) is a neuropeptide that has strong regulatory activity on intestinal immunity. With further clarity on the mechanism of the regulation of interleukin-10 expression by VIP in regulatory B cells in colitis patients, we believe that VIP can provide a novel strategy for the clinical treatment of ulcerative colitis.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a global health problem. It consists of two main types, Crohn's disease (CD) and ulcerative colitis (UC). UC is a chronic non-specific IBD that mainly involves the mucosa and submucosa of the rectum and colon. With a significant increase in the incidence of UC, it was identified as a modern refractory disease by the World Health Organization[1]. It is currently believed that UC is caused by an abnormal and continuous immune response to intestinal microorganisms and is facilitated by the genetic susceptibility of an individual[2].

The immune response of the human body includes innate and adaptive immunity. Innate immunity, which is the first defense of the body against pathogens, is non-specific and does not provide lasting immunity; it is mediated by pattern recognition receptors, including toll-like receptors (TLRs) on the cell surface and nucleotide-binding oligomerization domain-like receptors in the cytoplasm[3]. Adaptive immunity is a highly specific and adaptive type of immunity. T helper (Th) cells are the key to an adaptive immune response. Most studies suggest that the pathological characteristics of UC are mainly an abnormal Th2 reaction, whereas an abnormal Th1 reaction is mainly found in CD[2,4]. Therefore, studying the underlying mechanism of intestinal mucosal immune response disorders can help us better understand the pathogenesis of IBD. Herein, the role of vasoactive intestinal peptide (VIP) and regulatory B cells (Bregs) in the pathogenesis of UC as well as the interaction between these two are reviewed, in the context of the immune pathogenesis of UC.

ROLE OF BREGS AND INTERLEUKIN-10 IN INTESTINAL IMMUNITY IN UC PATIENTS

A study found that dextran sulfate sodium (DSS) treated mice showed more severe colitis without B cells, and the filtration metastasis of B cells alleviated the disease. During colitis, the number of regulatory T cells (Tregs) of gut-associated lymphoid tissue (GALT) in B-cell deficient mice decreased significantly. After B cells were transferred into these mice, the number of GALT Tregs was restored, indicating that B cells contribute to Treg homeostasis. The study also found that B cells can induce the proliferation of Tregs and then promote the differentiation of B cells into IgA-producing plasma cells. These results suggest that B cells and Tregs interact and cooperate to prevent excessive immune response, which can lead to colitis[5]. Strong stimulation of B cells by *Escherichia coli* leads to significant expression of inhibitory molecules on the surface of B cells and increases the production of anti-inflammatory

cytokines, such as interleukin (IL)-10. Bregs induced by the bacterium can effectively inhibit the maturation and function of dendritic cells (DCs), inhibit the proliferation and polarization of Th1 and Th17 cells, and promote the differentiation of Th2 cells. In addition, Bregs promote the development of Tregs, which may establish immune homeostasis through feedback cooperation. The number of Bregs is directly related to the severity of inflammation. These findings may provide new methods for B-cell control in the treatment of autoimmune diseases[6].

IL-10 is an anti-inflammatory cytokine produced by monocytes, B cells, T cells, as well as some other cells. It is an important cytokine that induces the immune response of Th2 cells. IL-10 is expressed in different cells involved in innate and adaptive immunity, including dendritic cells, macrophages, T cells, natural killer cells, and B cells[7,8]. On the one hand, IL-10 protects the host from tissue damage caused by excessive inflammation, inhibits antigen presentation and production, and suppresses antigen expression and the production of pro-inflammatory chemokines and cytokines. IL-10 is the key gatekeeper in maintaining distal immune homeostasis. When specific pathogens enter the intestinal track, distal immune homeostasis may be imbalanced[9]. On the other hand, it enhances the survival, proliferation, differentiation, and homotypic transformation of human B cells[10]. The reduction of this anti-inflammatory cytokine is an important feature of UC[11].

IL-10 helps to maintain tissue integrity and promotes intestinal tissue homeostasis through its anti-inflammatory, anti-apoptotic, and tissue regeneration properties. The new immune cytokine, F8-IL-10 (Dekavil), a targeted antibody associated with IL-10, is currently being evaluated in phase 2 clinical trials in patients with rheumatoid arthritis, potentially paving the way for its use in IBD patients[12]. Mizoguchi *et al*[13] first used the concept of Bregs to define these B cell populations with negative regulatory functions[13]. Yanaba *et al*[14] defined the B cell subsets, CDld hiCD 5+, that secrete IL-10 in the spleen of mice; B10 cells do as well[14]. At present, the mechanism of Bregs inhibiting immune inflammation is realized by the following aspects: Bregs mainly exert an immune regulatory function through the secretion of IL-10 and transforming growth factor-β. IL-10 produced by Bregs affects the disease process by regulating T cell differentiation[15]. For example, in IBD, the Th1/Th2 balance can be regulated to suppress the harmful immune response[16]. Macrophages and DCs are also the main targets of IL-10 by playing an inhibitory and regulatory role, which can reduce the release of inflammatory factors from macrophages or monocytes and reduce the inflammatory response[17].

Bregs play a regulatory role through intercellular contacts. Studies have shown that Bregs promote the proliferation and activity of Tregs through direct major histocompatibility complex and B7 pathways and inhibit the proliferation of effector T cells through Fas/FasL-mediated apoptosis[18,19]. Studies on the interactions between Bregs and other regulatory immune cells have shown that co-culture of Bregs and $CD4^+$ T cells can quantitatively reduce the proliferative capacity of $CD4^+$ T cells[20]. B10 cells induced by mannose-capped lipoarabinomannan (ManLAM) were transferred into IL-$10^{-/-}$ mice, and B10 cells induced by ManLAM can reduce the severity of colitis in mice. B10 cells down-regulated Th1 polarization of spleen and mesenteric lymph nodes in DSS treated mice. These results suggest that the production of IL-10 by B cells treated with ManLAM helps to maintain the balance between $CD4^+$ T cell subsets and protects mice against DSS-induced IBD[21].

Bregs generate antibodies, neutralize harmful soluble factors, inhibit the activity of DCs/macrophages through the immunoglobulin G/FcTRII interaction, enhance the clearance of apoptotic cells, and reduce the potential autoantigen activating autoreactive T cells. In a recent *in vitro* study, the secretion of IL-10 and IL-12 p40 was significantly decreased and IL-12 p40 was increased in phosphoinositide 3-kinase (PI3K) kinase (d910a/d910a) mice or wild-type B cells treated with PI3K specific inhibitors. Macrophages or $CD4^+$ T cells activated by co-cultured microorganisms did not inhibit inflammatory cytokines. *In vivo*, co-transferred wild-type B cells improved T-cell-mediated colitis, while PI3K kinase (d910a/d910a) B cells had no protective effect on mucosal inflammation. These results suggest that the PI3K pathway plays an important role in the induction and regulation of IL-10 production in B cells in intestinal homeostasis and inflammation[22].

In humans, B-cell depletion using anti-CD20 (rituximab) for various diseases has been reported either to aggravate colitis or lead to spontaneous colitis[23,24]. Feces were transplanted into sterile (GF) IL10 $^+$/EGFP reporting mice and IL$10^{-/-}$ mice. It was proved that the microbiota in specific pathogen-free mice mainly stimulated IL-10 to produce colon-specific B cells and T-regulatory-1 cells. IL-10, in turn, down-regulated the microbial activated inflammatory cytokines. These studies suggest that resident intestinal bacteria activate IL-10 producing B cells through the TLR2, MyD88, and PI3K

pathways. These B cells reduce the activation of colon T cells and maintain mucosal homeostasis under the action of intestinal microbiota[25]. The B cell subsets that produce IL-10 have different phenotypes and origins. Such B cell subsets appear in chronic inflammatory processes and inhibit the progression of intestinal inflammation by down-regulating the inflammatory cascade. In response to wounds, epithelial cells migrate and proliferate to cover the mucosal surface and repair barrier defects. This process is coordinated by immune cells and epithelial cells; however, the mechanism is not fully understood, and after mucosal injury, macrophage-derived IL-10 induces activation of epithelial cAMP response element binding protein (CREB) and then synthesis and secretion of Wnt1-inducible signaling protein 1 (WISP-1). WISP-1 induces epithelial cell proliferation and wound healing by activating the epithelial proliferation pathway. These findings suggest that macrophages are involved in regulation of the IL-10/CREB/WISP-1 signal axis and have broad significance in the relationship between innate immune activation and mucosal injury repair[26]. IL-10 is a key regulator of mucosal homeostasis. Some studies have shown that residents of *E. coli*-induced chronic colitis in mice have a rapid but temporary activation effect on the immune system in normal hosts, and the induced protection of IL-10 B cells and $CD4^+$ cells subsequently suppresses this reaction and modulates homeostasis in the mucosa[27].

In summary, IL-10 is the key factor for the negative regulatory function of Bregs. However, further studies are required to clarify the function of IL-10 secreted from Bregs. Whether it is expressed by specific transcription factors and whether immune tolerance to autoantigens is temporary or permanent needs to be determined.

ROLE OF VIP IN INTESTINAL IMMUNITY IN UC PATIENTS

VIP is a 28 amino acid neuropeptide that was originally isolated from the small intestine of pigs in 1970 by Sami Said and Victor Mutt. It belongs to the neuropeptide family that includes the pituitary adenylate cyclase activated polypeptide (PACAP). In the gastrointestinal tract, VIP occurs in endocrine cells and neurons of the enteric nervous system[28]. In established CD models, such as the colitis model induced by enteral injection of trinitrobenzene sulfonic acid (TNBS), VIP reduces the clinical and histopathological severity of the disease and reduces weight loss, diarrhea, and macroscopic and microscopic intestinal inflammation[29]. VIP treatment reduced the levels of various chemokines and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, IL-12, and IL-10[30]. In food allergy patients with enteritis, studies have found that VIP can increase the expression of TSP1, stabilize the expression of IL-10 in food allergy Bregs, and restore immune regulation function, thereby reducing the food allergy response. The results show that VIP has therapeutic potential for treating food allergy and other diseases related to Bregs immune regulation dysfunction[31].

However, the therapeutic effect of VIP in the management of UC has not been confirmed. Some studies have shown that the number of VIP positive nerve fibers in UC patients either decreases or remains unchanged[32-34]. Other studies have revealed that the level of VIP in the colon of UC patients increases with increased mRNA expression and VIP levels in neurons[34,35]. Similarly, plasma VIP level was also found to be elevated in UC patients[36]. Studies on the expression of VIP in UC patients are limited and contradictory, which might be due to the diversity of tissue sampling methods and patient populations. Jayawardena *et al*[37] reported that the VPAC1 receptor might mediate the pro-inflammatory effect of VIP, and VPAC2 might mediate the anti-inflammatory effect of DSS-induced colitis[37]. In addition, recent studies have shown that VIP and its receptor, VPAC1, cannot be detected in the tissues of IBD patients with severe mucosal barrier disorders[38]. These results suggest that the VIP response disorder may be one of the causes of IBD.

VIP plays a key role in protecting the colon epithelium from pathogenic bacteria. Impaired crypt cell dynamics in VIP knockout mice, including reduced proliferation and migration of intestinal epithelial cells (IEC) and their increased apoptosis, resulted in a percolating intestinal barrier susceptible to DSS- and TNBS-induced colitis[13]. In addition, impaired proliferation and increased apoptosis of IEC may be one of the reasons for impaired epithelial regeneration in VIP knockout mice. Similarly, in IBD patients, the inflammatory changes in VIP^+ neurons and their receptors may lead to occurrence of the disease through the loss of VIP-mediated epithelial homeostasis regulation[32,38,39].

VIP plays a critical role in the development and maintenance of the integrity of

colonic epithelium and the mucus barrier, possibly by activating caudal type homeobox transcription factor 2. VIP regulates the proliferation, migration, maturation, and secretion of bioactive cup-cell peptides in Cogu crypt cells to promote tissue repair and homeostasis, thereby controlling the susceptibility to colitis.

VIP REGULATES THE EXPRESSION OF IL-10 IN BREGS

VIP is a peptide secreted by nerve and immune cells. As a cytokine/chemokine, VIP performs a wide range of immune functions. It affects the innate and adaptive immunity through interactions with specific receptors, VPAC1, VPAC2, chemoattractant receptor-homologous molecule 2, and PAC1[40]. Both T and B lymphocytes express the VIP-related receptors[41], suggesting that B lymphocytes may regulate their immune response through VIP partly[42]. The levels of VIP in neurons and non-neurons were lower in UC patients. In UC, substance P and VIP decreased simultaneously. Further study is needed to determine the role of the neuropeptide in UC[43].

In innate immunity, VIP inhibits the production of inflammatory cytokines (interferon-γ, TNF-α, IL-6, and IL-17) and chemokines by immune cells (monocyte chemotactic protein-1, nuclear factor kappa-B), reduces the expression of co-stimulatory molecules (B7-1, B7-2 and CD40) on antigen-presenting cells, and reduces the stimulation of antigen-specific $CD4^+$ T cells. In addition, in adaptive immunity, VIP promotes the Th2 immune response and reduces the inflammatory Th1 response[44]. Although the exact mechanism remains to be clarified, VIP seems to regulate the Th1/Th2 balance in several ways as discussed below. First, VIP inhibits the production of the Th1-related cytokine, IL-12[45]. Second, VIP was reported to induce the expression of CD86 in resting mouse DCs, which played an important role in the development of Th2 cells[46]. Third, VIP inhibited CD95 (FasL) and granulase B-mediated apoptosis of mouse T2 cells but not of Th1 effector cells[47]. Lastly, VIP induced Th2 major transcription factors, c-MAF, GATA-3, and JUNB, during the differentiation of $CD4^+$ T cells in mice to inhibit T-bet, which is necessary for the differentiation of Th1 cells[48]. Therefore, VIP regulates the Th1/Th2 balance by directly acting on T cell differentiation and indirectly regulating the antigen presenting cell function.

Previous studies have shown that exogenous VIP can alleviate the histopathological symptoms of TNBS-induced colitis[29]. Studies have shown that rVIPa (VIP analogue) can improve TNBS-induced colitis in rats and effectively protect intestinal mucosal barrier function, and rVIPa can be used as a new alternative therapy for the treatment of intestinal inflammatory diseases[49]. VIP reinstated immune tolerance by down-regulating the inflammatory response and inducing Tregs[50]. VIP and PACAP inhibit IL-10 through direct transcriptional events. Unlike IL-2, IL-4 and IL-10 act as pro-inflammatory or anti-inflammatory cytokines, depending on the type of immune response they are involved in[51]. Specific VPAC1 receptors mediate the stimulation of VIP/PACAP, and cAMP is the main second messenger involved. VIP/PACAP increased lipopolysaccharide (LPS) to stimulate IL-10 mRNA in cells, and the effects of transcription and protein synthesis inhibitors suggested the *de novo* production of IL-10 protein. It may work together with pro-inflammatory cytokines, such as IL-6 and TNF-α, *in vivo* to reduce the intensity of the immune response. It has been proved that lentivirus VIP can down-regulate the expression of pro-inflammatory TNF mRNA and protein and up-regulate the expression of anti-inflammatory IL-10 mRNA and protein. In addition, VIP reduced the expression of TNF in mouse macrophages stimulated by LPS through the protein kinase C and protein kinase A pathways.

$CD5^+$ B cells are regulatory immune cells. Some studies have found that the expression level of TLR9 in colon *lamina propria* (LP) $CD5^+$ B cells is higher than that in CD5- B cells. Compared with LP CD5- B cells, colon LP $CD5^+$ B cells stimulated by TLR ligand produced more IL-10. Acute intestinal inflammation can temporarily reduce the frequency of colon LP $CD5^+$ B cells, while chronic inflammation can lead to the continuous decrease of colon LP $CD5^+$ B cells and lead to the state of mainly CD5- B cells. This study concluded that the continuous changes in mucosal B cells caused by chronic intestinal inflammation may be involved in the pathogenesis of IBD[52]. However, IL-10 secreted by Bregs may also be regulated by VIP and may affect the immune balance in intestinal inflammation, but this needs to be confirmed in future studies[53]. Recently, we found in human peripheral blood samples and in animal experiments that insufficient VIP levels in the UC intestinal microenvironment accelerated the degradation of IL-10 mRNA, leading to dysfunction of Bregs[54]. VIP plays an important role in stabilizing the expression of IL-10 mRNA in Bregs. The

administration of VIP can effectively inhibit experimental colitis in mice, suggesting the transformation potential of VIP in the treatment of UC.

In summary, VIP is a neuropeptide present in the lymphoid microenvironment and is a potent anti-inflammatory drug that inhibits the function of activated macrophages and Th cells. VIP may regulate the expression of IL-10 through different pathways, and IL-10 produced by B10 cells has been shown to play an important role in the Th2 immune regulation of UC. Stimulation of the transcription of IL-10 by VIP/PACAP could have important therapeutic potential (Figure 1). To date, VIP (Avidil in clinical application) has been successfully used in pulmonary hypertension and sarcoidosis. VIP in its native form, using sterically stabilized micelles treatment significantly reduced the mRNA levels of pro-inflammatory cytokines and showed significant histological recovery. Therefore, these results indicate that as a nanodrug, the anti-inflammatory and anti-diarrhea effects of VIP can be achieved in a single dose. Therefore, the study suggested the development of VIP SSM as a potential therapeutic tool for UC[55].

CONCLUSION

Usually, peripheral blood samples are collected from UC patients. Bregs are isolated from these samples and their immune regulatory function (serum IL-10 and VIP levels) is analyzed. Although an increasing number of studies have focused on Bregs in recent years, many problems concerning Bregs still need to be resolved. It remains unclear which antigen can promote the development of Bregs. Moreover, although TLRs, CD40, and CD80/CD86 are known to be involved in the activation of Bregs, whether other important co-stimulatory molecules or cytokines are involved in this process is unclear. In addition, IL-10 is a key factor in the negative regulatory function of Bregs, and further studies are needed to determine how IL-10 secreted by Bregs plays its role, whether it is expressed by specific transcription factors and whether immune tolerance to autoantigens is temporary or permanent. With further clarity on the mechanism of the regulation of IL-10 expression by VIP-Breg axis of colitis patients, we believe that VIP would provide a novel strategy for the clinical treatment of UC.

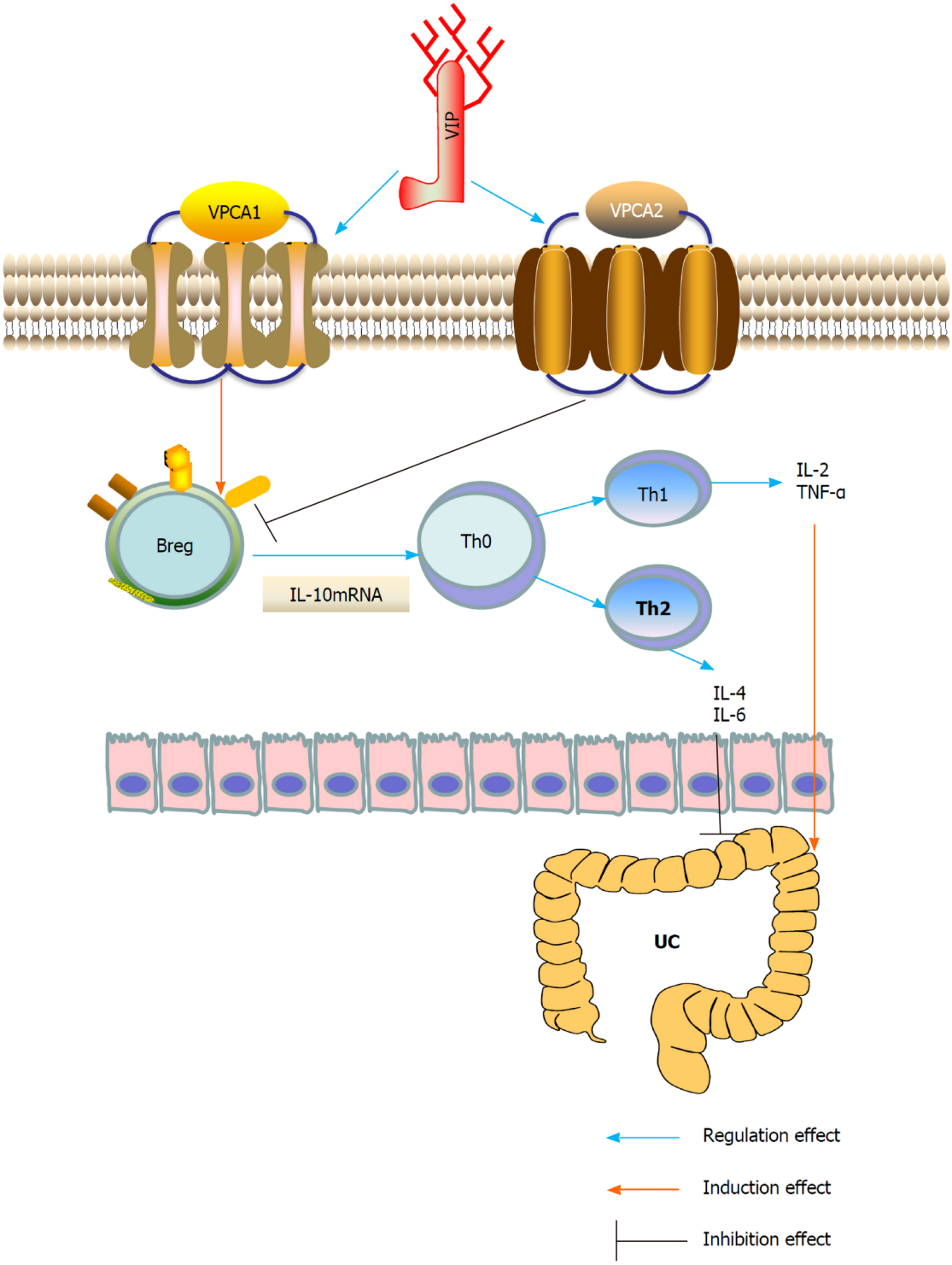

Figure 1 Underlying mechanisms of vasoactive intestinal peptide interference of ulcerative colitis by regulating the expression of interleukin-10 in regulatory B cells. Breg: Regulatory B cell; IL: Interleukin; Th: T helper; TNF-α: Tumor necrosis factor-α; UC: Ulcerative colitis; VIP: Vasoactive intestinal peptide.

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MINIREVIEWS

Gut microbiota mediated molecular events and therapy in liver diseases

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Abstract

Gut microbiota is a community of microorganisms that reside in the gastrointestinal tract. An increasing number of studies has demonstrated that the gut-liver axis plays a critical role in liver homeostasis. Dysbiosis of gut microbiota can cause liver diseases, including nonalcoholic fatty liver disease and alcoholic liver disease. Preclinical and clinical investigations have substantiated that the metabolites and other molecules derived from gut microbiota and diet interaction function as mediators to cause liver fibrosis, cirrhosis, and final cancer. This effect has been demonstrated to be associated with dysregulation of intrahepatic immunity and liver metabolism. Targeting these findings have led to the development of novel preventive and therapeutic strategies. Here, we review the cellular and molecular mechanisms underlying gut microbiota-mediated impact on liver disease. We also summarize the advancement of gut microbiota-based therapeutic strategies in the control of liver diseases.

Key Words: Gut microbiota; Intrahepatic immunity; Metabolite; Fecal microbial transplantation; Probiotic; Antibiotic

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Core Tip: Accumulating evidence shows that gut microbiota plays a critical role in liver pathophysiology and targeting gut microbiota is a potential treatment option for chronic liver disease. Herein, this review explores the cellular and molecular mechanisms of how gut microbiota contributes to liver diseases, including alcohol-induced and nonalcohol-induced liver fatty liver diseases, liver fibrosis, cirrhosis, and cancer. This review also summarizes the current gut microbiota-based therapeutic strategies and discusses future directions in promoting gut microbiota-based therapy for liver diseases.

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INTRODUCTION

The growing evidence of gut microbial roles in human diseases attracts researchers' attention in exploring gut microbiota-mediated therapy. The gut microbiota is defined as the entire community of microorganisms residing in the gastrointestinal tract, and it is dominated mainly by bacteria[1]. In the earlier stages of investigation, people focused solely on the gut microbiota's function regarding modulation of human nutrition and metabolism, on which a healthy status relies. However, dysbiosis of the gut microbiome may cause inflammatory bowel disease and bacterial infection in addition to impairing human alimentation[2]. Nowadays, growing evidence has emerged to demonstrate how gut microbiota profoundly and systemically influences human health and disease through various mechanisms, particularly influencing cancer. It has been discovered that gut microbiota and their associated metabolites are closely related to malignant tumor generation by developing chronic inflammation and immune surveillance dysregulation.

More interestingly, due to the close anatomical and physiological connection between the gut and liver, the role of gut microbiota and its associated metabolites were found in liver diseases from many studies (discussed in the following sections). By focusing our attention on the interactions between gut microbiota and liver diseases, we are pulling back the curtain on how gut microbiota and its associated metabolomes influence liver health and disease. However, it seems as though these findings are but the tip of the iceberg. Many questions still remain. Some cause-specific and disease severity-specific microbiota in liver diseases have been reported, but the underlying mechanisms are still unclear or only show a piece of the whole picture. For example, there are distinct explanations for antibiotics cocktail (ABX) mediated suppression of hepatocellular carcinoma (HCC) tumor progression. Therefore, it is not surprising that many different independent studies targeted the same question to attain different answers, as gut-mediated effect on the liver is involved by a wide range of factors. This further confirms that interactions amongst the gut microbiota and liver work through multiple pathways. These interactions may have developed over a long period as a result of evolution. A systemic review of recent research findings in the gut-liver axis helps to dig the underlying mechanism of how the gut modulates liver function and disease treatment.

Here, we discuss the current findings in correlations between gut microbiota and categorized liver diseases. The perspectives of underlying mechanisms will be reviewed and summarized. Development in gut-microbiota-manipulation-based preventive and therapeutic strategies in liver diseases also will be considered. Given all the current progress, gaps in understanding the influence of gut microbiota on liver diseases are proposed, and the feasible directions are prospectively assumed.

DYSBIOSIS OF GUT MICROBIOTA FACILITATES LIVER DISEASES

Nonalcoholic fatty liver disease and alcoholic liver disease

Nonalcoholic fatty liver disease (NAFLD) and the advanced stage nonalcoholic steatohepatitis (NASH) are currently the most common types of liver disease in humans. Studies have indicated that alteration of gut microbiota is involved in the development of NAFLD and NASH[3,4]. Endotoxemia induced by increased gut permeability has been observed in patients with NAFLD, suggesting that gut permeability-induced inflammatory pathways contribute to NAFLD pathogenesis[5]. Small intestinal bacterial overgrowth (SIBO) is another often observed dysbiosis of gut microbiota occurring in NAFLD/NASH[3,6]. Along with the altered intestinal microbiota profiles, SIBO was seen in most patients with cirrhosis[7]. The presence of SIBOs may partially explain why the permeability of the gut increases. Gut permeability and SIBOs enhance the hepatic expression of Toll-like receptor 4 (TLR-4) and the production of interleukin 8 (IL-8). Therefore, determining the specific pathogenic bacteria species has been made a very appealing question. Clinical studies revealed that patients with NASH exhibited significantly lower concentrations of *Bacteroidetes* in their gut compared to healthy individuals[4,8]. Meanwhile, another cohort study showed that high-alcohol-producing *Klebsiella pneumoniae* was associated with up to 60% of NAFLD patients[9]. Selective depletion of *K. pneumoniae* before fecal microbiota transplant (FMT) from NASH patients into mice prevented NAFLD development in recipient mice. In contrast, the study on patients with alcohol-induced liver cirrhosis exhibits a decreased proportion of *Bacteroidaceae* family than healthy individuals[10]. Also, the analysis of the gut microbiota profiles in patients with cirrhosis revealed an increase in pathogenic bacteria and a decrease in beneficial bacteria, such as a decreased *Bacteroidetes* along with overgrowth of the *Proteobacteria* and *Enterobacteriaceae* species[11,12]. However, it remains unclear if this change drives disease or it comes from disease as a result. To answer this question, a well-designed, large-scale clinical and experimental investigation is needed. Moreover, in excessive alcohol intake induced gut microbiota dysbiosis, the intestinal innate immune responses, such as secreted antimicrobial molecules, may impact disease development. For example, the reduced bactericidal c-type lectins, Reg3b, and Reg3g in the small intestines were discovered after alcohol feeding, further causing SIBO and dysbiosis in mice[13].

HCC

HCC, the final stage of chronic liver disease, has been evidenced to have gut microbiota involved in its initiation and progression. In DEN/CCl_4 induced HCC murine model, germ-free mice presented fewer and smaller tumors compared to its syngeneic parallel[14], indicating that gut commensal flora is required for tumor progression and the activation of TLR-4 by lipopolysaccharide (LPS) promotes tumor generation. In HCC murine models, studies also demonstrated that the administration of ABX in tumor-bearing mice effectively slowed tumor progression[15,16]. Even though the underlying mechanisms were distinct in those studies, which will be further discussed in the following sections, we can assume modulating gut microbiota is favorable to prevent liver cancer. However, there is still a lack of clinical data to support it. In addition, obesity-induced gut microbiota dysbiosis that produces DNA damaging bacterial metabolites has been indicated to facilitate HCC development in high-fat diet (HFD)/carcinogen 7,12-dimethylbenz[a]anthracene treated murine model. Furthermore, the administration of antibiotics was able to reduce the prevalence of tumors in the chemically treated mice[17]. However, in those studies, the relevant specific bacteria species remain unclear. Clearly identifying either specific pathogenic or beneficial bacteria species may be the next mission.

MODULATION OF INTRAHEPATIC IMMUNITY, THE CENTRAL ROLE OF GUT MICROBIOTA IN LIVER DISEASE

Although etiologies vary in liver diseases, inflammation and liver fibrosis are the basis for most liver diseases. The intrahepatic immunity has been certified as an acting point, as gut microbiota can play different roles in liver inflammation and establishment of fibrosis through different liver infiltrating immune cells and resident hepatic cells[18]. Furthermore, the immune checkpoints emerged as a central pivot to modulate disease progression with gut microbiota-derived metabolites as informatic messengers (Figure 1) and various TLRs as informatic receivers.

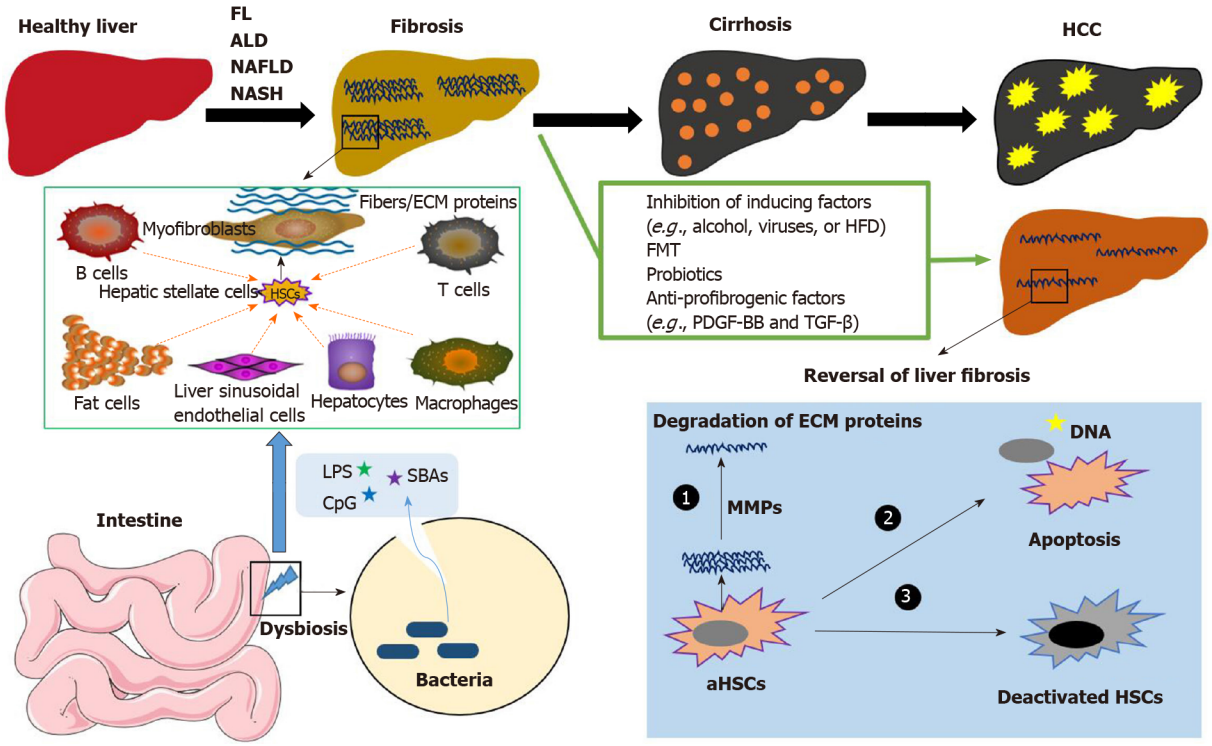

Figure 1 The development of liver diseases. Without effective treatment or preventive strategies, fatty liver disease, alcohol-induced liver disease, nonalcoholic fatty liver disease, and nonalcoholic steatohepatitis can result in liver fibrosis, cirrhosis, and hepatocellular carcinoma. Gut microbiota-derived molecules, including lipopolysaccharide, CpG, and secondary bile acids, initially activate liver resident cells to produce cytokines and chemokines. Quiescent hepatic stellate cells (HSCs) can be activated and transformed into myofibroblasts (MFBs), which is mediated by chemokines and cytokines released by liver-infiltrating macrophages, leukocytes, and other cell types, including fat cells and damaged hepatocytes. MFBs are the predominant source of collagen-producing cells and other extracellular matrix proteins (ECM). With effective treatment, such as fecal microbiota transplant, probiotics, and anti-profibrogenic factors, fibrosis is reversible. The treatments that induce apoptosis (or deactivation) of activated HSCs or MFBs and degrade the ECM proteins can reduce the stiffness of the liver, reverse liver fibrosis, and inhibit the progression of liver disease. FL: Fatty liver; ALD: Alcohol-induced liver disease; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; ECM: Extracellular matrix proteins; HFD: High-fat diet; FMT: Fecal microbiota transplant; LPS: Lipopolysaccharide; SBAs: Secondary bile acids; HSC: Hepatic stellate cells.

Hepatic resident and infiltrating cells

Hepatic cells are comprised of parenchymal hepatocytes and the non-parenchymal cells (NPCs), including quiescent hepatic stellate cells (HSCs), Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and diverse lymphocytes (*e.g.*, natural killer cells, NKT cells), which play crucial roles in the liver health and disease[19,20]. Here, we discuss how gut microbiota modulates intrahepatic immune responses by different hepatic cell types.

Hepatocytes: Hepatocytes consist of two-thirds of the total liver cell population and are responsible for the primary metabolic functions and engage in the immune responses by interacting with NPCs[21]. In different mouse models of liver fibrosis, either induced with alcohol or non-alcohol (*e.g.*, CCl_4 or HFD), injured hepatocytes release reactive oxygen species and fibrogenic mediators, which recruit lymphocytes and promote the activation of HSCs[22]. The Notch signaling pathway also plays a pivotal role in liver development, liver repair, and carcinogenesis[23]. Notch activation in hepatocytes can induce and promote liver fibrosis in normal chow diet and NASH diet-fed mice[24], respectively. Even though the mechanism by which the gut microbiota directly impacts the Notch signaling in hepatocytes is unclear, some studies have shown that dysbiosis of gut microbiota may increase the activation of the Notch signaling pathway, thus promoting tumor regression[25]. In addition, the TLR signaling pathway of hepatocytes is directly involved in the gut-liver axis, which will be discussed in the following context.

HSCs: HSCs reside in the space of Disse (perisinusoidal space) between the hepatocytes and LSECs. Their main functions include the storage of vitamin A and lipid droplets[26]. HSCs are the primary sources of liver myofibroblasts[27] and play a key role in the initiation, progression, and regression of liver fibrosis[28]. All other liver cells can directly or indirectly impact the activation, trans-differentiation of HSCs to

myofibroblasts, and their deactivation. There are multiple cellular and molecular signals involved in these reactions[29]. The interaction of HSCs with other hepatic cells is bidirectional. For instance, upon activation, HSCs, in turn, can further activate macrophages by secreting macrophage colony-stimulating factor (M-SCF), MCP-1, IL-6, RANTES, and so on[30].

KCs: Liver macrophages, including the liver resident KCs and circulating monocyte-derived macrophages, play essential roles in various liver diseases, including virus hepatitis, alcoholic liver disease (ALD), NAFLD, NASH, and HCC[31,32]. The purpose of macrophages in NAFLD and NASH and the underlying mechanisms involved in liver inflammation and liver fibrosis have been well summarized recently[33]. Gut-derived endotoxins (LPS), lipid metabolites, and hepatocyte damage-associated molecules are the main factors inducing macrophage activation in NAFLD[33]. Furthermore, the activated macrophages secrete chemokines (*e.g.*, CCL2) and cytokines (*e.g.*, TGF-β1), which leads to the activation of T cells and the transformation of HSCs into myofibroblasts.

LSECs: With the loss of fenestration, capillarized LSECs reduce the transfer of nutrients and other products from the blood to hepatic cells[34,35], which occurs with liver fibrosis in animals and humans[36]. The capillarized LSECs can secrete TGF-β1 and contribute to the accumulation of extracellular matrix proteins, including fibronectin and laminin in the liver, promoting the activation of HSCs and contributing to the formation of liver fibrosis[35]. Xie *et al*[37] reported that the reversal of LSEC differentiation with the use of BYY 60-2770, an activator of soluble guanylate cyclase (sGC), prevented the progress of rat liver cirrhosis. LSECs are directly exposed to dietary and bacterial products from the gut through the portal circulation. It has been shown that there is a link between the fenestration of LSECs and diet-induced changes in the gut microbiome. The increase of fenestration of LSECs was associated with a high abundance of *Firmicutes* and a low amount of *Bacteroidetes*[38].

NKT cells: *In vivo* animal studies showed that alteration of gut bacteria with ABX treatment induced a liver-selective anti-tumor effect with an increase of hepatic $CXCR6^+$ NKT cells and heightened IFN-γ production upon antigen stimulation[15]. NKT cell accumulation was regulated by CXCL16 expression of LSECs, which is controlled by gut microbiome-mediated primary-to-secondary bile acid (SBAs) conversion[15]. Similarly, Zhang *et al*[39] reported a significant increase of primary bile acids (PBAs) in the large intestine of mice treated with antibiotics such as vancomycin and imipenem. This antibiotic treatment was also associated with the selective suppression of several bacterial species such as *Clostridium,* responsible for deconjugating PBAs into SBAs. Selective depletion of intestinal bacteria *Firmicutes* increases PBA levels, which further promotes hepatic NKT cell accumulation and NKT-mediated anti-tumor effect, indicating that modulating gut microbiota is an optional strategy for HCC treatment.

Immune checkpoints

The efficacy of anti-immune checkpoints (PD-1 or CTLA4) in cancer treatment was markedly reduced in germ-free mice or specific-pathogen-free mice treated with broad-spectrum antibiotics[40]. For instance, the efficacy of anti-CTLA4 on melanoma immunotherapy in mice and patients was associated with *Bacteroides fragilis*, since gavage with *B. fragilis* or adoptive transfer of *B. fragilis*-specific T cells recovered the anti-tumor efficacy of CTLA4 blockade in antibiotic-treated or GM mice[41]. Similarly, the abundance of *Akkermansia muciniphila* was associated with the effectiveness of anti-PD-1 immunotherapy[42]. FMT from cancer patients who were not responding to anti-PD-1 treatment to tumor-bearing mice failed to ameliorate the anti-tumor effects of anti-PD-1. In contrast, oral supplementation of *A. muciniphila* restored the anti-tumor efficacy of anti-PD-1 in those mice[42]. Meanwhile, Gopalakrishnan *et al*[43] also reported the gut microbiota modulated therapeutic responses to anti-PD-1 immunotherapy in human melanoma patients. Iida *et al*[44] concluded that antibiotic-induced disruption of gut microbiota impaired both CpG oligonucleotide immunotherapy and platinum chemotherapy in their subcutaneous breast cancer murine model. Investigation on anti-immune checkpoints on HCC has recently begun[45,46]; however, the role of gut microbial effects on the blockage of immune checkpoints in liver diseases remains unclear. Notably, immune checkpoint inhibitors-associated hepatotoxicity should not be ignored[47,48]. For example, clinical trials have shown that alanine aminotransferase elevations in grade 3-4 levels are observed in approximately 20% of patients who are treated with ipilimumab (CTLA-4 inhibitor) and nivolumab (PD-1/PD-L1 inhibitor)[49].

Metabolites

Currently, the role of microbiota in liver diseases has been broadly investigated[50]. Gut microbiota-associated metabolites such as fatty acids, amino acids, and carbohydrates are a group of contributory factors involved in the modulation of intrahepatic immune responses[51]. Ma *et al*[15] observed that manipulation of gut microbiota by treatment with an ABX can suppress liver tumor growth *via* accumulated NKT cells, which increased anti-tumor immunity in the liver. Modulating commensal gut microbiota composition with ABX treatment changed the bile acid metabolism and significantly increased the PBA production in the liver microenvironment. This rise in PBA production elevated CXCL16 expression on LSECs, which recruited more CXCR6 specific NKT cells to the liver. Conversely, SBAs have been shown to reverse this phenomenon and reduce CXCL16 expression on LSECs. As such, alteration of the ratio of PBAs to SBAs resulted in the growth of hepatic NKT cell accumulation in the ABX treated mice compared to control mice. In addition, the conversion of PBAs to SBAs can be significantly inhibited with the treatment of antibiotics. SBAs, such as deoxycholic acid, activated mTOR to promote the HCC development in NASH mice fed with a steatohepatitis-inducing high-fat diet (STHD-01)[52]. Accumulation of free fatty acids in hepatocytes can also induce lipotoxicity, which causes hepatocellular injury and NAFLD progression[53]. Similarly, excess carbohydrates from dietary intake can be *de novo* synthesized to lipids in the liver, causing lipotoxic liver injury[54]. The characteristics of metabolites of gut bacteria with the association of liver diseases found in recent studies are summarized in Table 1. The mechanisms of how microbiota-derived metabolites might influence liver diseases or their potential roles as diagnostic markers have been summarized when we were drafting this review[55-61].

TLRs

The TLR family is one of the best-characterized families of pattern recognition receptors[62], which can activate the innate immune system by recognizing pathogen-associated molecular patterns and damage-associated molecular patterns[63]. There are a total of 13 TLRs in mammals; only TLR1 to 10 exist in humans[64]. TLRs, excluding TLR10, can bind with different bacteria-derived molecules to participate in the inflammatory and immune responses[65]. The gut-liver axis defines the close anatomic connection, through the circulation of the portal vein and biliary tract, and the functional interaction of the gastrointestinal tract and the liver[66]. Through the portal vein, bacteria-derived products, including LPS and CpG-containing DNA, might regularly activate TLR signaling pathway in the hepatocytes and other hepatic cells. Current studies have demonstrated that TLR signaling pathways play pivotal roles in liver inflammation, fibrosis, regeneration, and carcinogenesis[67,68]. TLRs are broadly expressed in hepatic cell populations, including hepatocytes, LSECs, KCs, lymphocytes, DCs, biliary epithelial cells, and HSCs[68], which link the gut-liver axis. In this review, we summarize the recent findings of the role of TLRs in liver diseases.

TLR1, TLR6, and TLR10: Currently, TLR10 is the only member of the human TLR family without a definite known ligand, function, and localization[69]. Phylogenetic analysis shows that TLR10 is most related to TLR1 and TLR6, both of which mediate immune responses in cooperation with TLR2[70]. The study of chimeric receptors of TLR10 indicated that it can sense triacylated-lipopeptides and other microbial-derived agonists, the ligands of TLR1[70]. We will not describe the separate roles of these three TLRs at this point.

TLR2: Liver injury resulting from adenovirus or CCl_4 promoted more robust liver inflammation by releasing the TLR2/TLR4 ligands, including HSP60, gp96, HMGB1[71]. The amplified non-autoimmune hepatitis (AIH)-inflammation could result in the initiation of AIH, a severe autoimmune liver disease, which can lead to fibrosis, cirrhosis, and HCC. Conversely, TLR2 deficiency promoted HCC development in C57BL/6 mice associated with the increase of $Ly6C^{high}IL18R\alpha^{+}$ myeloid-derived suppressor cells and with impaired $CD8^{+}$ T cells function[72]. This result further confirmed the previous finding that TLR2 knockdown by siRNAs inhibited the proliferation of HCC cell line BLE-7402, which was associated with a reduction of IL-6 and IL-8 production[73].

TLR3: TLR3 activation by agonist double-stranded RNA BM-06 or poly(I:C) induced apoptosis of HepG2.2.15 HCC cells[74]. The analysis of human HCC tissue also indicated that the expression of TLR3 positively correlated with apoptosis of HCC cells and negatively correlated with the proliferation of HCC cells and angiogenesis[75], suggesting that TLR3 expression may serve as a prognostic marker of HCC.

Table 1 Summary of metabolites associated with liver diseases

Liver disease	Sample sources	Metabolites	Gut microbiota	Functions in liver disease	Ref.
NAFLD	Serum	3-(4-hydroxyphenyl) lactate	Abundance in bacteria *Firmicutes*, *Bacteroidetes*, *Proteobacteria*	It is positively associated with liver fibrosis	[56]
NAFLD	Serum	Eight lipids (5α-androstan-3β monosulfate, pregnanediol-3-glucuronide, androsterone sulfate, epiandrosterone sulfate, palmitoleate, dehydroisoandrosterone sulfate, 5α-androstan-3β disulfate, glycocholate), one amino acid (taurine) and one carbohydrate (fucose)	Without special bacterial species	Glycocholate is positively associated with advanced liver fibrosis	[57]
FL	Stool	Tryptamine and I3A	Microbiota-dependent without exact bacterial species	They inhibited the pro-inflammatory cytokines in macrophages and hepatocytes. I3A attenuated inflammatory responses under lipid loading and reduced the expression of fatty acid synthase and sterol regulatory element-binding protein-1c	[58]
FL	Stool	Gly-MCA	Increases in the ratio of *Bacteroidetes*/*Firmicutes*	Gly-MCA is an intestinal FXR antagonist, which inhibits HFD-induced fatty liver	[59]
HCC	Stool	Secondary bile acids, such as deoxycholic acid	Increase of *Bacteroides* and *Clostridium* cluster XVIII. Low of *Streptococcus*, *Bifidobacterium*, and *Prevotella*	Bile acids derived from the increased microbiota promote the progression of HCC	[52]
HCC	Stool	Bile acids, such as primary bile acid CDCA	Increase in *Clostridium* species	Removing gram-positive bacteria by antibiotic treatment with vancomycin, which contains the bacteria mediating primary-to-secondary bile acid conversion, was sufficient to induce hepatic NKT cell accumulation and decrease liver tumor growth	[15]
Liver cirrhosis	Stool	Glutamic acid, fumaric acid, 4-aminobutyric acid, succinic acid, isoleucine, valine, lactic acid, mannitol, sorbitol, carbamide, 4-aminobutyric acid, 5-aminopylamine, glutamate, proline, hydroxyproline	High of *Enterobacteriaceae* and *Veillonella*. Low of *Bacteroidetes*	These metabolites are involved in the KEGG pathway in nitrogen metabolism alanine, aspartate, and glutamate metabolism, and valine, leucine, and isoleucine, pantothenate and CoA biosynthesis, glycolysis/ gluconeogenesis, fructose and mannose metabolism, arginine and proline metabolism	[60]
NAFL or NASH	Stool	1-pentanol and 2-butanone, and 4-methyl-2-pentanone	Abundance in *Oscillospira*, *Dorea*, and *Ruminococcus*	The results indicated that significantly lower levels of *Oscillospira* and higher levels of 1-pentanol and 2-butanone in NAFL patients compared to healthy ones. In NASH, lower levels of *Oscillospira* were associated with a higher abundance of *Dorea* and *Ruminococcus* and higher levels of 2-butanone and 4-methyl-2-pentanone compared to CTRLs	[4]
ALD	Stool, urine	*e.g.*, SCFAs butyrate and propionate	The main harmful bacterial species included altered *Bacteroides phylum* as well as *Bilophila*, *Alistipes*, *Butyricimonas*, *Clostridium*, *Proteus*, and *Escherichia coli*. *Faecalibacterium*	Metabolites are affected by chronic ethanol feeding or consumption, including amino acids, steroids and their derivatives, fatty acids and conjugates	[61]

NAFLD: Nonalcoholic fatty liver disease; FL: Fatty liver; I3A: Indole-3-acetate; Gly-MCA: Glycine-β-muricholic acid; HFD: High-fat diet; HCC: Hepatocellular carcinoma; CDCA: Chenodeoxycholic acid; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; ALD: Alcohol-induced liver disease; SCFAs: Short-chain fatty acids.

TLR4: TLR4 is one of the most well-known TLRs implicated in the liver inflammation-fibrosis-cancer axis. TLR4 Ligands, including LPS, can induce liver fibrosis by promoting the transformation of HSCs to myofibroblasts through the TLR4-MyD88-

NF-κB signaling pathway[76]. Dapito *et al*[14] reported that TLR4 was required for HCC progression but not the initiation, which is relative not limited to the increase of hepatomitogen epiregulin. Suppressing the TLR4 signaling pathway in HSCs by Dectin-1, the major progenitors of myofibroblasts, inhibited hepatic fibrosis and hepatocarcinogenesis[77]. Moreover, blockage of TLR4 also potentially inhibited the LPS induced inflammatory reaction and other immune responses[78].

TLR5: TLR5 knockout can attenuate CCl_4-induced liver fibrosis in wild-type C57BL/6 mice and the activation of HSCs *via* inhibiting NF-κB and MAPK signaling pathways[79]. Through the same signaling pathway, TLR5 knockdown also can ameliorate hyperammonemia (HA)-induced liver injury in rats by inhibiting hepatocyte apoptosis, inflammation, and oxidative stress[80].

TLR7: TLR7 activation by its natural ligand imiquimod can induce autophagy and release of insulin-like growth factor (IGF-1) and inhibit lipid accumulation in hepatocytes induced by unsaturated fatty acid (arachidonic acid: oleic acid = 1:1) *in vitro*[81]. More so, *in vivo* experiments also showed that TLR7 knockout prevented NAFLD progression *via* inducing autophagy and the release of IGF-1 from the liver.

TLR8: Even though not many studies about the role of TLR8 are reported currently, its role in liver disease should not be ignored. Stimulation of liver intrasinusoidal cells with TLR agonist 1/2, 2, 2/6, 3, 4, 5, 7, 8, or 9 (respectively Pam3CSK4, HKLM, FSL-1, poly(I:C), LPS, flagellin, imiquimod, ssRNA40, or CpG ODN2216) indicated that only the TLR8 agonist ssRNA40 selectively can activate the innate immune cells to produce IFN-γ[82].

TLR9: Tak1ΔHep mice with hepatic deletion of transforming growth factor-β-activated kinase 1 (Tak1ΔHep) can develop a spontaneous liver injury, inflammation, fibrosis, and HCC, mimicking the progression of human HCC[83]. Ablation of TLR9 or TLR4 suppressed the spontaneous process liver inflammation-fibrosis-cancer axis in Tak1ΔHep mice. The inhibition of HCC progression was associated with downstream signaling molecules MyD88 and TNFR.

MANIPULATION OF GUT MICROBIOTA, A PROMISING THERAPEUTIC STRATEGY IN LIVER DISEASE

We have previously highlighted the strong correlations between liver disease and the intestinal microbiota. Further, the modulation of gut microbiota and its products may prove to be a promising target for liver disease and HCC treatment. For example, the abundance of *Enterococcus faecalis* was associated with liver disease severity and the mortality of patients with alcoholic hepatitis due to cytolysin production[84]. Treatment with *E. faecalis*-targeted bacteriophages can ameliorate alcohol-induced liver disease in humanized mice by reducing liver cytolysin. The intestinal microbiome contains seven-fold more genetic material than the human genome, providing an abundance of potential therapeutic targets. Here, we focus on current studies involving probiotics, FMT, and antibiotics in liver disease treatment.

Probiotics, a new use of an old remedy against liver diseases

Probiotics, prebiotics, and synbiotics have recently undergone investigation for their ability to influence the gut microbiota composition[2]. Research has long established that probiotic yogurt can positively impact gut health[85], and more so, the prebiotic insulin was shown to reduce hepatic lipogenesis and plasma triglyceride concentrations in human patients[86]. Further, the use of probiotics to combat the altered gut microbiota profile in liver disease is currently being investigated in clinical trials[87]. One recent randomized, double-blind, placebo-controlled study investigated the effects of probiotic treatment on intrahepatic fat fractions in 68 patients with NAFLD. They reported that patients exhibited decreased intrahepatic fat fractions after 12 wk of probiotic treatment (from 16.3% ± 15.0% to 14.1% ± 7.7%, P = 0.032), with an overall mean decrease of 2.61% when compared to baseline measurements[88]. Although the argument that this statistical significance may not be clinically significant, the authors propose a mechanism by which specific probiotic agents may aid in correcting dysbiosis rather than directly decreasing hepatic fat. Moreover, this may lead others to investigate the use of probiotics for correcting dysbiosis in liver disease.

FMT, a star of tomorrow to control patient's gut microbiota in personalized therapy

FMT is also an old yet underrepresented area of investigation. This technique has gained considerable attention after its effectiveness in the last resort treatment of antibiotic-resistant infections such as *Clostridium difficile*[89]. The advantage of FMT from healthy persons to patients is the resetting of gut microbiota composition, returning the flora to a "healthy" state[87]. To investigate the effects of FMT in liver disease, one study used fecal material from patients with ALD to transplant into germ-free mice through oral gavage. The investigators reported increased intestinal permeability and bacterial translocation, resulting in severe liver inflammation in the microbiome repopulated mice[90]. Similarly, a pilot study containing eight male patients with severe alcoholic hepatitis were treated with nasoduodenal FMT over the course of 7 d. Results revealed the resolution of ascites and hepatic encephalopathy (HE) within one week in most FMT treated patients, along with significantly improved 1-year survival rates (87.5% *vs* 33.3%; $P = 0.018$) compared to historical controls[91]. Taken together, these results demonstrate the importance of the intestinal microbiome population in the development and treatment of liver diseases. While FMT provides distinct challenges for its commercial use in the clinical setting, its profound therapeutic effects can no longer be ignored when current first-line therapies lack adequate efficacy.

Antibiotics, perhaps another resolution in fighting liver diseases

Treatment with antibiotics is the most obvious approach for modulating the intestinal bacterial profile and its products. It has been well documented that microbiota depletion with ABX results in a significant reduction of tumor number, size, and fibrosis severity in several HCC murine models[14,15,17,76]. While ABX treatment is unfit for human studies, antibiotics have undergone investigation to treat liver disease in many clinical trials. The overwhelming majority of current antibiotic studies utilize the antibiotic rifaximin. Further, this section will primarily report current findings related to rifaximin treatment and liver disease; however, ABX and norfloxacin treatments will also be briefly discussed.

Rifaximin: Rifaximin is a highly favorable antibiotic due to its safety profile and broad-spectrum effects against gram-positive and gram-negative aerobic and anaerobic bacteria. Its classification of a non-absorbable antibiotic makes it well suited for targeting the intestinal microbiome rather than treating systemic infections. Unique to most antibiotics, rifaximin works by binding to the beta-subunit of bacterial DNA-dependent RNA polymerase, consequently blocking bacterial transcription[92]. Further, this results in endotoxin-lowering and anti-inflammatory effects rather than diminishing the gut flora composition[93-95]. Multiple studies have correlated rifaximin treatment with reduced risk of death and cirrhotic complications in patients with HE[96-98]. More so, a phase 3, open-label maintenance study revealed long-term treatment (≥ 24 mo) of rifaximin (550 mg, twice daily) showed no increased adverse events in HE patients compared to placebo controls, further confirming its safeness of use[98]. However, patients with cirrhosis lack average BA concentrations, resulting in decreased efficacy of rifaximin. To combat this issue, rifaximin soluble solid dispersion (SSD) was introduced. Unlike the previous generation, rifaximin SSD is water-soluble and no longer dependent on the patient's intestinal BA concentrations. Despite this distinct advantage, few clinical trials have implemented rifaximin SSD into their liver disease studies.

It is widely theorized that liver cancer progression is partly mediated by gut dysbiosis and other downstream effects. Further, we believe a longitudinal study should be performed to assess the preventative effects of rifaximin and rifaximin SSD on liver cancer development in a high-risk patient population. While it is highly unlikely that rifaximin would provide a therapeutic effect in patients who have already developed tumors, the benefits of long-term, preventative rifaximin treatment on patients with a high risk of developing HCC may provide a better response than the current treatment options.

ABX: Besides enhancing anti-tumor immunity in the liver, antibiotics may also weaken the tumor-promoting impact of specific intestinal bacteria. One study investigated how antibiotic alteration of the gut microbiota impacted liver carcinogenesis by depleting the intestinal flora of mice through regular treatment of ABX. Further, results indicated a significant reduction in tumor number, size, and liver-body weight ratio compared to the untreated control mice[14]. More so, the authors observed that antibiotics mitigated tumorigenesis in a healthy liver but did not impact tumors that had already developed. These results suggest that the gut microbiota plays a role in preventing hepatic proliferation and fibrogenesis, leading to liver carcinogenesis. And

also, there was a decreased expression of NF-kB regulated genes and increased apoptosis rates in gut-sterilized mice[14], indicating that the gut flora and its products may promote liver inflammation and carcinogenesis *via* activating hepatic TLR-4, further exerting pro-survival signals on hepatocytes.

Norfloxacin: Norfloxacin antibiotics may also attenuate pro-inflammatory phenomena exerted by the immune system. Zapater *et al*[99] observed that selective intestinal decontamination, facilitated by the antibiotic norfloxacin, in patients with cirrhosis reduced serum and ascitic concentrations of pro-inflammatory cytokines TNF-α, IFN-γ, and IL-12 by blunting the activation of serum NF-κB. Furthermore, norfloxacin treated patients exhibited both decreased levels of neutrophilic oxidative burst and apoptotic events. The authors did not describe a precise mechanism by which the fluoroquinolone mitigated immunological processes, though they did propose that norfloxacin exerted a direct effect on cellular function.

CONCLUSION

Here we have described many reported mechanisms that the gut-liver axis plays in liver disease. Further, we aim to spread awareness of the existing knowledge gap that could be used to modulate the gut-liver axis to alter the disease state. The modulation of the intestinal microbiome has transitioned to clinical trials for its use in both preventative and therapeutic approaches in human liver disease. A summary of the current ongoing clinical trials can be found in Table 2. It should be recognized that alteration of the gut microbiota is not a one size fits all solution due to its highly individual nature and convoluted intricacies. However, the bilateral relationship of the gut-liver axis cannot be ignored when its alteration possesses abundant therapeutic potential.

To aid in furthering the use of gut microbiota-based therapy against liver diseases, we will give brief suggestions for future directions in this field. Lacking from clinical data are well-designed, large-scale investigations of the gut flora profile for liver disease progression (*e.g.*, NAFLD, NASH, HCC, *etc.*). Understanding the underlying mechanisms of how the gut microbiota or its metabolic products alter liver immunity over time could help clarify liver disease development. Such profiles could provide a model to test preventative treatments or therapeutic approaches with antibiotics, probiotics, and FMT. Establishing which bacteria to eliminate and which to nurture would allow for tailored probiotics and specific antibiotics for chronic liver disease therapy. Continuing with this strategy comes the need for longitudinal studies to assess the preventative and therapeutic effects of antibiotics (*e.g.*, rifaximin and rifaximin SSD) on liver disease development. As stated previously, rifaximin SSD would be an ideal drug to study its preventative measures against high-risk liver cancer populations. Pre-, pro-, and synbiotic strategies are underwhelming in clinical studies due to a lack of high-quality study design. Our review highlighted how a decreased level of *Bacteroidetes* is seen in patients with NASH, cirrhosis, and other liver diseases. Finding commonalities in the abovementioned gut profiles (*e.g.*, decreased *Bacteroidetes*) will prompt the use of gut microbiome repopulation therapies in clinical trials. Lastly, the use of FMT treatment desperately needs a more substantial safety investigation. Conducting a proper prospective cohort study with a large study size and a longer observation period is crucial for its safe implementation in the clinical setting.

Collectively, modulating the population of the gut microbiome by biological products may be a new generation therapeutic strategy for liver disease. For now, we must wait to see the results of ongoing and scheduled clinical trials. As we wait in anticipation for the results of mentioned clinical trials, we hope that our current knowledge of the gut-liver-axis inspires new ideas for the next potential therapies in liver disease.

Table 2 Gut microbiota-based clinical trials

Study	Mechanism	Trial phase	Patients (*n*)	ClinicalTrials.gov identifier	Estimated study completion date
Nivolumab (Anti-PD1), Tadalafil and Oral Vancomycin in People with Refractory Primary Hepatocellular Carcinoma or Liver Dominant Metastatic Cancer from Colorectal or Pancreatic Cancers	Immunotherapy + Antibiotic	Phase II	27	NCT03785210	2022
Administration of Rifaximin to Improve Liver Regeneration and Outcome Following Major Liver Resection (ARROW)	Antibiotic	Phase II	96	NCT02555293	2020
Efficacy of the Combination of Simvastatin Plus Rifaximin in Patients with Decompensated Cirrhosis to Prevent ACLF Development (2018-001698-25)	Antibiotic	Phase III	240	NCT03780673	2021
Comparative Study of Rifaximin Versus Norfloxacin in the Secondary Prophylaxis of Spontaneous Bacterial Peritonitis (SBP)	Antibiotic	Phase III	100	NCT02120196	2023
Efficacy of Antibiotic Therapy in Severe Alcoholic Hepatitis Treated with Prednisolone (AntibioCor)	Antibiotic	Phase III	280	NCT02281929	2019
Rifaximin Reduces the Complications of Decompensated Cirrhosis: A Randomized Controlled Trial	Antibiotic	Phase IV	200	NCT02190357	2019
Steady-State Pharmacokinetics of Rifaximin 550 mg Tablets in Healthy and Hepatically Impaired Subjects	Antibiotic	Phase IV	18	NCT03818672	2019
Efficacy, Safety, And Pharmacokinetics of Rifaximin In Subjects with Severe Hepatic Impairment and Hepatic Encephalopathy	Antibiotic	Phase IV	100	NCT01846663	2021
Rifaximin Soluble Solid Dispersion (SSD) Tablets Plus Lactulose for the Treatment of Overt Hepatic Encephalopathy (OHE) (OHE)	Antibiotic	Phase II	325	NCT03515044	2019
Mastiha Treatment for Obese with NAFLD Diagnosis (MAST4HEALTH)	Prebiotic	Early Phase I	52	NCT03135873	2019
Study on the Optimal Strategy for Acute-on-chronic Liver Failure with Integrative Treatment	Prebiotic	N/A	510	NCT03577938	2020
Efficacy of Albumin Therapy with Standard Medical Treatment (SMT) as Compared to Standard Medical Treatment (SMT) in Improving Patient Survival and Immune Modulation in Patients with Acute on Chronic Liver Failure (ASIA Trial).	Prebiotic	N/A	200	NCT03754400	2020
Impact of a Specific Micronutrient-probiotic-supplement on Fatty Liver of Patients After Mini-Gastric Bypass Surgery (FMG-01)	Probiotic	Phase III	60	NCT03585413	2019
Probiotics in the Treatment of NAFLD	Probiotic	N/A	58	NCT02764047	2018
Probiotics in NASH Patients - PROBILIVER TRIAL (NASH)	Probiotic	N/A	46	NCT03467282	2021
Profermin®: Prevention of Progression in Alcoholic Liver Disease by Modulating Dysbiotic Microbiota (SYN-ALD)	Probiotic	N/A	40	NCT03863730	2031
Novel Therapies in Moderately Severe Acute Alcoholic Hepatitis (NTAH-Mod)	Probiotic	N/A	130	NCT01922895	2019
Probiotics in the Prevention of Hepatocellular Carcinoma in Cirrhosis	Probiotic	N/A	280	NCT03853928	2023
Dietary Modulation of Intestinal Microbiota as Trigger of Liver Health: Role of Bile Acids - "A Diet for Liver Health" (ADLH)	Synbiotic	N/A	84	NCT03897218	2020
Investigation of Synbiotic Treatment in NAFLD (INSYTE)	Synbiotic	N/A	100	NCT01680640	2019
Fecal Microbiota Transplantation (FMT) in Nonalcoholic Steatohepatitis (NASH). A Pilot Study	FMT	Phase I	5	NCT02469272	2018
Fecal Microbiota Transplantation for the Treatment of Non-Alcoholic Steatohepatitis (FMT-NASH)	FMT	Phase I	15	NCT03803540	2021
Fecal Microbial Transplant for Alcohol Misuse in Cirrhosis	FMT	Phase I	20	NCT03416751	2019
FMT in Cirrhosis and Hepatic Encephalopathy	FMT	Phase II	100	NCT03796598	2023
Fecal Microbiota Transplant as Treatment of Hepatic Encephalopathy	FMT	Phase II	30	NCT03420482	2021
Fecal Microbiota Transplantation (FMT) in the Management of	FMT	Phase II	10	NCT02255617	2019

Hepatic Encephalopathy (HE): A Pilot Study					
Fecal Transplant for Hepatic Encephalopathy	FMT	Phase II	30	NCT03439982	2021
Trial of Faecal Microbiota Transplantation in Cirrhosis (PROFIT)	FMT	Phase III	32	NCT02862249	2019
To Assess the Role of Fecal Microbiota Transplant in Acute Liver Failure	FMT	N/A	40	NCT03363022	2018

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ORIGINAL ARTICLE

Basic Study

Pretreatment with intestinal trefoil factor alleviates stress-induced gastric mucosal damage *via* Akt signaling

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Abstract

BACKGROUND

Stress-related gastric mucosal damage or ulcer remains an unsolved issue for critically ill patients. Stress ulcer prophylaxis has been part of routine intensive care, but uncertainty and controversy still exist. Co-secreted with mucins, intestinal trefoil factor (ITF) is reported to promote restitution and regeneration of intestinal mucosal epithelium, although the mechanism remains unknown.

AIM

To elucidate the protective effects of ITF on gastric mucosa and explore the possible mechanisms.

METHODS

We used a rat model of gastric mucosal damage induced by water immersion restraint stress and lipopolysaccharide-treated human gastric epithelial cell line to investigate the potential effects of ITF on damaged gastric mucosa both *in vivo* and *in vitro*.

RESULTS

ITF promoted the proliferation and migration and inhibited necrosis of gastric mucosal epithelia in vitro. It also preserved the integrity of gastric mucosa by upregulating expressions of occludin and zonula occludens-1. In the rat model, pretreatment with ITF ameliorated the gastric mucosal epithelial damage and facilitated mucosal repair. The protective effects of ITF were confirmed to be

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exerted *via* activation of Akt signaling, and the specific inhibitor of Akt signaling LY249002 reversed the protective effects.

CONCLUSION

ITF might be a promising candidate for prevention and treatment of stress-induced gastric mucosal damage, and further studies should be undertaken to verify its clinical feasibility.

Key Words: Intestinal trefoil factor; Water immersion restraint stress; Gastric mucosa; Epithelium integrity; Akt signaling pathway

Core Tip: Stress-related gastric mucosal damage remains an issue for critical care patients. As an endogenous peptide, intestinal trefoil factor was found to alleviate both macroscopic and microscopic gastric mucosal damage *in vivo* induced by acute stress stimulation and promote mucosal epithelial cell survival, accelerate wound closure, and preserve mucosal integrity *in vitro*. Akt signaling pathway could play an essential role. Therefore, intestinal trefoil factor is a promising candidate for prevention and treatment of stress-induced gastric mucosal damage.

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INTRODUCTION

Stress-related gastric mucosal damage is one of the most common complications for critically ill patients in the intensive care unit (ICU), and it may evolve to ulceration and bleeding[1]. It can be induced by strong stimulation or chronic stress, including severe trauma, shock, infection, burns, surgery, or other etiological factors that involve complex pathophysiological changes. Most ICU patients are believed to develop gastrointestinal ulcers, and most of these ulcers are superficial and asymptomatic. Only a small proportion (2%-5%) progress to upper gastrointestinal bleeding, leading to increased morbidity or mortality[2,3]. However, the underlying mechanisms of the typically acute gastric mucosal lesions remain incompletely understood, as decreased blood flow, local tissue hypoxia, oxidative stress, ischemia, and reperfusion injury may contribute to the pathophysiology. Therefore, prevention and intervention of gastric mucosal injury has been the most common concern from both clinical and basic research perspectives.

Stress ulcer prophylaxis has been regarded as part of routine critical care for ICU patients[4]. However, pharmacotherapy of such gastric mucosal lesions is not consistent, and recommendations in some guidelines are conflicting. Recently, some new clinical trials and updated systematic reviews on the benefits and harms of the two most commonly prescribed agents, proton pump inhibitors and H2-receptor antagonists, have been reported, but the quality of evidence has limited their clinical decision-making value[5]. Therefore, further clinical trials, in-depth mechanism studies, and novel strategies are needed for gastric mucosal damage.

In the gastrointestinal tract, mucus plays an important role in protecting epithelial cells against mechanical damage, infection, or other stimuli and maintaining stability of the luminal microenvironment[6]. Co-secreted with mucins, trefoil factor family (TFF) peptides (TFF1, TFF2, and TFF3) are recognized as integral constituents of the mucus barrier. TFF3, also known as intestinal trefoil factor (ITF), is a small protease-resistant protein (59 amino acids) that is predominantly expressed in mucin-secreting goblet cells of the small intestine and colon[7]. Previous studies have demonstrated that ITF plays pivotal roles in maintaining epithelial integrity of the intestines through regulation of restitution and regeneration of the intestinal epithelium, which involves

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regulation of E-cadherin function in epithelial cells, epidermal growth factor receptor signaling, and the extracellular signal-regulated kinase and janus kinase/signal transducer and activator of transcription 3 pathways[8,9]. Additionally, ITF is upregulated in various pathological conditions such as infection and carcinogenesis. It may promote malignant progression by activating the epithelial–mesenchymal transition process in colorectal cancer and facilitate other tumors, including prostate and breast cancer[10-12]. Intriguingly, in normal gastric mucosa, expression of ITF is too low to detect, but recent studies have indicated that serum ITF levels could be an independent prognostic factor in patients with gastric cancer, and ITF downregulation could inhibit both proliferation and invasion of gastric cancer cells *in vitro*[13].

Our previous study demonstrated that ITF can protect gastric epithelial cells (GES-1) from non-steroidal anti-inflammatory drugs *in vitro*, leaving the therapeutic potential and mechanisms to be elucidated[14]. Here, we aimed to investigate the effects of ITF on gastric mucosal lesions induced by stress both *in vivo* and *in vitro*, and possible mechanisms have also been preliminarily examined.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (8-10 wk old, 180–200 g) and their formula diet were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). Rats were housed individual cages in a room with controlled conditions (22 ± 2 °C, relative humidity of 50% ± 5%), a 12 h light/12 h dark cycle, and free access to food and drinking water. Water immersion restraint stress (WIRS) model was adopted in the present study as previously described, with some modifications[15]. Briefly, in the stress exposure session, each rat was restrained individually in a plastic cage and immersed up to its xiphoid in temperature-controlled water (23 °C) for 16 h.

The animal study was approved by the Institution Animal Committee of Jinling Hospital, Medical School of Nanjing University, and the rats were maintained in accordance with the guidelines for the care and use of laboratory animals.

Experimental design

Sprague–Dawley rats were randomly divided into four groups (n = 10), and all rats were fasted for 24 h with free access to water before modelling. (1) Control group: The rats received intraperitoneal injection of saline without stress exposure; (2) WIRS group: The rats received intraperitoneal injection of saline with stress exposure; (3) WIRS + ITF group: The rats received intraperitoneal injection of ITF (0.1 mg/kg, PeproTech, Rocky Hill, NJ, United States) for 3 d before stress exposure; (4) WIRS + ITF + LY group: The rats received intraperitoneal injection of ITF (0.1 mg/kg) for 3 d before stress exposure and received additional injection of LY294002 (20 mg/kg, Cell Signaling Technology, Danvers, MA, United States) prior to stress exposure.

Histological examination

All the rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg, Sigma Aldrich, St. Louis, MO, United States) and sacrificed by cervical dislocation 24 h after the stress exposure session. Stomach samples of each rat were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Paraffin sections were then cut to a thickness of 5 μm and stained with hematoxylin and eosin for histological examination according to standard procedures. The histological damage was assessed and scored [gastric lesion index (GLI)] by two experienced pathologists as described previously[16].

Cell culture and administration

Human gastric mucosal epithelial cell line GES-1 were obtained from American Type Culture Collection (ATCC, Rockville, MD, United States). Cells were cultured as an adherent monolayer in cell culture flasks (25 cm^2, Cat. No. 430639, Corning, Corning, NY, United States) using high glucose-Dulbecco's modified Eagle medium (Thermo Scientific, Waltham, MA, United States) with 10% fetal bovine serum (Gibco, Gaithersburg, MD, United States) at 37 °C in a humidified incubator with 5% CO_2. When GES-1 cells reached 80% confluence, they were routinely passaged using 0.25% trypsin and were diluted 1:2 at each passage. Cells treated with optimum concentration of ITF (100 ng/mL), LY294002 (15 μmol/L), and lipopolysaccharides (LPS, 10 μg/mL, Sigma Aldrich) were used in the following experiments as reported

in our previous study[17].

Cell viability assay

Cells were placed on a 6-well culture plate at 2 × 10^6 cells/mL in 2 mL culture medium, then incubated at 37 °C in an atmosphere of 95% air and 5% CO_2 for 24 h, 48 h, and 72 h. After treated at different times, cells in each group were plated on a 96-well culture plate at 2 × 10^4 cells/well in 100 μL culture medium; after incubated for 12 h, the culture medium was removed, and 100 μL free-serum medium was added with 10 μL Cell Counting Kit-8 solution (CCK-8, Cat. No. CK04-11, Dojindo Laboratories, Kumamoto, Japan) to each well of culture plate. After incubated for 4 h, absorbance (optical density) was measured at 450 nm with a multi- detection micro plate reader (VersaMax, Molecular Devices, San Jose, CA, United States).

Cell migration assay

The migration ability of GES-1 cells was determined using Transwell and wound healing migration assay. Briefly, the former was performed in Transwell chambers (8 μm pore size, Cat. No. 3422, Corning) according to the manufacturer's instructions. Cells were suspended in serum-free culture medium at a concentration of 4 × 10^5 cells/mL and then added to the upper chamber (at 4 × 10^4 cells/well). Simultaneously, 0.5 mL of culture medium with 10% fetal bovine serum containing ITF (100 ng/mL) or LY294002 (15 mol/L) was added to the lower compartment. The cells were allowed to migrate in a humidified CO_2 incubator at 37 °C for 12 h. After incubation, cells that had entered the lower surface of the filter membrane were fixed with 90% ethanol for 30 min at room temperature, washed three times with distilled water, and stained with 0.1% Crystal Violet in 0.1 mol/L borate and 2% ethanol for 30 min at room temperature. Cells remaining on the upper surface of the filter membrane were gently scraped off with a cotton swab. Images of penetrated cells were captured by a photomicroscope (BX51, Olympus, Tokyo, Japan). Cell migration ability was quantified in a blinded manner by counting the number of penetrated cells on the lower surface of the membrane with five fields (100 × magnification) per chamber. During wound healing migration assay, the confluent monolayers of cells were wounded by scratching lines with a sterile micropipette tip. After removing the cellular debris with phosphate buffered saline (PBS), cells were cultured for 72 h with serum-free medium. The cells migrated to the wounded region were observed by inverted microscope (CK-2 L, Olympus) and photographed (100 × magnification), and the percentages of wound closure were calculated.

Western blotting analysis

Proteins were obtained from tissue specimens or GES-1 cells, and western blot analysis was performed as previously described. Briefly, the isolated protein samples (30 μg) were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel to perform electrophoresis. The separated proteins were then transferred to polyvinylidene fluoride membranes using standard procedures. For immunoblotting, the membranes were incubated at 37 °C for 1 h in blocking buffer [0.1% Tween 20, 1% bovine serum albumin (BSA), and 5% non-fat milk in PBS]. The primary antibodies were added to the membranes and incubated at 4 °C overnight. After three-times washing with PBS, the membranes were incubated with 1:10000 diluted secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG, Boster, Wuhan, China) at 37 °C for 1 h. After additional washing with tris-buffered saline containing 0.1% Tween 20 detergent, the target proteins on the blot membrane were visualized using the enhanced chemiluminescence system (Cat. No. 345818, Merck, Darmstadt, Germany). The Odyssey Scanning System (LI-COR, Lincoln, NE, United States) was used for image capture. Equal loading of proteins was confirmed by visualization of β-actin. Band intensities were quantified by densitometry using Image J Software (version 1.41). The primary antibodies were employed as follows: Rabbit anti-Akt, rabbit anti-pAkt (Cell Signaling Technology), rabbit anti-occludin, rabbit anti-zonula occludens (ZO)-1, and mouse anti-β-actin (Abcam, Cambridge, United Kingdom).

Immunofluorescent staining

For studying the protective effect of ITF on maintaining integrity of GES-1 cells and investigating the regulation of Akt signaling pathway, 10 μg/mL LPS was added into cultured GES-1 cells. After 4 h, 100 ng/mL ITF was added, and then treated GES-1 cells were cultured in this condition for 48 h. Immunofluorescence analysis of GES-1 cells was performed as described previously. Briefly, cells were first fixed with 4% paraformaldehyde for 10 min. To block unspecific binding sites, the cells were

incubated with PBS containing 2% BSA for 1 h at 37 °C. After blocking of the non-specific staining, the cells were incubated with the primary antibodies rabbit anti-occludin and rabbit anti-ZO-1 (Abcam) at a dilution of 1:200 at 4 °C overnight. After three washes with PBS with 0.2% Triton X-100, GES-1 cells were incubated with a secondary antibody (goat anti-rabbit/mouse Alexa Fluor 594 or 488, Life Technologies, Carlsbad, CA, United States) at a 1:400 dilution in 2% BSA for 1 h at 37 °C in dark. Then cells were stained with 10 μg/mL 4′, 6′-diamidino-2-phenylindole (Biyuntian, Nantong, China) for 10 min to identify cellular nuclei. The images were captured using a confocal fluorescence microscope (FV3000, Olympus).

Fluorescein diacetate/propidium iodide staining for morphologic evaluation

The integrity of the cell membrane was detected using fluorescein diacetate and propidium iodide staining. Cells were placed on a 6-well culture plate at 1×10^6 cells/mL in 2 mL culture medium. After 12 h, cells were treated with LPS (10 μg/mL), ITF (100 ng/mL), and LY294002 (15 μmol/L) for 24 h, and then plated at a density of 2×10^5 cells/well onto 96-well plates, stained with 5 μg/mL propidium iodide and 4 μg/mL fluorescein diacetate, and observed under a fluorescent microscope (Nikon ECLIPSE TE2000-S, Tokyo, Japan).

Statistical analysis

All data were analyzed using SPSS 21.0 software (Armonk, NY, United States). Experimental results are expressed as mean ± standard deviation (m ± SD) and were compared by one-way analysis of variance followed by SNK-Q test. $P < 0.05$ was defined as indicating a statistical significance.

RESULTS

ITF alleviated gastric mucosal damage induced by stress in vivo

The stomachs of the WIRS group presented with severe gastric mucosal lesions, and part of the lesion area developed severe edema, bleeding, and ulceration (Figure 1A). Compared with the WIRS group, the gastric lesions in the WIRS + ITF group were almost negligible, and the GLI was significantly decreased (7.50 ± 2.10 *vs* 28.50 ± 3.20, $P < 0.01$) (Figure 1B), although the GLI was still higher than that of the control group (without stress exposure). More-detailed histological examination showed that the WIRS group displayed extensive destruction of the gastric mucosa. The pathological changes, including epithelial necrosis, congestion, bleeding, inflammatory cell infiltration, dilating and congesting vessels, and edema, were also present in the submucosa. Minimal damage was observed in the WIRS + ITF group (Figure 1C). Integrity of the mucosa is the basis of its barrier function, and we investigated expression of epithelial tight junction markers occludin and ZO-1. Occludin and ZO-1 were significantly downregulated in the stomach of the WIRS group (Figure 1D). The potential mechanisms that may be involved in this wound healing process were also preliminarily investigated. Akt/protein kinase B is a widely recognized vital regulatory factor responsible for maintaining cell viability and survival. After treatment with ITF, the Akt signaling pathway was activated and expression of pAkt in the gastric specimens was increased in the WIRS + ITF group (Figure 1D).

ITF promoted proliferation and migration and inhibited necrosis of gastric mucosal epithelia

When treated with LY294002, cell viability was inhibited compared with the LPS + ITF group but was still significantly increased at 48 and 72 h compared with the LPS group ($P < 0.01$) (Figure 2A). Necrosis was induced in GES-1 cells by LPS and was attenuated by ITF, while the number of necrotic cells was increased when treated with LY294002 (Figure 2B). Epithelial cell migration and wound closure were promoted by ITF but hindered by LPS (Figure 2C and D). Inhibition of the Akt pathway by LY294002 suppressed ITF-induced cell migration.

ITF maintained integrity of the gastric mucosa via the Akt signaling pathway

To explore the effects of ITF on gastric mucosal epithelial integrity, expression of tight junction makers was detected. Immunofluorescence and western blotting demonstrated that LPS induced epithelial tight junction damage and decreased expression of occludin and ZO-1, whereas ITF reversed the decreased expression of occludin and ZO-1 (Figure 3A and B). Expression of Akt phosphorylation in GES-1

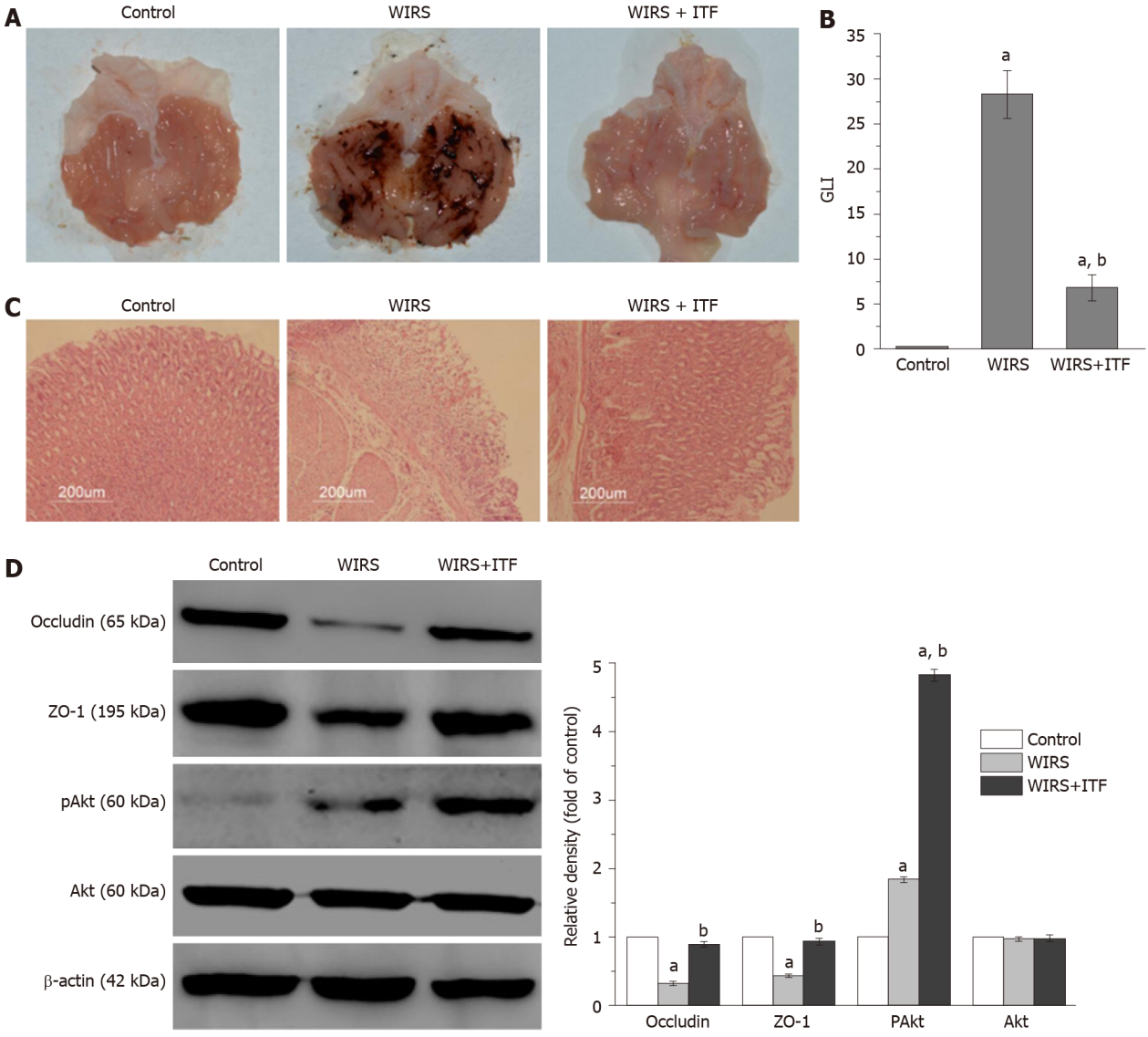

Figure 1 Intestinal trefoil factor alleviated gastric mucosal damage induced by acute stress. Gastric mucosal changes and protein expressions were presented. A: Macroscopic appearance; B: Gastric lesion index; C: Histopathological analysis (Hematoxylin and eosin staining, magnification: 100 ×); D: Western blotting analysis of occludin, zonula occludens-1 (ZO-1), Akt, and pAkt. $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* water immersion restraint stress (WIRS) group. ITF: Intestinal trefoil factor.

cells was increased when treated with ITF (Figure 3B), but LY294002 inhibited activation of the Akt signaling pathway induced by ITF and suppressed the protective effects of ITF on epithelial integrity (Figure 3A and B). These results indicated that ITF had protective effects on gastric mucosal epithelial integrity by activating Akt signaling, while the inhibitor of Akt signaling pathway, LY294002, eliminated the protective effects of ITF.

ITF ameliorated gastric mucosal epithelial damage via activation of the Akt signaling pathway in vivo

To confirm the gastroprotective effects of ITF and involvement of Akt signaling in this process, another group of rats (WIRS + ITF + LY group) was enrolled and treated with Akt signaling pathway inhibitor LY294002. Stress exposure caused formation of gastric lesions in all groups except for the control group with intact mucosa (Figure 4A). Rats from the WIRS and WIRS + ITF + LY groups developed severe gastric mucosal lesions and the typical macroscopic signs including hyperemia, hemorrhage and edema. On the contrary, only minimal morphological lesions and reduced areas of gastric ulcer formation were observed in the WIRS + ITF group. Correspondingly, the GLI in the WIRS + ITF group was markedly decreased compared with that in the WIRS group (5.50 ± 1.20 *vs* 32.0 ± 2.50, $P < 0.01$) (Figure 4B), but this trend was reversed by LY294002 (GLI: 22.0 ± 1.50). Microscopic examination showed that ITF prevented

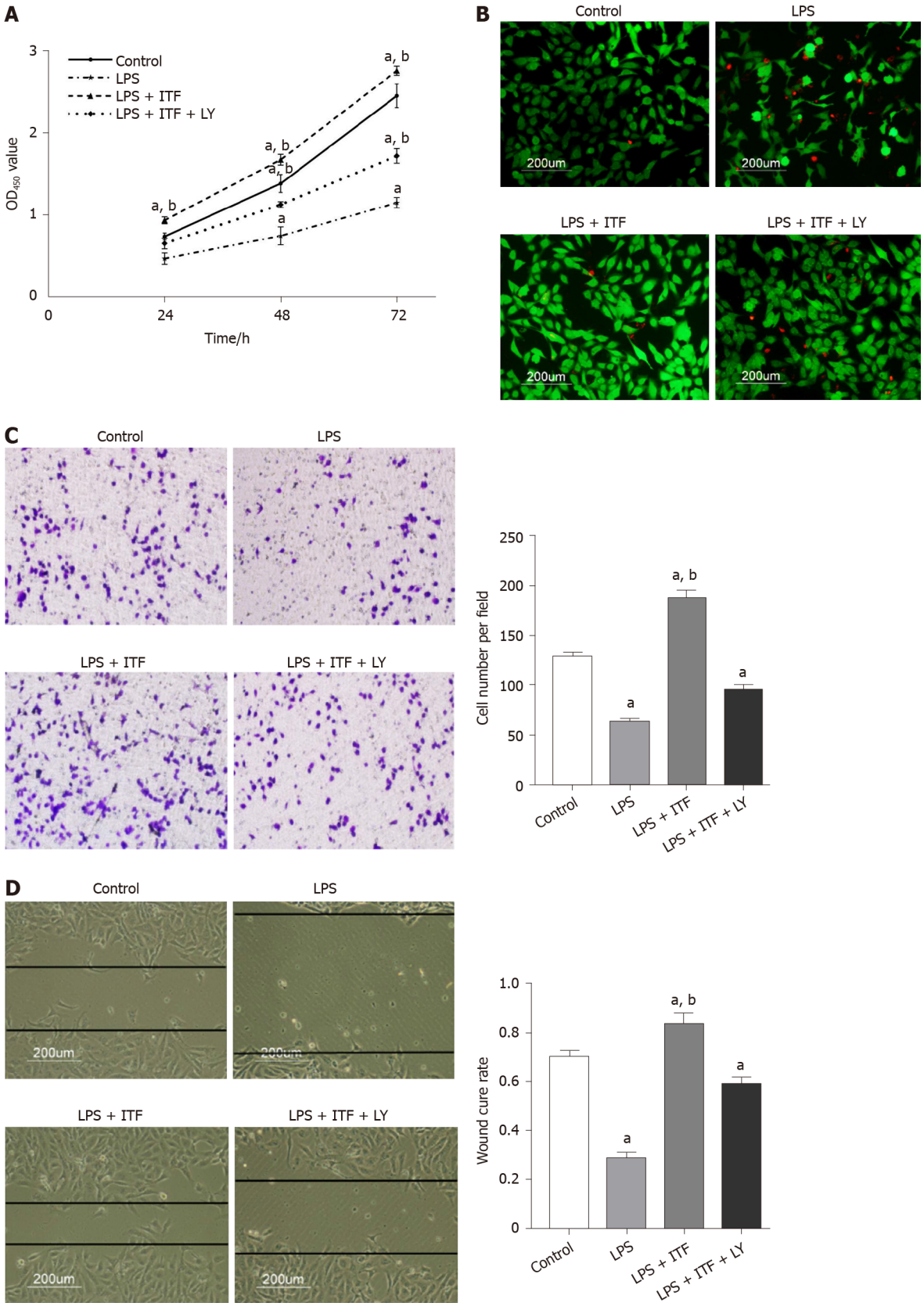

Figure 2 Intestinal trefoil factor promoted the proliferation and migration and inhibited necrosis of GES-1 cells. A: Intestinal trefoil factor (ITF) promoted proliferation of GES-1 cells, and LY294002 decreased the cell viability; B: Fluorescent images of treated cells following fluorescein diacetate (FDA)/propidium iodide (PI) staining. Viable cells were stained with FDA (green), and necrotic cells were stained with propidium iodide (red), the scale bar = 200 μm; C: Transwell migration; and D: Wound healing assay analyzed the migration of GES-1 cells treated with lipopolysaccharides (LPS), ITF, or LY294002. The images are representative of three independent experiments; [a]*P* < 0.05 *vs* control cells; [b]*P* < 0.05 *vs* LPS-treated cells.

stress-induced histological changes in the gastric mucosa with inflammation infiltration, congestion, and hemorrhage. Administration of LY294002 interfered with the protective effects of ITF, and the mucosal damage was slight compared with that in the WIRS group (Figure 4C).

Expression of occludin, ZO-1, Akt, and pAkt in gastric mucosa was determined. Expression of occludin and ZO-1 in the gastric tissues was significantly enhanced by ITF, and the effects were partially inhibited by LY294002 (Figure 4D). Akt phosphorylation was upregulated when challenged by stress exposure and was further increased by ITF. However, the inhibitor of the Akt pathway reduced phosphorylation in the WIRS + ITF + LY group compared with the WIRS + ITF group ($P < 0.05$) (Figure 4D).

DISCUSSION

The gastrointestinal epithelial barrier is crucial for the maintenance of homeostasis, and it is also constantly exposed to various stimuli and susceptible to those threats. It is not surprising that critically ill patients are often confronted with the risk of mucosal damage, alterations in epithelial barrier function, and various complications. Despite the increased risk of myocardial ischemia, Clostridium difficile enteritis and hospital-acquired pneumonia, prophylactic medication for stress ulcers is still widely adopted in the ICU, along with treating the primary diseases[18]. Novel approaches have been explored to control and prevent mucosal damage or promote epithelial restitution.

As an endogenous peptide and a key constituent of mucus barrier, ITF is a potential choice for mucosal damage prophylaxis and treatment. In recent decades, changes in ITF expression level have been recognized in various diseases, especially in gastrointestinal disorders. Serum levels of ITF are significantly increased after skeletal trauma in humans, which could enhance motility and migration of mesenchymal progenitor cells and promote skeletal repair[19]. For critically ill children, where the body is in a stressed state, serum ITF concentration is associated with gastrointestinal failure and prognosis[20]. Another study has suggested that urinary ITF can help diagnose and predict the disease course in necrotizing enterocolitis at the early stage in newborns[21]. Previous studies have also paid close attention to the application of ITF to inflammatory bowel disease, showing that ITF could be used as a biomarker to predict disease activity and assess mucosal healing in ulcerative colitis[22,23]. All these reports suggest the therapeutic potential of ITF in gastrointestinal mucosal damage, but there is an absence of direct evidence.

However, few studies have explored the roles of ITF in stress-induced gastric mucosal injury. Previously, we have reported the ITF-mediated protection of gastric epithelial mucosa cells from NSAIDs *in vitro* without mechanistic explanation and further investigation *in vivo*[14]. In the current study, we initially found that pretreatment with ITF attenuated gastric lesions and alleviated local inflammation in the WIRS rat model, and the altered expression of epithelium tight junction markers indicated the ability of ITF to maintain or restore epithelial integrity. Preliminary results also suggested the activation of Akt kinase. In the following experiments, the cytoprotective effects of ITF were verified using LPS to induce GES-1 cell injury, and ITF promoted cell proliferation and migration, inhibited LPS-induced necrosis, and preserved the intercellular tight junction *in vitro* without signaling. Intriguingly, when treated with LY294002, a specific inhibitor of the Akt signaling pathway, these protective effects of ITF were attenuated significantly *in vitro*. The results with the GES-1 cell line suggested that Akt signaling was essential for ITF to function biologically, and this was validated in another set of WIRS rat models.

The Akt signaling pathway is reported to play essential roles in the process of cell proliferation, differentiation, apoptosis, and migration. It has also been shown to preserve epithelial integrity during inflammation, which was confirmed in the current study[24]. Our results *in vitro* and *in vivo* demonstrated that ITF was a rapid responder to stress stimuli. Although mucosal restitution is an intrinsic function of gastrointestinal epithelial cells, ITF still exerted promising wound healing effects.

Several issues involved in the subtle regulatory networks still need to be discussed. Whether ITF responsiveness requires a receptor is unclear. Belle and co-workers found that the leucine rich repeat receptor and nogo-interacting protein 2 (LINGO2) is essential for ITF-mediated functions, and ITF–LINGO2 interactions derepress inhibitory LINGO2–epidermal growth factor receptor complexes, allowing ITF to drive wound healing and immunity[25]. ITF has also been reported to interact with other receptors including chemokine CXC receptor 4 and 7, protease-activated

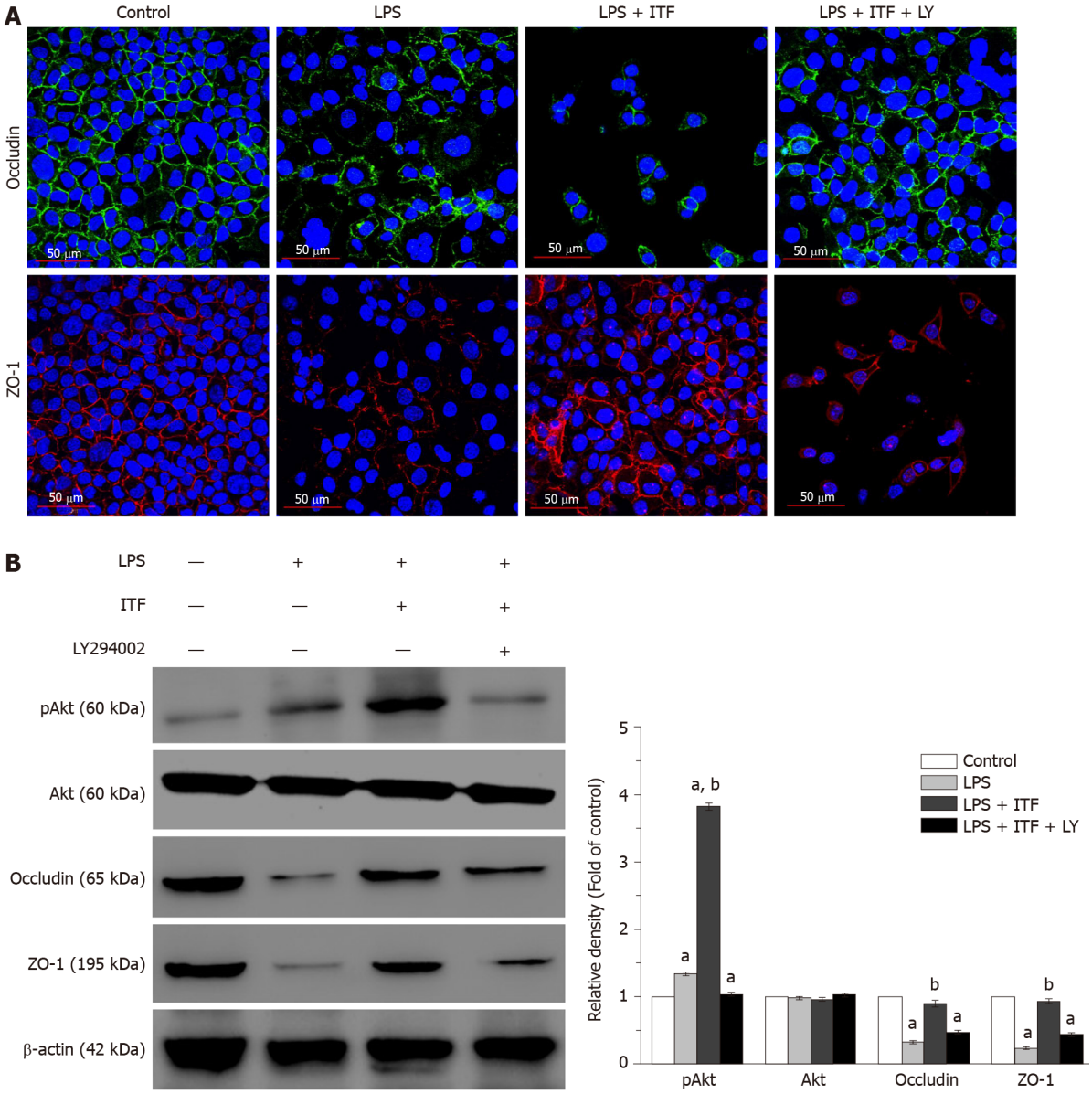

Figure 3 Intestinal trefoil factor preserved integrity of the gastric mucosa by activating the Akt signaling pathway. A: Immunofluorescence staining of tight junction markers [occludin and zonula occludens-1 (ZO-1)] demonstrated that intestinal trefoil factor (ITF) maintained the integrity of GES-1 cells after treated with lipopolysaccharides (LPS). LY294002 undermined the protective effects of ITF *via* inhibiting the Akt signaling pathway *in vitro*; and B: Expression of occludin, ZO-1, Akt and pAkt were detected by Western blotting, suggesting that activation of the Akt signaling was essential for ITF to protect epithelium from damage induced by LPS. $^{a}P < 0.05$ *vs* control cells; $^{b}P < 0.05$ *vs* LPS-treated cells.

receptors, and classic signaling pathways[26-28].

The histological examination in our study indicated that ITF could exert anti-inflammatory effects *in vivo*. These effects were also demonstrated when the microglial cells were cultured in the presence of ITF, and subsequent reduced expression and secretion of pro-inflammatory cytokines after LPS stimulation were detected[29]. ITF derived from human breast milk can downregulate proinflammatory cytokines and upregulate human β-defensin expression *via* regulating intracellular Ca^{2+} activity, and the significance of ITF as an immunomodulator should be given more emphasis[27]. Recombinant human ITF is reported to protect mucosal barrier function in rats with nonalcoholic steatohepatitis and reduce inflammatory injury by reducing expression of Toll-like receptor 4 and nuclear factor-κB[30]. Supplementation of ITF also rescued Toll-like receptor 2-deficient mice from increased inflammatory-stress-induced mucosal damage linked to innate immune protection[31]. Additionally, the TFFs, including ITF, are reported to share a divalent lectin activity that recognizes the N-acetyl-glucosamine-α-1, 4-galactose disaccharide and interact with mucosal glycoproteins relying on the glycosylation state. How their lectin activities might promote cell migration to achieve epithelial restitution remains unclear[32]. Accumulating evidence

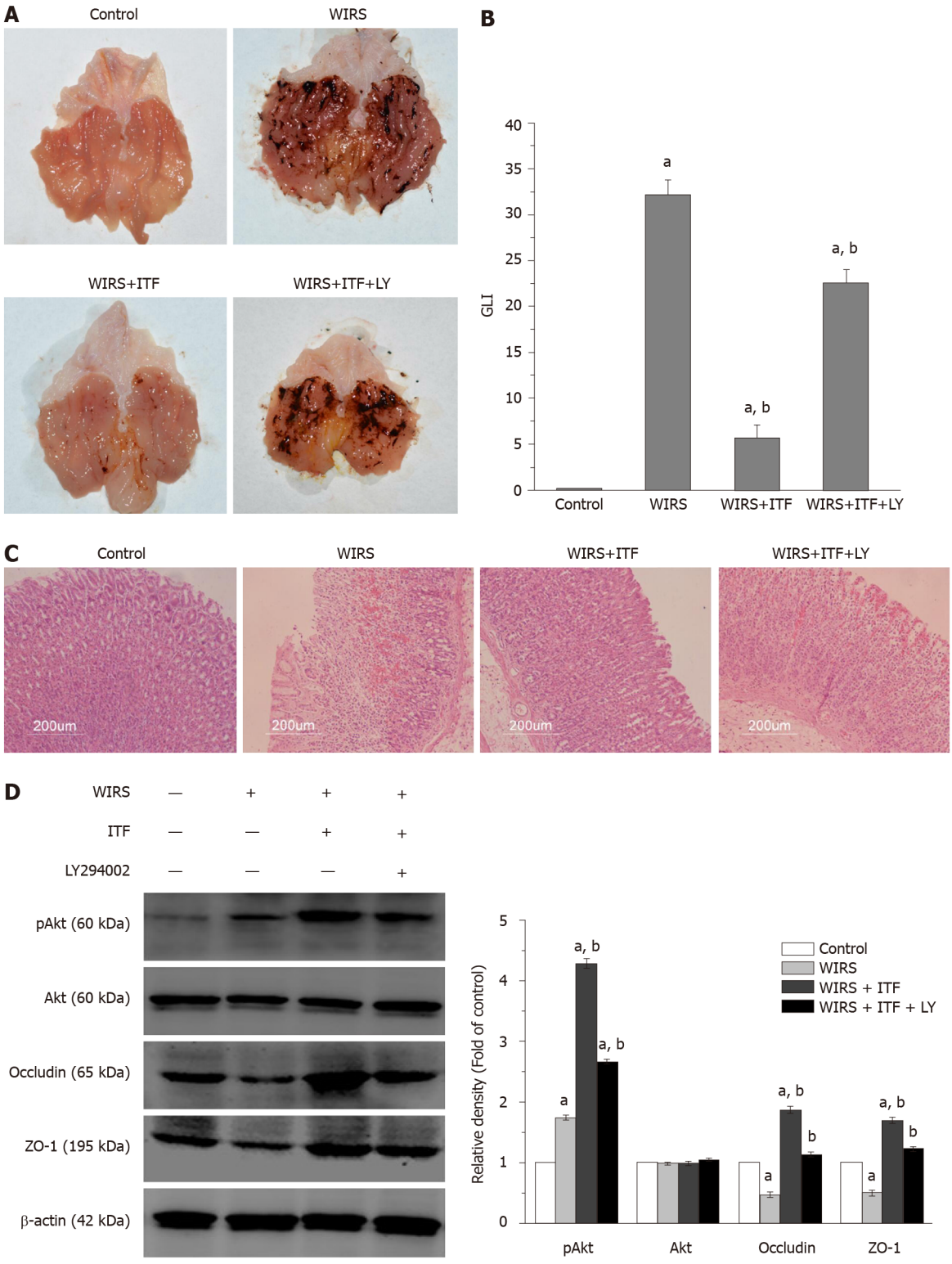

Figure 4 Inhibition of the Akt signaling pathway attenuated the protective effects of intestinal trefoil factor *in vivo*. Gastric mucosal changes were presented in each group. A: Gross morphological appearance; B: Gastric lesion index (GLI); C: Histopathological analysis (Hematoxylin and eosin staining, magnification: 100 ×); and D: The expression of occludin, zonula occludens-1 (ZO-1), Akt, and pAkt were determined. Inhibition of the Akt signaling pathway by LY294002 significantly attenuated the protective effects of intestinal trefoil factor (ITF) *in vivo*. $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* water immersion restraint stress (WIRS) group.

has implicated glycosylation as an underappreciated post-translational modification, and glycan alterations have an important impact on the mucus layer, glycan–lectin interactions, and mucosal immunity[32-34].

With regard to clinical implications of ITF in adult critically ill patients, the latest research shows that plasma ITF levels are sustained elevated in abdominal sepsis patients, and higher ITF levels are associated with shock and multiple organ (≥ three)

failure. However, elevated ITF level is not an independent risk factor for 30 d mortality[35]. These results suggest that gastrointestinal injury contributes to the pathogenesis of critical illness, and ITF could be a potential therapeutic agent. ITF has been utilized for enema in patients with mild-to-moderate left-sided ulcerative colitis in a clinical trial, but this well-tolerated regimen did not provide any benefit above that of adding 5-aminosalicylic acid alone[36]. A novel delivery method, use of the systemic route, and adjustment of medication duration should be taken into consideration in subsequent trials. The latest study found that protein disulfide isomerase A1 can directly catalyze dimerization of ITF, and changes in the modification protein disulfide isomerase A1 reduce its activity, resulting in a corresponding decrease in ITF dimerization and delayed intestinal mucosal repair during sepsis. This work suggests novel mechanisms for the inhibition of mucosal repair and promising targets for the prevention and treatment of sepsis[37]. The aforementioned potential adverse effects of conventional antiulcer agents such as proton pump inhibitors and H2-receptor antagonists and the current results support the prospect of clinical translation of ITF, especially for critically ill patients who are in a of stress.

CONCLUSION

In conclusion, ITF can alleviate both macroscopic and microscopic gastric mucosal damage *in vivo* induced by acute stress stimulation and promote mucosal epithelial cell survival, accelerate wound closure, and preserve mucosal integrity *in vitro*. Akt signaling pathway could play essential roles in this process (Figure 5). Further studies should be implemented to explore the feasibility of clinical application of ITF for prevention and treatment of stress ulcer and gastric mucosal damage caused by other factors.

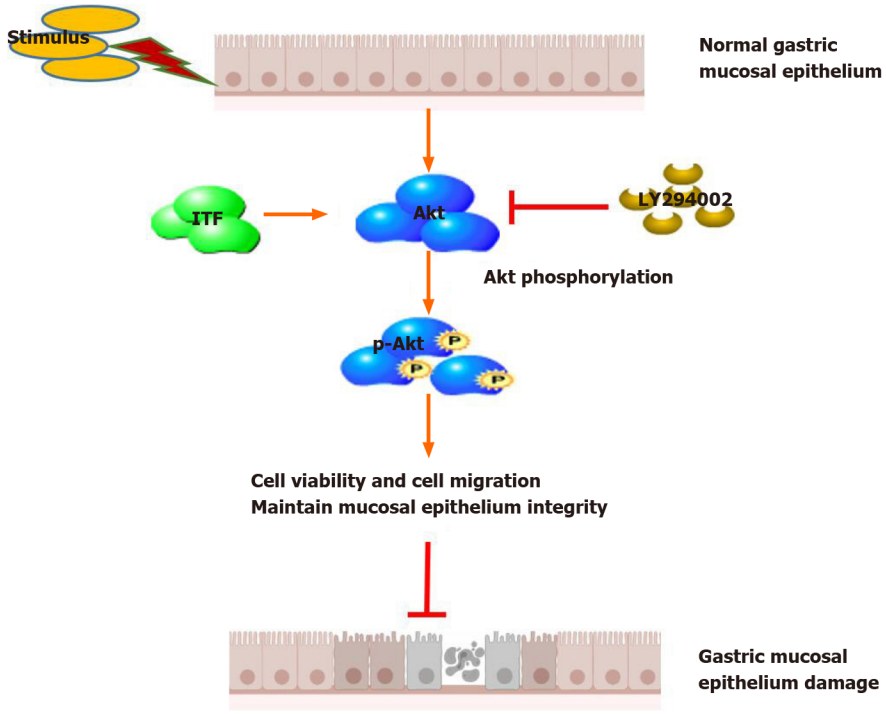

Figure 5 Schematic diagram of the protective effects of intestinal trefoil factor on gastric mucosal epithelium. Intestinal trefoil factor (ITF) promoted the proliferation and migration of gastric epithelial cells and preserved integrity of the mucosal epithelium *via* activating the Akt signaling pathway.

ARTICLE HIGHLIGHTS

Research background

Stress-related gastric mucosal damage is a prevalent complication in critically ill patients in the intensive care unit, and it may evolve to ulceration and bleeding. Stress ulcer prophylaxis has been common in routine intensive care, but with controversy. Co-secreted with mucins, intestinal trefoil factor (ITF) is reported to promote the restitution and regeneration of intestinal mucosal epithelium, but the mechanism is unknown.

Research motivation

As an endogenous peptide, ITF harbors innate advantages over conventional anti-ulcer agents, and might be a new candidate for stress ulcer prophylaxis.

Research objectives

To investigate the protective effects of ITF on gastric mucosa and explore the underlying mechanisms.

Research methods

We utilized water immersion restraint stress-induced gastric mucosal damage rat model and lipopolysaccharide-induced gastric epithelium cell damage model to investigate the potential functions of ITF on damaged gastric mucosa both *in vivo* and *in vitro*.

Research results

We found that ITF promoted proliferation and migration and inhibited necrosis of gastric epithelium cells and preserved the integrity of gastric mucosa by increasing expression of occludin and zonula occludens-1. Additionally, pretreatment with ITF ameliorated the gastric mucosal epithelial damage and promoted mucosal repair *in vivo*. We found that the protective effects of ITF were exerted by activation of Akt signaling, and the specific inhibitor of this pathway, LY249002, abolished the protective effects.

Research conclusions

Pretreatment with ITF alleviated stress-induced gastric mucosal damage by activation

of Akt signaling.

Research perspectives

This study provides insight into the translational potential of ITF as a promising candidate for prevention and treatment of stress-induced gastric mucosal damage.

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ORIGINAL ARTICLE

Case Control Study

Gegen Qinlian decoction enhances immunity and protects intestinal barrier function in colorectal cancer patients *via* gut microbiota

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Author contributions: Jia YT and Li ZX made substantial contributions to the conception and design of the study; Li Y searched and reviewed published articles and wrote the manuscript; Li Y, Xie CY and Lv J collected patient data and prepared the tables; Li Y and Fan J tested the relevant experiments in the manuscript, statistically analyzed the data, and prepared the figures; Lv J, Xu XJ and Kuai WT critically reviewed the article and made revisions to the manuscript; all authors approved the final version.

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

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Abstract

BACKGROUND

We previously showed, using the Traditional Chinese Medicine System Pharmacology Database, that Gegen Qinlian decoction (GQD) had a direct antitumor effect, and was combined with programmed cell death protein (PD)-1 inhibitors to treat microsatellite stable (MSS) tumor-bearing mice. However, the effect of GQD on patients with colorectal cancer (CRC) is not clear.

AIM

To determine the therapeutic mechanism of GQD in improving immune function, reducing inflammation and protecting intestinal barrier function.

METHODS

Seventy patients with CRC were included in this study: 37 in the control group and 33 in the treatment group. The proportions of $CD4^+$T, $CD8^+$T, natural killer (NK), NKT and T regulatory cells were measured by flow cytometry. Levels of the cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-

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6, IL-10 and serotonin (5-hydroxytryptamine; 5-HT) in serum were assessed by enzyme-linked immunosorbent assay (ELISA). The expression of zonula occludens (ZO)-1, occludin, nuclear factor (NF)-κB and TNF-α in tumor and normal tissues was measured by immunohistochemistry. The composition of gut microbiota from patients in the treatment group was assessed using 16S rDNA analysis.

RESULTS

There were no adverse events in the treatment group. The proportion of $CD4^+$ T cells and NKT cells in the post-treatment group was significantly higher than that in the pre-treatment and control groups ($P < 0.05$). The level of TNF-α in the post-treatment group was significantly lower than that in the pre-treatment and control groups ($P < 0.05$). The concentration of 5-HT in the post-treatment group was significantly lower than that in the pre-treatment group ($P < 0.05$). The expression of ZO-1 and occludin in tumor tissues in the treatment group was significantly higher than that in the control group ($P < 0.05$). The expression of ZO-1 in normal tissues of the treatment group was significantly higher than that in the control group ($P = 0.010$). Compared with the control group, expression of NF-κB and TNF-α in tumor tissues of the treatment group was significantly decreased ($P < 0.05$). Compared with the pre-treatment group, GQD decreased the relative abundance of *Megamonas* and *Veillonella*. In addition, GQD increased the relative abundance of *Bacteroides*, *Akkermansia* and *Prevotella*.

CONCLUSION

GQD enhances immunity and protects intestinal barrier function in patients with CRC by regulating the composition of gut microbiota.

Key Words: Colorectal cancer; Gegen Qinlian decoction; Immunity; Inflammation; Intestinal barrier function; Gut microbiota

Core Tip: On the basis of our previous study, this study revealed that Gegen Qinlian decoction (GQD) repaired intestinal barrier function in patients with colorectal cancer (CRC) by regulating gut microbiota, thereby improving immune status and reducing inflammation. Our findings highlight the therapeutic potential of GQD in modulating the gut microbiota and protecting intestinal barrier function in CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide[1]. Economic and social development and changes in lifestyle in China have resulted in a rapid increase in the incidence of CRC. At present, the main treatments for CRC are surgery, radiotherapy and chemotherapy, but the overall effect is not satisfactory. With the advent of immune checkpoint inhibitors, there is a new dawn in the treatment of malignant tumors such as melanoma[2]. However, the therapeutic effect of immune checkpoint inhibitors in CRC is not optimistic. The reason for this is that the tumor microenvironment is closely related to the effect of CRC treatment[3,4]. Based on the particular anatomy, the microenvironment of CRC is composed of intestinal microorganisms, their metabolites or secretions, and the intestinal barrier.

It has been shown that the number of immune cells in patients with CRC is important for prognosis[5]. There must be sufficient immune cell infiltration in the tumor to ensure the killing effect. The proportion of T and natural killer (NK) cells in

peripheral blood of CRC patients decreases, while the proportion of regulatory T (Treg) cells increases significantly, and the percentage of NKT cells is independently correlated with disease-free survival[6]. It has been found that cytokines such as interferon (IFN)-γ and interleukin (IL)-2 can improve the therapeutic effect of CRC by recruiting lymphocytes[7,8]. In addition, the increase in tumor necrosis factor (TNF)-α can promote the occurrence of chronic inflammation, but the inhibition of immune cells can be eliminated by blocking the signal transduction function of TNF-related receptors in T cells. In recent years, it has been found that serotonin (5-hydroxytryptamine, 5-HT) is not only known to regulate intestinal movement and secretion, but is also an important immunomodulator[9,10]. However, the relationship between 5-HT and poor prognosis, metastasis and recurrence of CRC is still controversial[11].

The tight junction components are composed of occludin, zonula occludens (ZO)-1, ZO-2, ZO-3, claudin and junction adhesion molecules. The decreased expression of ZO-1 is related to cancer invasion[12]. The intestinal barrier function of patients with CRC is destroyed by decreased expression of ZO-1 and occludin[13]. The expression of ZO-1 and occludin is also decreased in inflammatory bowel diseases, while the level of nuclear factor (NF)-κB, TNF-α and IL-1 is significantly increased[14]. Evidence suggests that proinflammatory factor TNF-α can induce tumor invasion and metastasis by reducing the expression of E-cadherin and ZO-1[15].

The uniqueness of CRC suggests that its occurrence and development, and destruction of the intestinal barrier may be inseparable from gut microbiota. There are approximately 1000 species of bacteria in the human intestinal system, more than 10 times the number of human eukaryotic cells[16]. If intestinal diseases do not take into account the role of bacteria, it is difficult to know the real cause of the disease. Recent studies have shown that changes in the type and number of gut microbiota play an important role in the occurrence and development of CRC[17]. It has been found that the combined action of *Escherichia coli* and *Bacteroides fragilis* can promote the occurrence of CRC[18]. One study found that familial adenomatous polyposis eventually becomes cancerous due to the formation of bacterial biofilms that are mainly composed of the above two bacteria[19]. Other studies have also found that *Bacteroides fragilis* (*B. fragilis*) can activate the NF-κB pathway, leading to inflammation and ultimately carcinogenesis[20]. Yachida *et al*[21] showed that the decreased abundance of *Prevotella* and increased abundance of *Megamonas* were significantly associated with progression of CRC. The Gustave Roussy Cancer Campus in France found that the increase in the relative abundance of *Akkermansia muciniphila* can promote the infiltration of immune cells into tumor tissues, which in turn improves the efficacy of immune checkpoint inhibitors[22-25]. The increase in harmful bacteria can inhibit the expression of mucin, thus weakening the protective effect of the intestinal barrier, which increases damage to the intestinal epithelium by intestinal toxic substances.

Regulation of gut microbiota is expected to become an adjuvant therapy for CRC. Traditional Chinese medicine has unique advantages in this respect. According to traditional Chinese medicine, CRC originates from the damp-heat syndrome, which is roughly similar to the imbalance of gut microbiota in western medicine. Gegen Qinlian decoction (GQD) is a classical traditional Chinese medicine for the treatment of damp-heat syndrome, which has a history of more than 2000 years. The formula contains four types of medicinal materials: Radix Puerariae, Radix Scutellariae, Rhizoma Coptidis and Radix Glycyrrhizae. GQD can be used in the treatment of type 2 diabetes and ulcerative colitis (UC)[26,27]. In our previous study, using the Traditional Chinese Medicine System Pharmacology Database, we found that GQD had a direct antitumor effect, and was combined with programmed cell death protein (PD)-1 inhibitors to treat microsatellite stable (MSS) tumor-bearing mice, which synergistically enhanced anti-PD-1 immunotherapy[28].

However, the effect of GQD on intestinal mucosal barrier function and gut microbiota in patients with CRC has not been reported. In this study, based on previous animal studies, we determined the changes in immune cells, cytokines and intestinal barrier function in patients with CRC after GQD administration to assess the therapeutic mechanism of GQD in improving immune function, reducing inflammation and protecting intestinal barrier function.

MATERIALS AND METHODS

GQD preparation

The herbal formula GQD is a combination of four medicinal herbs: Radix Puerariae (15 g), Scutellariae Radix (9 g), Coptidis Rhizoma (9 g), and liquorice (6 g) at a ratio of

5:3:3:2 (w/w/w/w). GQD was prepared at the pharmacy of the Fourth Hospital of Hebei Medical University, and was identified by two experienced pharmacists.

Patient variables and medication standard

Seventy patients with colon or rectal cancer diagnosed for the first time in the Fourth Hospital of Hebei Medical University were selected. Diagnosis and tumor-node-metastasis (TNM) classification were made according to the Seventh Edition of TNM classification criteria issued by the Union for International Cancer Control (UICC). On admission, the patients were randomly divided into the control group (n = 37) and the treatment group (n = 33). The study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University and followed the ethical standards stipulated in the Declaration of Helsinki. All patients gave informed consent. The control group received routine treatment and elective surgery after admission. The treatment group received routine treatment and oral GQD for 7 d (250 mL, twice daily) before surgery. The subjective and abdominal symptoms of the patients were observed and recorded.

Flow cytometry

Peripheral blood was collected at the beginning of the study in the control group, and was collected in the treatment group before and after medication. All peripheral blood was collected from veins with EDTA-Li micro-anticoagulant tubes. Blood was stained with anti-human CD45-FITC (REFA07749), anti-human CD3-PC5, anti-human CD4-RD1, anti-human CD8-ECD, anti-human CD(16+56)-PE (A07735), anti-human CD4-FITC (REFA07750), anti-human CD25-PE (REFA07774) and anti-human CD127-PC5 (REFA64617) (Beckman Coulter, CA, United States), which was divided into three tubes for testing. Measurement was carried out on a Fortessa Flow Cytometer (BD, San Jose, CA, United States). Analysis was performed with Flow Jo version 10 (Tree Star Inc., Ashland, OR, United States).

Enzyme-linked immunosorbent assay

Peripheral blood was collected at the beginning of the study in the control group, and was collected before and after medication in the treatment group. All sera were obtained by centrifugation and stored at -80°C. Commercially available enzyme-linked immunosorbent assay (ELISA) kits (ABclonal Biotechnology, Wuhan, China) were used to detect the levels of TNF-α, IFN-γ, IL-2, IL-6 and IL-10 in the serum of patients with CRC. 5-HT was detected using another ELISA kit (GeneTex, Hsinchu City, Taiwan).

Immunohistochemistry

Tumor and normal tissues in the control group and treatment group were removed after surgery. The colon and rectum specimens were harvested and embedded in paraffin blocks and cut into 4-μm-thick tissue sections. The morphological changes in normal tissues were confirmed by hematoxylin and eosin staining. For immunohistochemical staining, the paraffin-embedded slides were dewaxed using xylene and rehydrated using alcohol of graded concentrations. Endogenous peroxidase activity was eliminated by 3% H_2O_2 for 15 min. The slides were then blocked with 5% goat serum for 20 min at 37°C, followed by primary antibody incubation overnight at 4°C. The next day, each sample was incubated with horseradish-peroxidase-labeled secondary antibody for 1 h at room temperature, followed by staining with the ready-to-use reagent DAB kit (ZSGB-BIO, Beijing, China). After dehydration and drying, the tissue sections were mounted with neutral gum and observed under a microscope (Olympus, Tokyo, Japan). Three high power visual fields were randomly selected for image acquisition, and image quantitative analysis was carried out with Image-Pro Plus 6.0 software. The average optical density of each protein was finally expressed by IOD value.

Analysis of fecal 16S rDNA

Feces were collected from the treatment group before and after medication for gut microbiota analyses by 16S rDNA. Microbial genomic DNA was extracted from fecal samples using a QIAamp DNA Stool Mini Kit (MoBio Laboratories Inc., Carlsbad, CA, United States). The 16S rDNA V4 region was amplified using the 515F primers (515F-GTGCCAGCMGCCGCGGTAA) and 806R primers (GGACTACHVGGGTWTCTAAT). PCR product quantification, qualification and purification were performed. Library preparation and sequencing were performed on the MiSeq platform (Beijing Genomics Institute, Shenzhen, China). The 16S rDNA sequencing data were quality filtered using

FLASH (Fast Length Adjustment of Short reads, version 1.2.11). Operational taxonomic units (OTUs) were picked at a 97% sequence similarity cut-off, and the identified taxonomy was then aligned using Silva (Release128 http://www.arb-silva.de). The RDP classifier (version 2.2) was used to classify OTUs at a given taxonomic rank.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (Chicago, IL, United States). Measurement data are expressed as mean ± standard deviation. Comparisons between two groups were assessed using Student's unpaired *t* tests. The Student's paired *t* test was used to compare the results between pre-treatment and post-treatment. If it did not conform to the normality test, the rank sum test was used. $P < 0.05$ was selected as the point of minimal statistical significance in every comparison.

RESULTS

Comparison of clinical data between the control and treatment groups

We compared clinical data between the control group and treatment group, including sex, age, tumor location, T stage, lymph node, and TNM stage (Table 1). The results showed that the clinical data of the two groups were consistent.

Safety evaluation of GQD

In the treatment group, nine patients had abdominal pain and distension, and 14 had diarrhea. Twelve patients complained of tenesmus. Following the administration of GQD, 12 of these 14 patients stated that their abdominal symptoms were better than those before treatment, including alleviation of diarrhea and reduced defecation, from five to three times per day. Another seven patients stated that the symptoms of tenesmus improved after taking GQD. No related adverse events were observed (Table 2).

GQD enhanced immunity

To determine the effect of GQD on immune function in patients with CRC, we measured immune cells in the control and treatment groups. There was no difference in the proportion of peripheral immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, NK cells ($CD3^-CD16^+CD56^+$), NKT cells ($CD3^+CD16^+CD56^+$) and Treg cells ($CD4^+CD25^+CD127^{dim}$) between the control and pre-treatment group ($P > 0.05$) (Figure 1). However, compared with the control group and pre-treatment group, the proportion of $CD4^+$ T cells was significantly increased in the post-treatment group ($P < 0.05$) (Figure 1A and B). There was no significant difference in $CD8^+$ T cells and NK cells among the three groups ($P > 0.05$) (Figure 1C-F). The proportion of NKT cells in the control group and pre-treatment group was 1.28% and 1.58%, respectively, and increased to 2.58% in the post-treatment group ($P < 0.05$) (Figure 1E and H). There was almost no difference in the proportion of Treg cells among the three groups ($P > 0.05$) (Figure 1G and I).

GQD reduced inflammation

To establish whether GQD reduced inflammation in patients with CRC, serum cytokine levels were measured with ELISA. The level of TNF-α in the post-treatment group was significantly lower than that in the control and pre-treatment groups. The average value of TNF-α in the control and pre-treatment groups was 12.85 pg/mL and 12.47 pg/mL, respectively, and the average value in the post-treatment group was 9.88 pg/mL ($P < 0.05$) (Figure 2A). The levels of other cytokines including IFN-γ, IL-2, IL-6 and IL-10 did not change significantly among the three groups ($P > 0.05$) (Figure 2B-E). In addition, compared with the pre-treatment group, GQD significantly reduced the level of 5-HT in the post-treatment group ($P < 0.05$). Although the level of 5-HT was also lower than that in the control group, it did not reach statistical significance (Figure 2F).

GQD enhanced intestinal barrier function

In the normal tissues of patients with CRC, the inflammatory reaction in the control group was more severe than that in the treatment group, in terms of the distribution of lymph nodes and destruction of intestinal mucosa (Figure 3A and B). To detect the effect of GQD on intestinal barrier function in patients with CRC, the expression of ZO-1, occludin, NF-κB and TNF-α in tumor and normal tissues was detected by

Table 1 Clinical data of patients with colorectal cancer in the two groups

Groups	Control	Treatment	χ^2 value	*P* value
Gender			3.503	0.061
Female	29	19		
Male	8	14		
Age (yr)			0.479	0.489
≤ 60	16	17		
> 60	21	16		
Tumor location			0.243	0.622
Colon	18	18		
Rectum	19	15		
T stage			1.425	0.233
T1-T2	4	7		
T3-T4	33	26		
Lymph node			0.391	0.532
Positive	24	19		
Negative	13	14		
TNM stage			2.700	0.259
I	3	6		
II	21	13		
III	13	14		
Total	37	33		

TNM: Tumor-node-metastasis.

Table 2 Safety evaluation in patients with colorectal cancer in the treatment group

Observation index (patients)	Unchanged	Alleviation	Aggravation
Stomachache, Bloating (9)	7 (78%)	2 (22%)	0
Diarrhea (14)	2 (14%)	12 (86%)	0
Tenesmus (12)	5 (42%)	7 (58%)	0

immunohistochemistry (Figure 4A). The expression of ZO-1 in tumor and normal tissues in the treatment group was significantly higher than that in the control group ($P < 0.05$) (Figure 4B). The expression of occludin in tumor tissues in the treatment group was significantly higher than that in the control group ($P < 0.05$), but there was no change in normal tissues between the control and treatment groups ($P > 0.05$) (Figure 4C). Similarly, compared with the control group, expression of NF-κB and TNF-α in tumor tissues of the treatment group was significantly reduced ($P < 0.05$) (Figure 4D and E). There was also no significant change in normal tissues between the control group and treatment group ($P > 0.05$) (Figure 4D and E). GQD prevented destruction of the intestinal barrier in patients with CRC.

GQD regulated the gut microbiota

To determine the influence of GQD on the gut microbiota, 16S rDNA was used to detect the changes before and after treatment. Under 97% similarity, the number of OTUs in each sample was obtained. A Venn diagram showed 143 different species between the pre-treatment and post-treatment groups (Figure 5A). Partial least squares discriminant analysis demonstrated a notable clustering effect in the gut microbiota pre-treatment and post-treatment (Figure 5B). By visualizing the landscape of the gut

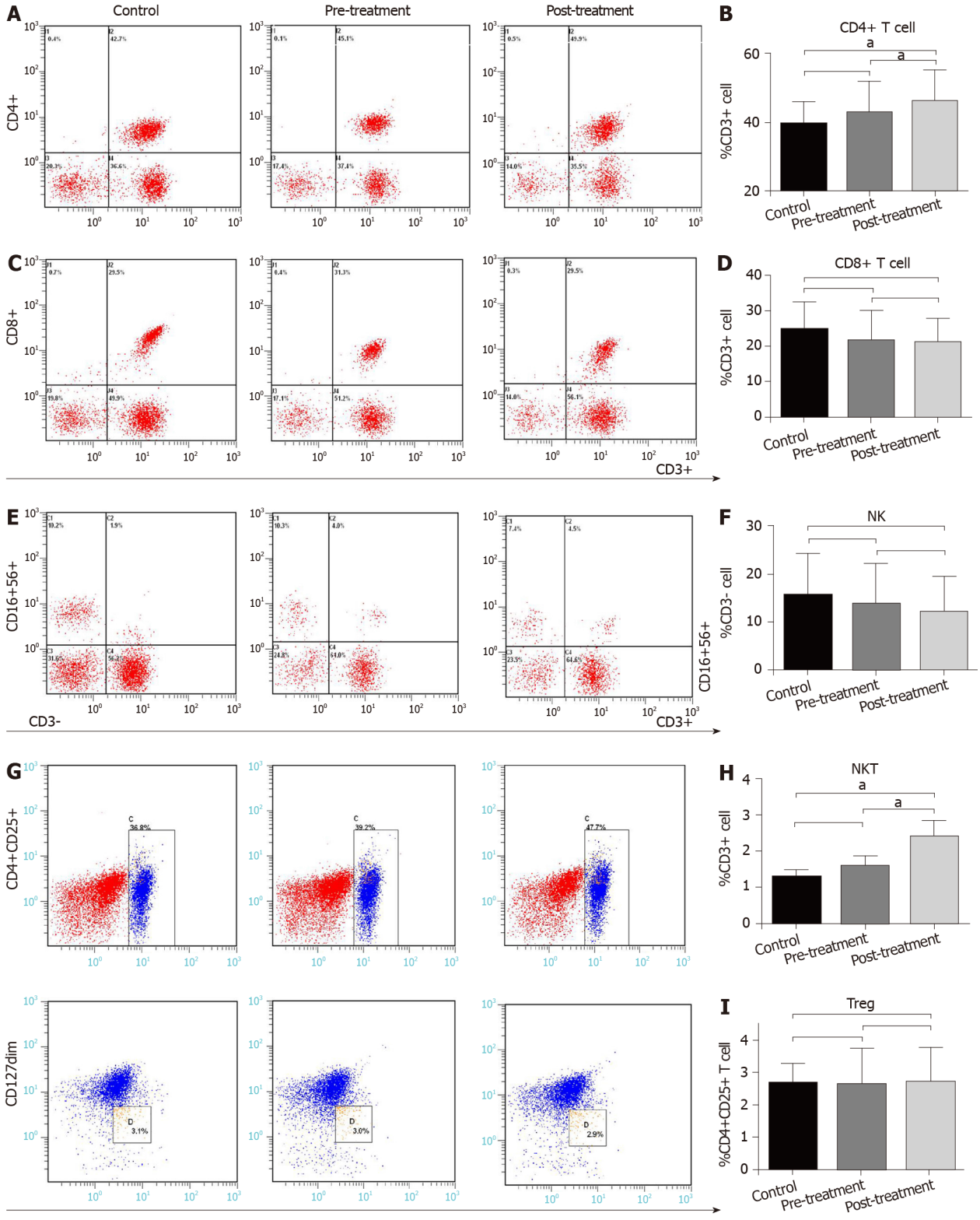


Figure 1 Gegen Qinlian decoction enhanced the immunity. A, C, E and G: Flow cytometry analysis of the proportions of $CD4^+$T, $CD8^+$T, natural killer (NK), NKT and Treg cells in the control group, the pre-treatment group and the post-treatment group of patients with colorectal cancer; B, D, F, H and I: Changes in the proportions of $CD4^+$T, $CD8^+$T, NK, NKT and Treg cells ($^aP < 0.05$).

microbiota in all feces samples, we found that the alpha and beta diversity of the gut microbiota was significantly lower in the post-treatment group than in the pre-treatment group based on Ace, Chao and Shannon indices (Figure 5C and D).

We analyzed the gut microbiota for differences between the pre-treatment and post-treatment groups. Four dominant phyla were identified. Compared with pre-treatment, the abundance of Bacteroidetes was increased, while the abundance of Firmicutes, Proteobacteria and Verrucomicrobia was decreased post-treatment

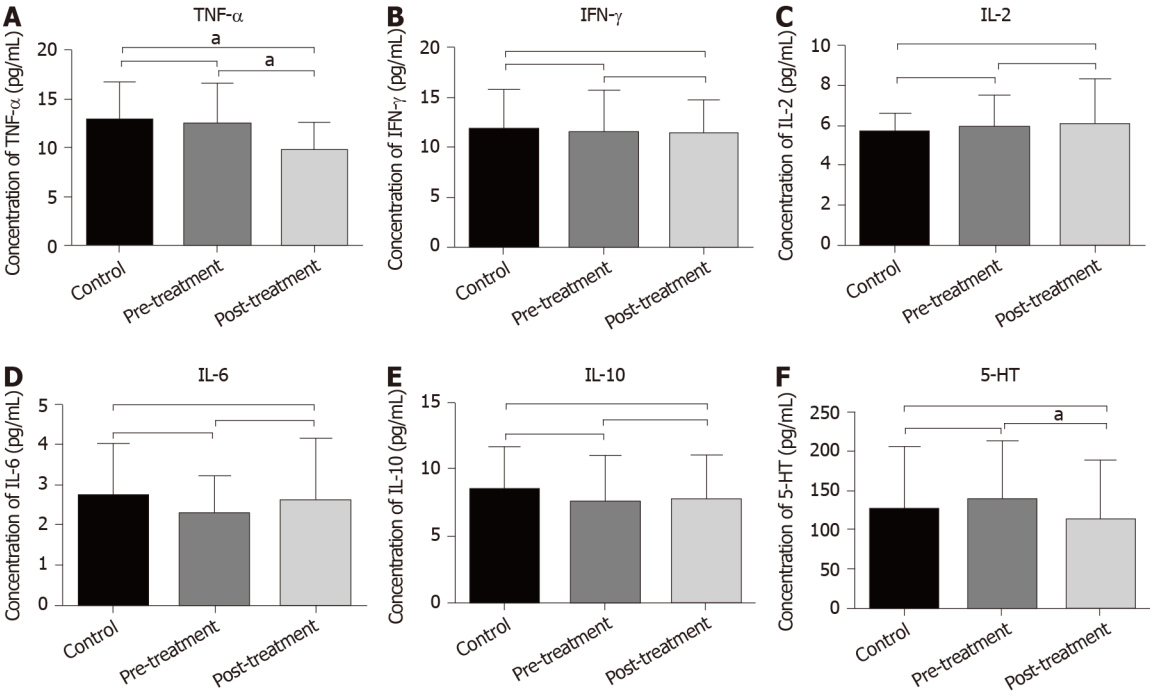


Figure 2 Gegen Qinlian decoction reduced inflammation. A-E: Changes in the concentration of cytokines [including tumor necrosis factor-α, interferon-γ, interleukin (IL)-2, IL-6, and IL-10] in serum among the control group, pre-treatment group and post-treatment group of patients with colorectal cancer ($^aP < 0.05$); F: Changes in neurotransmitter 5-HT levels in serum among the three groups ($^aP < 0.05$). TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; IL: Interleukin; 5-HT: 5-Hydroxytryptamine.

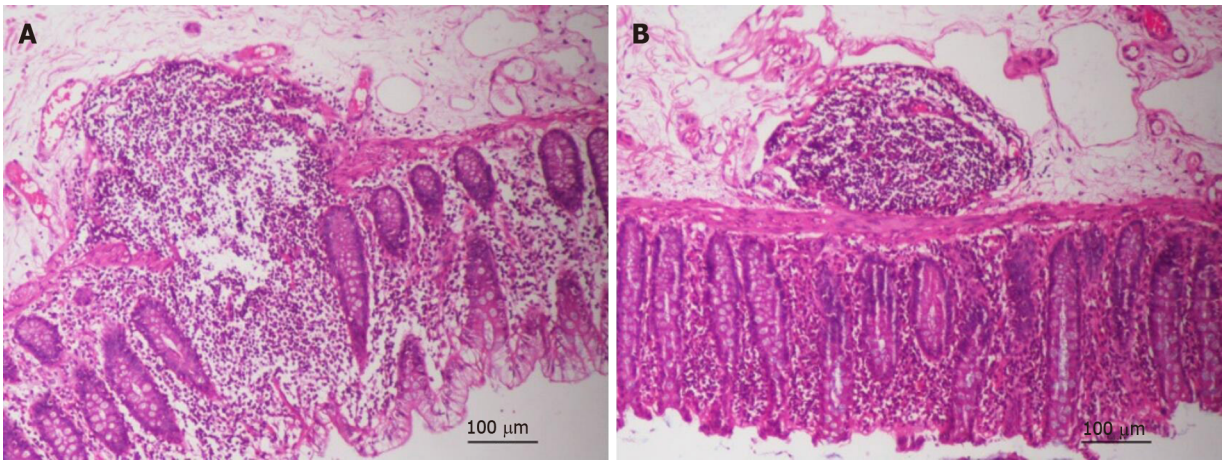
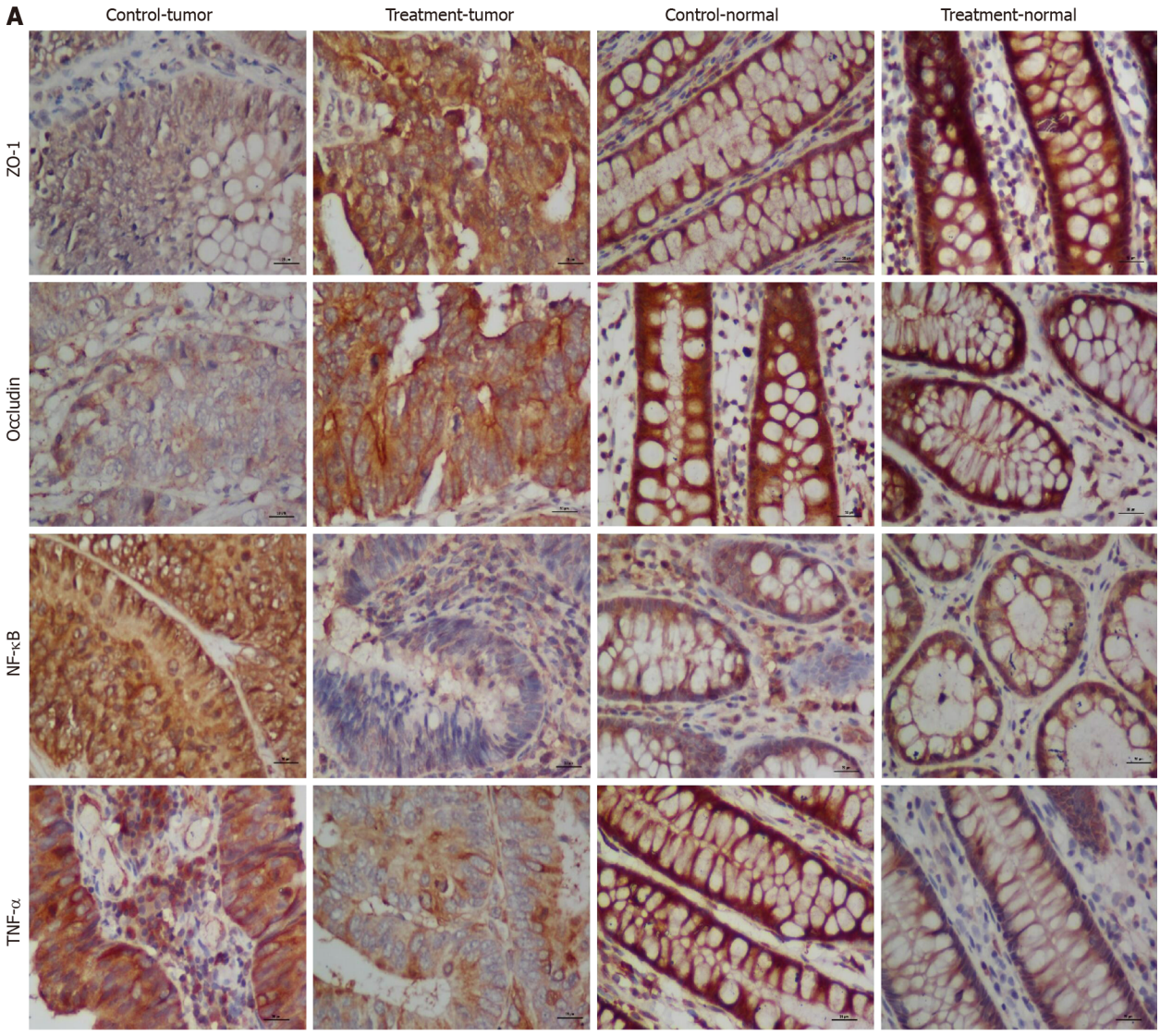


Figure 3 The morphological features of normal tissues in the control and treatment groups by HE staining (× 100 magnification). A: The control group; B: The treatment group.

(Figure 5E). When we compared the phylogenetic composition of common bacterial taxa at the genus level, we found that *Bacteroides*, *Akkermansia* and *Prevotella* were enriched and the abundance of *Megamonas* and *Veillonella* was decreased in the post-treatment group (Figure 5F and G).

To analyze these findings further, the differential genes of gut microbiota between the two groups were enriched by KEGG function. We found functional differences in the gut microbiota between the pre-treatment and post-treatment groups, which included energy metabolism, immune system, nervous system and cancer ($P < 0.05$) (Figure 6). We suggest that GQD changed the functional state of patients with CRC *via* the gut microbiota.


A
Control-tumor
Treatment-tumor
Control-normal
Treatment-normal
ZO-1
Occludin
NF-κB
TNF-α
B
ZO-1
IOD value
400
300
200
100
0
a
a
Control-tumor
Treatment-tumor
Control-normal
Treatment-normal
C
Occludin
IOD value
300
200
100
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a
Control-tumor
Treatment-tumor
Control-normal
Treatment-normal

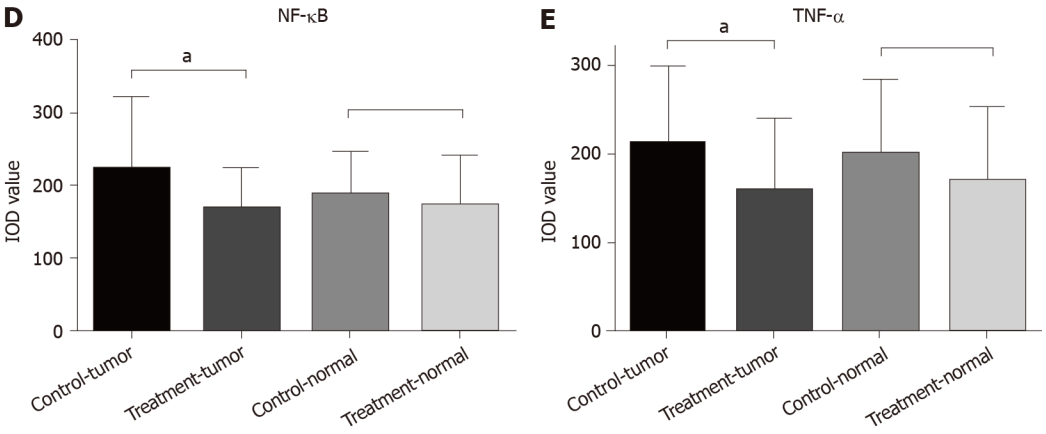


Figure 4 Gegen Qinlian decoction enhanced intestinal barrier function. A: The expression of zonula occludens (ZO)-1, occludin, nuclear factor (NF)-κB and tumor necrosis factor (TNF)-α in tumor and normal tissues between the control and treatment groups determined by immunohistochemistry (× 400 magnification); B-E: Histogram showing the expression of ZO-1, occludin, NF-κB and TNF-α ($^aP < 0.05$). ZO-1: Zonula occludens-1; NF-κB: Nuclear factor-κB; TNF-α: Tumor necrosis factor-α.

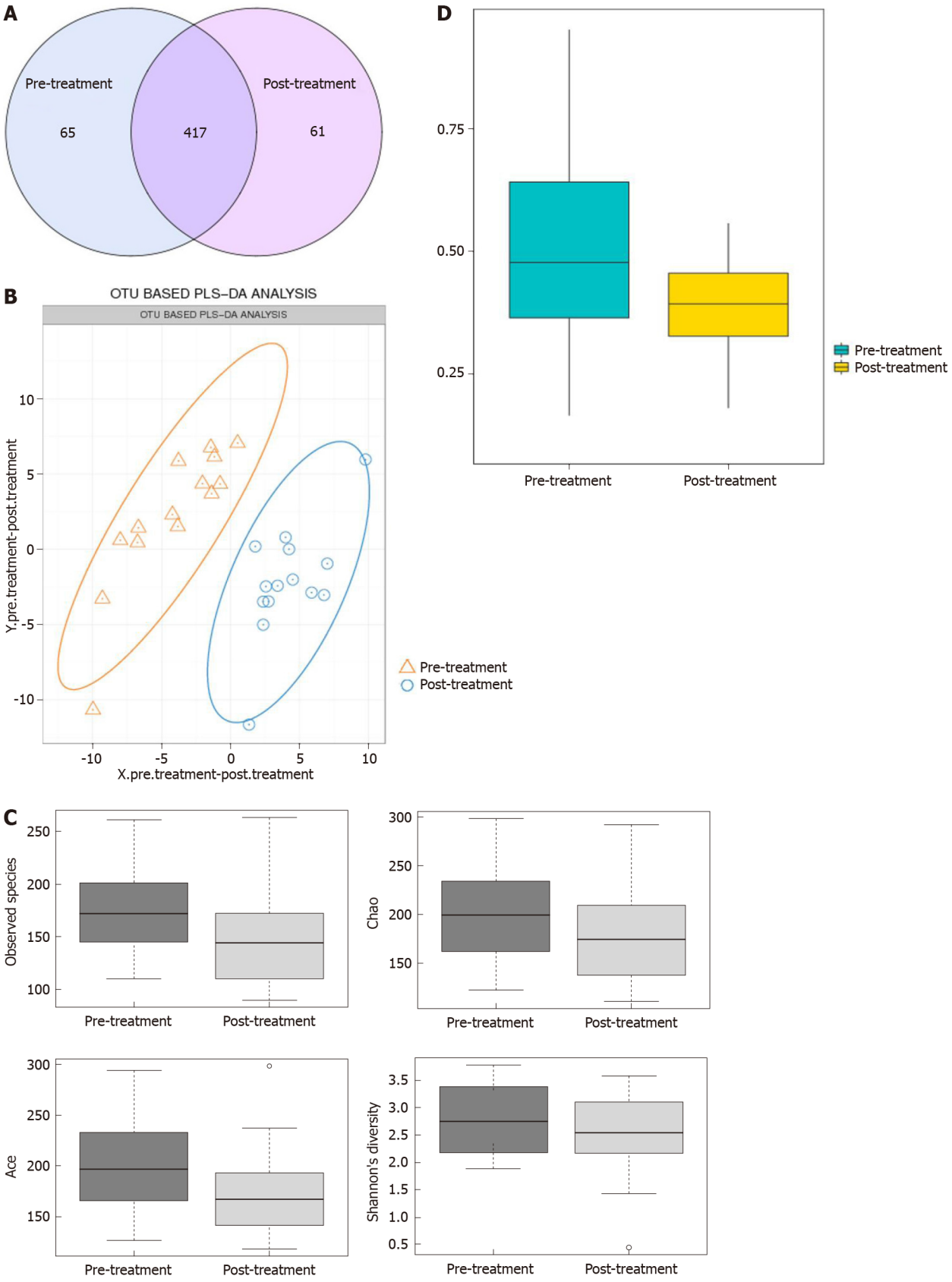
DISCUSSION

GQD is composed of Radix Puerariae, Radix Scutellariae, Rhizoma Coptidis and Radix Glycyrrhizae, the heat-clearing and detoxifying effects of which are well known. Wang *et al*[29] reported that GQD combined with other traditional Chinese medicine can prolong progression-free survival of patients with cholangiocarcinoma, and they also found that GQD restricts tumor growth in patients with CRC, but did not clarify the mechanism. In our study, we analyzed the immune status of peripheral blood and found that GQD increased the number of $CD4^+$ T cells and NKT cells; both of which contribute to the immune response against tumor cells. NKT cells are a group of special T cells with both T cell and NK cell receptors. Although the number is small, it can produce a large number of cytokines, but also reflects the state of immune metabolites, and can fight tumor cells together with $CD4^+$ T cells. Mossanen *et al*[30] found that $CD4^+$ T cells and NKT cells can work together against tumor cells. It has been shown that the decrease in the proportion of NK and NKT cells and the increase in the proportion of Treg cells in patients with CRC lead to poor efficacy of comprehensive treatment[31]. GQD regulates immune balance by inhibiting the differentiation of Treg cells in mice with influenza[32]. In this study, GQD had no significant effect on NK and Treg cells in peripheral blood, which may be related to the small sample size and activation state. Even though the proportion of NK and Treg cells in lymphocytes does not change significantly, their activation state may be changed. Future studies will increase the sample size and measure the activation state of immune cells to explore the effect of GQD on immune function.

Chronic inflammation is one of the main risk factors for CRC. Patients with inflammatory bowel diseases, including UC and Crohn's disease, have a higher risk of CRC than the general population[33,34]. Recently, it was reported that GQD has anti-inflammatory and anti-infective effects. A variety of active components of GQD, such as baicalin, licorice flavonoids and berberine, can significantly reduce inflammation and oxidative stress. GQD also decreases diarrhea in piglets by reducing the levels of TNF-α and IL-6[35]. Wu *et al*[36] found that GQD extract could resist the increase in cytokines IL-1β, cyclo-oxygenase-2, intercellular adhesion molecule-1 and TNF-α induced by irinotecan, indicating that GQD has anti-inflammatory effects. In addition, TNF-α is an important inflammatory factor, which can significantly promote tumor progression. We found that GQD decreased the level of TNF-α, which verified that GQD could also reduce the inflammatory response in patients with CRC.

Studies have shown that 5-HT is obviously associated with diarrhea[37]. GQD was originally used to treat diarrhea. The main components of GQD, such as puerarin and berberine, reduce secretion of 5-HT[38,39]. In addition, berberine improves visceral hypersensitivity in rats with diarrhea and irritable bowel syndrome by reducing the levels of 5-HT, substance P and calcitonin gene peptide. We found that the level of 5-HT in patients with CRC after taking GQD was significantly lower than before treatment. We speculate that the reduction of diarrhea in most patients with CRC may be closely related to a decrease in 5-HT by GQD.

The intestinal barrier can prevent harmful substances from entering the blood, thus


A
Pre-treatment
Post-treatment
65
417
61
B
OTU BASED PLS-DA ANALYSIS
OTU BASED PLS-DA ANALYSIS
Y.pre.treatment-post.treatment
X.pre.treatment-post.treatment
Pre-treatment
Post-treatment
C
Observed species
Chao
Ace
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D
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Post-treatment

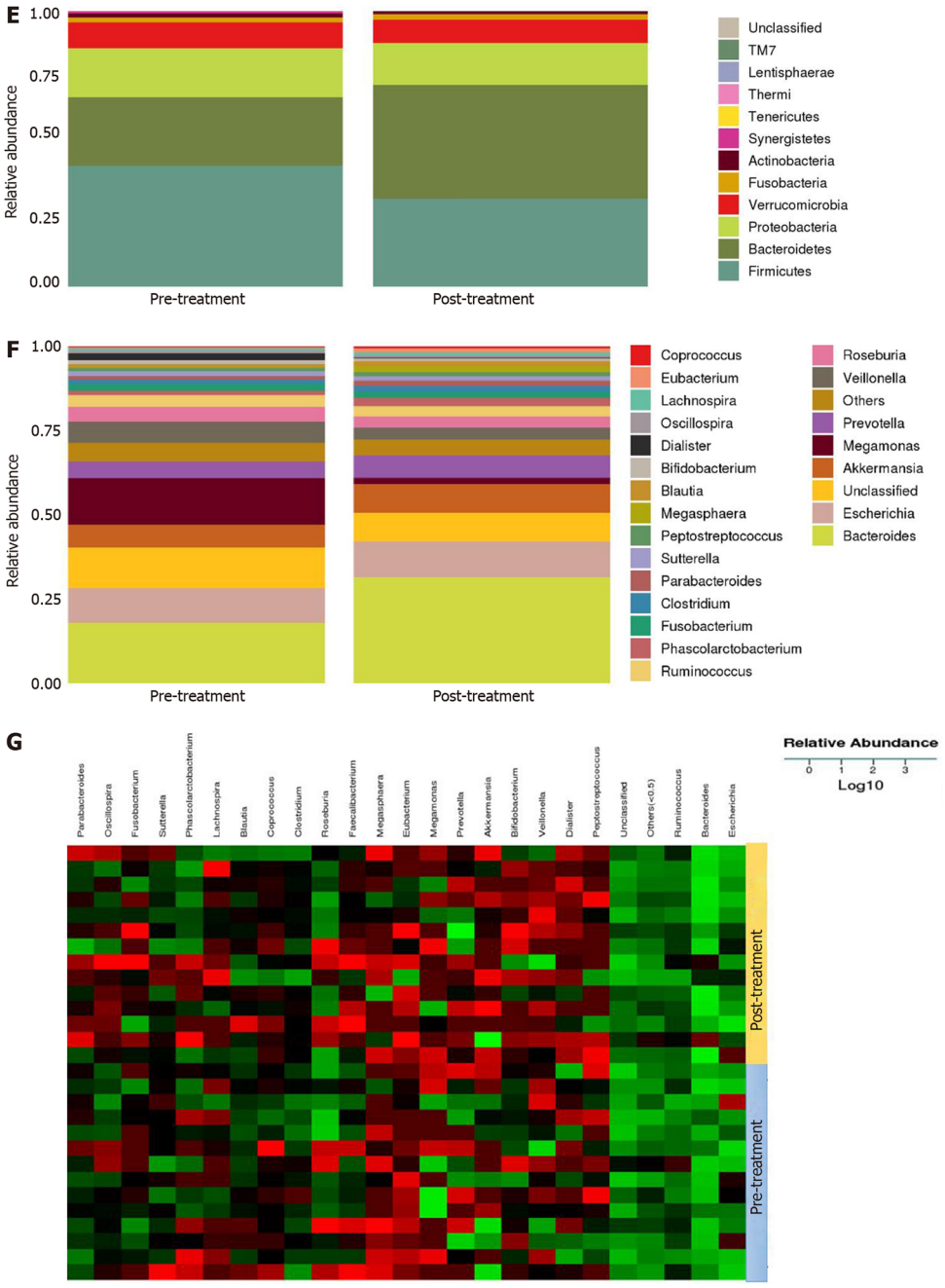

Figure 5 Gegen Qinlian decoction regulated the gut microbiota. A: Venn diagram of the total number of species shared between the pre-treatment and post-treatment groups; B: Principal coordinate analysis at the operational taxonomic unit level; C and D: Alpha diversity and beta diversity of the gut microbiota in the pre-treatment and post-treatment groups; E and F: Cumulative column chart of relative species abundance at the phylum and genus levels in the gut microbiome between the pre-treatment and post-treatment groups; G: Heatmap showing the changes in gut microbiota at the genus level between the pre-treatment and post-treatment groups. Columns represent samples and rows represent species. OUT: Operational taxonomic unit; PLS-DA: Principal coordinate analysis.

avoiding a series of pathophysiological changes. It was found that the intestinal barrier function in patients with CRC was damaged, which was manifested by the decrease in intestinal tight junction proteins (ZO-1 and occludin)[40,41]. In addition, expression of ZO-1 and occludin in cancer tissues was significantly lower than that in paracancerous tissues in a colon cancer model induced by dextran sulfate sodium, suggesting that the intestinal barrier function of cancer tissue was destroyed, which eventually led to tumor progression, while the occurrence and development of colon tumor was significantly inhibited after restoration of intestinal barrier function[40]. Baicalin and puerarin, the main components of GQD, can reverse the epithelial–mesenchymal transition process of hepatocytes by upregulating the expression of ZO-1, occludin and claudin[42]. In our study, the expression of ZO-1 and occludin in CRC tissues after GQD administration was significantly higher than that in the control group. GQD also significantly promoted the expression of ZO-1 in normal tissues, while the expression

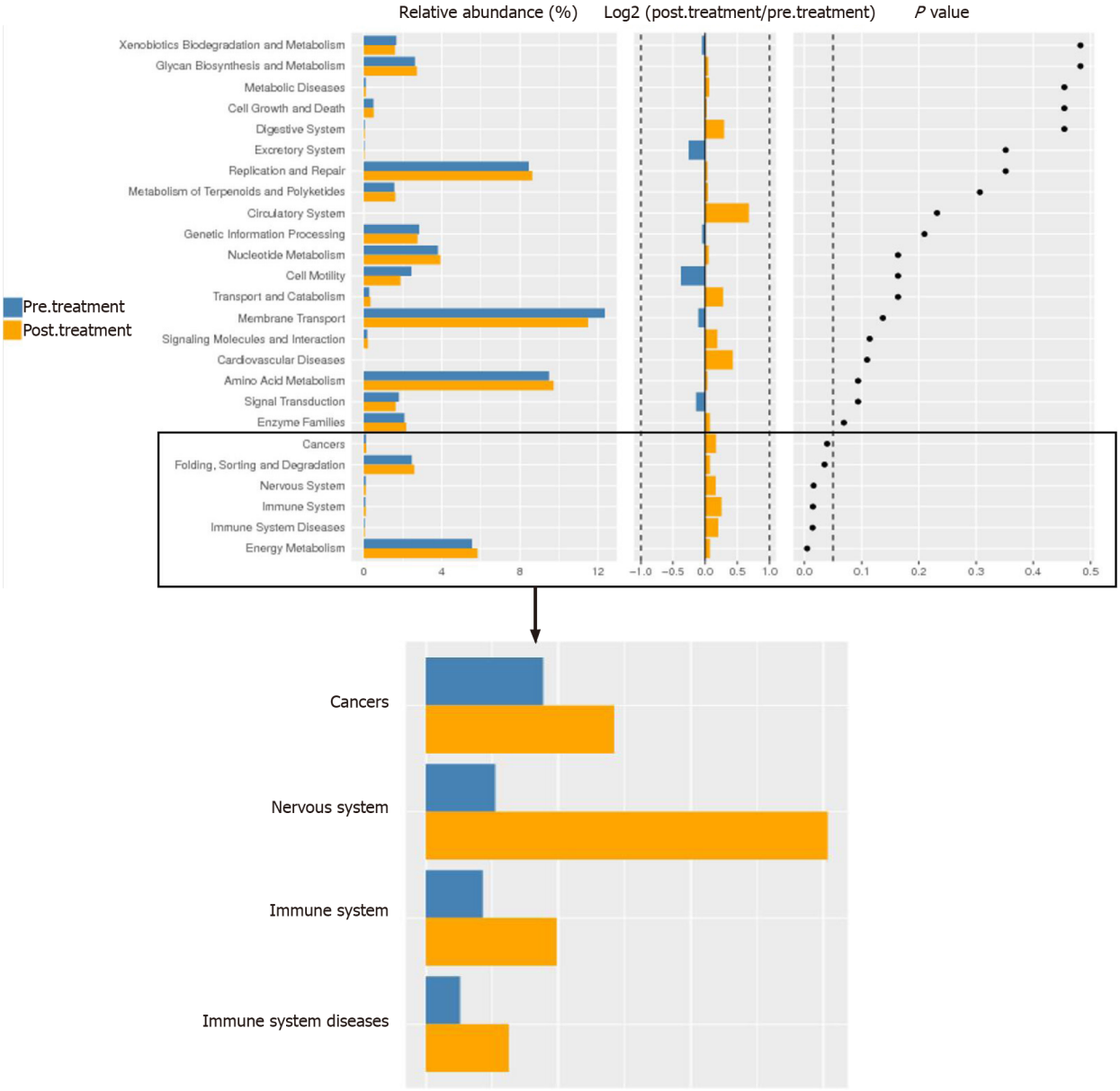

Figure 6 KEGG functional enrichment of differential genes in the gut microbiota of patients with colorectal cancer between the pre-treatment and post-treatment groups.

of occludin in normal tissues was higher in both the control and treatment groups. These results suggest that GQD promotes the recovery of intestinal barrier function, which may be one of the reasons for the relief of diarrhea and other clinical symptoms.

Inflammatory factors not only cause destruction of the intestinal barrier, but are also related to tumorigenesis. The decrease in TNF-α regulates intestinal epithelial permeability by upregulating the expression of ZO-1 and occludin proteins. It can increase the expression of adhesion molecules in endothelial cells and neutrophils, thus reducing the inflammation caused by migration, finally delaying tumor occurrence. In our study, consistent with the results of inflammatory factors in the peripheral blood, the expression of TNF-α and NF-κB in CRC tissues in the treatment group, which is significantly related to inflammation, was significantly lower than that in the control group. Although expression of TNF-α and NF-κB in the normal tissues of the treatment group was not significantly different from that of the control group, there was a decreasing trend. Therefore, we believe that GQD may upregulate intestinal tight junction proteins in the tumor microenvironment, improve intestinal inflammation, and protect the integrity of the intestinal barrier function, thus relieving clinical symptoms such as diarrhea in patients with CRC.

Intestinal microecological disorders are closely related to the occurrence and development of CRC[43]. Because of its special location, the gut microbiota is an

important part of the microenvironment of CRC. The abundance of harmful bacteria such as *Escherichia coli*, *Bacteroides fragilis* and *Fusobacterium nucleatum* increased, while the abundance of beneficial bacteria such as *Akkermansia* and *Prevotella* decreased. 16S rDNA sequencing was used to analyze the gut microbiota of feces from patients with CRC before and after GQD administration. Compared with pre-treatment, the abundance of Bacteroidetes was increased, while the abundance of Firmicutes, Proteobacteria and Verrucomicrobia was decreased post-treatment. The increase in Firmicutes and the decrease in Bacteroidetes have been proved to contribute to the development of cancer. At the genus level, the abundance of *Bacteroides*, *Akkermansia* and *Prevotella* was enriched and the abundance of *Megamonas* and *Veillonella* was decreased in the post-treatment group. It was reported that the abundance of *Ruminococcus* and *Prevotella* in the intestine of rats with CRC was significantly lower than that of healthy rats, which indirectly indicates that the abundance of these two bacteria is negatively related to the occurrence and development of CRC[44]. *Megamonas* can promote the invasion and metastasis of CRC[18]. We demonstrated that GQD reduces harmful bacteria and increases beneficial bacteria, which is consistent with our previous animal experiments and literature reports, confirming that GQD may delay the development of CRC by regulating gut microbiota.

Many scientists have found that the role of gut microbiota is not only limited to the intestinal tract, but also has an important impact on the normal function of the immune system[45]. A clinical study found that the abundance of *Fusobacterium nucleatum* in the intestine of CRC patients was inversely proportional to the density of $CD3^+$ T cells. This suggests that gut microbiota may be involved in immune regulation. It was reported that *Akkermansia* plays a vital role in intestinal homeostasis, and its abundance is also proportional to the effect of PD-1 inhibitors on CRC[23]. In addition, the abundance of *Megamonas* is closely related to immune function and the inflammatory response. NKT cells play an important role in intestinal immunity, which can regulate immune cells, including NK cells, dendritic cells, $CD4^+$ T cells and $CD8^+$ T cells[46]. However, animal experiments have shown that *B. fragilis* inhibits NKT cell activation through sphingolipids.

The increase in short chain fatty acids (SCFAs), metabolites of gut microbiota, helps to reduce inflammation. Furthermore, *Akkermansia*, *Butyricicoccus*, *Clostridium* and *Ruminococcus* of the gut microbiota reduce the intestinal-related chronic inflammation by reducing IL-6, IL-22, IL-1β and TNF-α, and play an immunomodulatory role to inhibit tumorigenesis[47]. Liu *et al*[48] found that GQD can regulate the gut microbiota of animals with diarrhea and increase the relative abundance of *Akkermansia*, *Bacteroides* and *Ruminococcus*[35]. Researchers have shown that certain gut microbiota in human feces can increase the content of $CD8^+$ T cells, and metabolites of gut microbiota such as SCFA can reduce 5-HT[49,50]. Combined with these changes, we consider that GQD may reduce the level of 5-HT by regulating the gut microbiota, thereby improving diarrhea symptoms in patients with CRC.

Our previous animal experiments showed that GQD increased the content of $CD8^+$ T cells and reduced the inflammatory response by increasing the Bacteroidales S24-7 group[28]. In this study, Bacteroidetes, *Prevotella* and *Ruminococcus* in the gut microbiota were significantly increased by GQD, while the abundance of *Megamonas* was significantly decreased. It is precisely these changes in the abundance of gut microbiota that enhance the immune function and reduce the inflammatory response of patients with CRC. Low-grade chronic inflammation and damage to the intestinal barrier function are not only related to the changes in intestinal microorganisms, but also to the occurrence and development of CRC. In this study, the abundance of *Akkermansia* of patients with CRC after GQD administration was increased. Although *Akkermansia* use mucin as the source of energy, a large number of observations have confirmed that *Akkermansia* has a positive regulatory effect on the thickness of the gut mucous layer and integrity of the intestinal barrier[51].

Puerarin, the main component of GQD, can increase the abundance of *Akkermansia* to promote the expression of ZO-1 and occludin, thereby protecting the intestinal barrier function[52]. Activation of the LPS–TLR4–NF-κB pathway has been shown to be involved in inflammatory processes and malignant transformation[53]. SCFAs are present in metabolites of *Bacteroides* and *Prevotella*. SCFAs (especially butyrate) protect the intestinal barrier function by increasing the expression of claudin-1, ZO-1 and mucin[54,55]. SCFAs can also reduce the expression of NF-κB and IL-18 to inhibit inflammation and regulate the intestinal microecology[56]. These studies show that the gut microbiota and its metabolites play a pivotal role in protecting the integrity of the intestinal tract. Although we did not detect the metabolites of gut microbiota, GQD increased the abundance of *Bacteroides*, *Prevotella* and *Akkermansia*, which increase the expression of ZO-1 and occludin, inhibit the NF-κB inflammatory signaling pathway,

and reduce the inflammatory factor TNF-α in blood and tumor tissues, thereby protecting the intestinal barrier function and inhibiting the development of intestinal inflammation.

KEGG function is significantly enriched in the immune system, energy metabolism, nervous system, and cancer, which indicates that the gut microbiota remodeled by GQD is related to the above functions. Our research shows that GQD can increase the number of immune cells, especially $CD4^+$ T cells and NKT cells, reduce the inflammatory response, and protect the intestinal barrier function. The above-mentioned effects of GQD are likely to be achieved by regulating the gut microbiota, and it has good clinical application prospects.

CONCLUSION

GQD improves intestinal barrier function by reducing the systemic inflammatory reaction and enhancing immune function in patients with CRC. These functions may be achieved through regulation of the gut microbiota.

ARTICLE HIGHLIGHTS

Research background

According to traditional Chinese medicine, colorectal cancer (CRC) originates from the damp-heat syndrome, while Gegen Qinlian decoction (GQD) is a classical traditional Chinese medicine for the treatment of damp-heat syndrome. We previously showed that GQD had a direct antitumor effect on tumor-bearing mice.

Research motivation

GQD can be used in the treatment of type 2 diabetes and ulcerative colitis (UC). However, the effect of GQD on patients with CRC is not clear.

Research objectives

This study aimed to determine the therapeutic mechanism of GQD in patients with CRC in improving immune function, reducing inflammation and protecting intestinal barrier function.

Research methods

The patients were divided into the control group and the treatment group. The proportions of T, natural killer (NK), NKT and Treg cells were measured by flow cytometry. The levels of cytokines and serotonin in serum were detected by enzyme-linked immunosorbent assay. The expression of zonula occludens (ZO)-1, occludin, nuclear factor (NF)-κB and tumor necrosis factor (TNF)-α in tumor and normal tissues was measured by immunohistochemistry. The composition of gut microbiota from patients in the treatment group was assessed using 16S rDNA analysis.

Research results

There were no adverse events in the treatment group. The proportion of CD4+ T cells and NKT cells in the post-treatment group was significantly higher than that in the pre-treatment and control groups ($P < 0.05$). The level of TNF-α in the post-treatment group was significantly lower than that in the pre-treatment and control groups ($P < 0.05$). The concentration of 5-HT in the post-treatment group was significantly lower than that in the pre-treatment group ($P < 0.05$). The expression of ZO-1 and occludin in tumor tissues in the treatment group was significantly higher than that in the control group ($P < 0.05$). The expression of ZO-1 in the normal tissues of the treatment group was significantly higher than that in the control group ($P = 0.010$). Compared with the control group, the expression of NF-κB and TNF-α in the tumor tissues of the treatment group was significantly decreased ($P < 0.05$). Compared with the pre-treatment group, GQD decreased the relative abundance of *Megamonas* and *Veillonella*. In addition, GQD increased the relative abundance of *Bacteroides*, *Akkermansia* and *Prevotella*. The differential genes of gut microbiota between the two groups were enriched by KEGG function and we found functional differences included energy metabolism, immune system, nervous system and cancer.

Research conclusions

GQD enhances the immunity and protects intestinal barrier function in patients with CRC by regulating the composition of gut microbiota.

Research perspectives

GQD has good clinical application prospects.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of colorectal cancer screening participation in remote northern Canada: A retrospective cohort study

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Abstract

BACKGROUND

Screening provides earlier colorectal cancer (CRC) detection and improves outcomes. It remains poorly understood if these benefits are realized with screening guidelines in remote northern populations of Canada where CRC rates are nearly twice the national average and access to colonoscopy is limited.

AIM

To evaluate the participation and impact of CRC screening guidelines in a remote northern population.

Informed consent statement: Our data collection was only retrospective and, therefore, no participant consent was required for ethics approval of this study.

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METHODS

This retrospective cohort study included residents of the Northwest Territories, a northern region of Canada, age 50-74 who underwent CRC screening by a fecal immunohistochemical test (FIT) between January 1, 2014 to March 30, 2019. To assess impact, individuals with a screen-detected CRC were compared to clinically-detected CRC cases for stage and location of CRC between 2014-2016. To assess participation, we conducted subgroup analyses of FIT positive individuals exploring the relationships between signs and symptoms of CRC at the time of screening, wait-times for colonoscopy, and screening outcomes. Two sample Welch *t*-test was used for normally distributed continuous variables, Mann-Whitney-Wilcoxon Tests for data without normal distribution, and Chi-square goodness of fit test for categorical variables. A P value of < 0.05 was considered to be statistically significant.

RESULTS

6817 fecal tests were completed, meaning an annual average screening rate of 25.04%, 843 (12.37%) were positive, 629 individuals underwent a follow-up colonoscopy, of which, 24.48% had advanced neoplasia (AN), 5.41% had CRC. There were no significant differences in stage, pathology, or location between screen-detected cancers and clinically-detected cancers. In assessing participation and screening outcomes, we observed 49.51% of individuals referred for colonoscopy after FIT were ineligible for CRC screening, most often due to signs and symptoms of CRC. Individuals were more likely to have AN if they had signs and symptoms of cancer at the time of screening, waited over 180 d for colonoscopy, or were indigenous [respectively, estimated RR 1.18 95%CI of RR (0.89-1.59)]; RR 1.523 (CI: 1.035, 2.240); RR 1.722 (CI: 1.165, 2.547)].

CONCLUSION

Screening did not facilitate early cancer detection but facilitated higher than anticipated AN detection. Signs and symptoms of CRC at screening, and long colonoscopy wait-times appear contributory.

Key Words: Gastroenterology; Rural health services; Public health; Colorectal neoplasms; Early detection of cancer; Northwest Territories

Core Tip: This 5-year retrospective cohort study evaluates the participation and impact of colorectal cancer (CRC) screening guidelines in a northern region of Canada. We evaluated CRC screening results of 6817 participants January, 2014 to March, 2019. We compared the stage and location of screen-detected CRC to clinically-detected CRC cases in 2014-2016. We observed no difference in screen-detected CRC *vs* clinically detected cases. During the 5-year observation period, we observed a higher incidence of advance neoplasia than anticipated, especially among patients presenting with signs and symptoms of cancer at the time of screening, who experienced long colonoscopy wait-times, and/or who identified as indigenous.

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INTRODUCTION

The benefits of colorectal cancer (CRC) screening have been well established in multiple prospective studies, including earlier detection, removal of pre-cancerous lesions, and reduction in CRC-associated mortality [RR 0.84, 95%CI: (0.78, 0.90)][1,2].

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While guidelines for CRC screening have been widely adopted, the extent to which the desired benefits of screening have been realized among remote northern populations remains poorly understood[3]. Remote northern populations experience multiple geographic and systemic barriers to health care which may impact CRC screening guideline implementation and adherence[4,5]. This is particularly important for indigenous populations who represent a high proportion of northern residents and are known to experience important sociocultural barriers to healthcare[6]. Significant disparities in CRC outcomes have been observed among remote and indigenous residents[6-9]. Therefore, it is imperative to evaluate if the benefits of CRC screening are realized in these regions.

The Northwest Territories (NWT) is a northern region of Canada with 44900 residents living in remote and isolated communities dispersed across 1.1 million km^2, of which, 50.7% of residents identify as indigenous. The population of the NWT has been shown to have a higher age-standardized incidence rate of CRC and a higher incidence of CRC-associated mortality than other areas of Canada; however, the reasons for these trends have not been explored[10]. Nonetheless, screening guidelines have been established since 2009 and recommend that average risk individuals age of 50-74 years undergo fecal immunochemical testing (FIT) every 1-2 years[11-13]. If the FIT is positive, the patient should receive a colonoscopy within 60 d. Higher risk individuals are advised to undergo regular screening colonoscopy (*i.e.*, those with a family history of CRC, relevant genetic syndrome, or inflammatory bowel disease). Semi-structured interviews with clinicians in the NWT indicate that implementation of these guidelines has been challenging particularly with regards to recruiting of participants, determining their eligibility for FIT, and arranging timely colonoscopy access for residents[4]. This study aims to understand the impact of screening guidelines in this remote population with a high incidence of CRC by assessing the participation and outcomes of CRC screening.

MATERIALS AND METHODS

This study was approved by the Aurora College Research Ethics Committee, protocol No. 20190404.

A population-based, retrospective cohort study was conducted of individuals who underwent CRC screening by FIT in the NWT between January 1, 2014 to March 30, 2019. Individuals were identified in the prospectively collected Public Health Registries, the most reliable form of capturing FIT participation in the NWT. We included all individuals who, at the time of testing, were between the ages of 50-74 and had a valid NWT health card. Of those included, we collected their demographic details including community of residence and indigenous status (based on health card data).

Individuals with a positive FIT result were included in further analysis of FIT follow-up, excluding individuals without accessible health records, or who moved out of territory during the observation period. FIT-eligibility and colonoscopy results was derived from the patient's chart using manual chart review and patient identifiers (name and health card number). FIT-eligibility was defined as per the NWT screening guidelines: Individuals age 50-74 who are average risk and asymptomatic. This definition excluded individuals with signs and symptoms concerning for CRC (rectal bleeding, melena, anemia, abdominal pain, change in bowel habits, and/or unexplained weight loss), candidates for high risk screening, and/or indications for surveillance colonoscopy[14].

For colonoscopy results, participants were classified by the highest-risk pathology identified (Table 1). Clinically-detected cases of CRC were identified using the most recent data available in the NWT Cancer Registry (only available prior to 2017). Individuals who were between the ages of 50-75 years at the time of diagnosis and were diagnosed between January 1, 2014 to December 31, 2016 were included. We collected data regarding the patient demographics, cancer pathology, stage, and location.

Statistical analysis

The screening participation rate was calculated using the Canadian Partnership Against Cancer definitions and the estimated cohort of screen eligible individuals age 50-75 from the NWT Bureau of Statistics[15,16]. To assess CRC screening impact, we compared screen-detected cases of CRC to clinically-detected cases. To assess CRC participation, we conducted a subgroup analysis of FIT positive individuals

Table 1 Adenoma and colorectal cancer classification

Classification	Description
LRA	One to three tubular adenomas or sessile serrated adenomas that are each < 10 mm diameter
HRA	Three or more tubular adenomas or an AA described as having at least one of the following features: Size > 10 mm diameter, villous or tubulovillous histology, and/or high-grade dysplasia
AN	Either an AA or cancer
CRC	Cancer identified including early stage (I and II), and late stage (III and IV)

These definitions are in keeping with the United States Multi-Society Task Force colorectal cancer guidelines for colonoscopy surveillance after screening and polypectomy[36,37]. LRA: Low risk adenoma; HRA: High risk adenoma; AN: Advanced neoplasia; CRC: Colorectal cancer; AA: Advanced adenoma.

comparing individual with signs and symptoms of CRC to FIT eligible individuals. Statistical analyses were conducted using Microsoft Excel (16.16.19) and RStudio (1.1463). The following tests were used to complete the statistical analyses found in this paper: Two sample Welch *t*-tests for normally distributed continuous variables, Mann-Whitney-Wilcoxon Tests for data without a normal distribution, Chi-Square Goodness of Fit Tests for categorical variables, and Fisher's Exact Tests for scenarios where categorical variables did not meet the requisite criteria for Chi-Square testing (*i.e.*, observed frequencies less than 5). Relative risks were calculated using the "epitools" package in R-Studio, which uses the Wald unconditional maximum likelihood estimation and has the option of a small sample adjustment (where appropriate); age, gender and other potentially confounding factors and/or comorbidities were not considered in the calculation of relative risk. A P value of < 0.05 was considered to be statistically significant; all confidence intervals are reported at a 95 percent confidence interval.

RESULTS

Between 2014-2019, 6817 FITs were completed by individuals between the age of 50-74 years, translating to an estimated biannual screening rate of 25.04% on average, 843 (12.37%) FITs were positive after 56 were excluded due to incomplete records or moving out of territory (Figure 1). We compared included and excluded individuals and observed a higher number of excluded individuals identified as Inuit (18 *vs* expected 5.17), and/or were from the Beaufort Delta region (22 *vs* expected 7.41). Fewer excluded patients were from Yellowknife (11 *vs* expected 27.26) (Supplementary Table 1). There was no significant difference in mean age or sex of individuals of included and excluded individuals.

When comparing cases of screen-detected cancer to clinically-detected cancer in 2014-2016 (Table 2), we observed no differences in age, sex, or indigenous status of individuals. In comparing the histology, location, and stage, we did not observe any statistically significant differences between the screen-detected and the clinically-detected cancers (Table 2).

Of the 843 FIT positive individuals who were FIT positive between 2014-2019, 629 (74.61%) underwent a colonoscopy after waiting a median of 133.00 d (IQR 166.5; SD 236.40; mean 207.20). On colonoscopy exam, 380 (60.41%) were found to have adenomas, of which, 120 were AA(s) (Figure 2). Thirty-four individuals were found to have a cancer. This translated to a positive predictive value (PPV) for FIT of 24.48% for AN.

At the time of referral for colonoscopy, 802 individuals were referred for a colonoscopy, of which, 398 (49.62%) met at least one exclusion criteria for FIT screening (Supplementary Figure 1). Among these individuals, we identified 288 (35.91%) with signs or symptoms concerning for CRC. In our first subgroup analysis, we compared symptomatic individuals to FIT eligible individuals, and observed symptomatic individuals were, on average, older than FIT eligible individuals at the time of FIT [61.18 *vs* 60.15 years, P = 0.047; 95%CI of difference (0.01, 2.06)] (Table 3). Indigenous patients were 49.04% more likely to have symptoms at the time of referral than non-indigenous patients [95%CI of RR: (1.248, 1.780)]. When comparing by the region of residence, we observed that the region of residence was not independent of FIT eligibility (P < 0.01): More patients than expected were referred from Fort Smith with symptoms, and fewer patients than expected from Yellowknife, but neither

Table 2 Screen detected *vs* clinically detected cancer, 2014-2016

Baseline characteristics	Screen detected (*n* = 19)	Clinically detected (*n* = 34)	*P* value
Age, mean years (SD)	63.2 (7.47)	59.1 (7.27)	0.06[1]
Sex, female (%; Std. Res)	6 (31.58; -0.56)	15 (68.42; 0.45)	0.37
Indigenous (%; Std. Res)	11 (33.33; -0.24)	22 (66.66; 0.18)	0.62
Region of residence (%; Std. Res)			0.89[2]
Beaufort Delta	< 5 (21.05; -0.31)	9 (26.47; 0.23)	
Dehcho	N/A	N/A	
Fort Smith	< 5 (5.26; -0.59)	< 5 (11.76; 0.44)	
Hay River	< 5 (15.79; 0.31)	< 5 (11.76; -0.23)	
Sahtu	< 5 (0; -0.85)	< 5 (5.88; 0.63)	
Tilcho	< 5 (10.53; 0.16)	< 5 (8.82; -0.12)	
Yellowknife	9 (47.37; 0.54)	12 (35.29; -0.40)	
Histology type, *n* (%; Std. Res)			0.13[2]
Adenocarcinoma	18 (94.74; 0.37)	28 (82.35; -0.28)	
Carcinoid	< 5 (5.26; 1.07)	< 5 (0; -0.80)	
Mucinous	< 5 (0; -0.85)	< 5 (5.88; 0.63)	
Other	< 5 (0; -1.20)	< 5 (11.76; 0.90)	
Location, *n* (%; Std. Res)			0.25[2]
Proximal	< 5 (10.53; -1.23)	11 (32.35; 0.92)	
Distal	17 (89.47; 0.70)	23 (67.65; -0.53)	
Stage, *n* (%; Std. Res)			0.18
Early	12 (63.16; 0.75)	15 (44.12; -0.56)	
Late	7 (36.83; -0.76)	19 (55.88; 0.57)	

[1]Mann-Whitney-Wilcoxon Test.
[2]Fisher's Exact Test for Count Data. Std. Res: Standardized residual. N/A: Not applicable.

observation was statistically significant (Table 3 and Supplementary Figure 2). Similar proportions of individuals underwent a colonoscopy however, patients who were symptomatic waited, on average, significantly longer than patients who were asymptomatic [199.5 *vs* 149.0 d, $P < 0.05$; 95%CI of difference: (5.07, 41.92)]. Symptomatic individuals were at least 22.8% more likely to have cancer identified on colonoscopy than screen eligible individuals [95%CI of RR: (1.228, 4.754)] (Figure 3).

When looking at the outcomes of FIT eligible individuals alone who underwent a colonoscopy, 229 of 333 had adenomas, of which, 130 were higher risk adenomas (HRAs). We identified 13 FIT eligible individuals who were diagnosed with CRC, the majority of which were early stage (I or II, 63.1%). The PPV for FIT among asymptomatic eligible individuals was 42.9% for HRA and advanced neoplasia (AN) combined, 23.4% for AN and 3.9% for cancer. We conducted a second subgroup analysis to look at only FIT eligible individuals comparing those with AN to those without. We observed no significant difference in sex ($P = 0.30$), or age (59.69 years with AN *vs* 59.96 years without; $P = 0.746$). However, we did observe that indigenous patients experienced an estimated 49.2% higher relative risk of AN compared to non-indigenous patients [95%CI of RR (adjusted for small sample): (1.0145, 2.194)] (Figure 4). We also observed that those with AN, on average, experienced a longer wait-time for colonoscopy than those without AN [194.54 *vs* 148.09 d; 95%CI of difference: (20.04, 72.85), $P = 0.0007$]. We found that that the relative risk of having an AN for FIT eligible patients who wait more than 180 d is estimated to be 68.21% more than those who wait less than 180 d [95%CI (adjusted for small sample size): (1.138, 2.487)]. The availability of colonoscopy services within the patients' community of residence was not associated with a diagnosis of AN.

Table 3 Demographics and outcomes of screen eligible *vs* symptomatic individuals

Baseline characteristics	FIT eligible (*n* = 390)	Symptomatic (*n* = 288)	*P* value
Age, mean years (SD)	60.15 (6.55)	61.18 (6.82)	0.0474[1]
Sex, female, *n* (%; Std. Res)	157 (40.26; -0.52)	127 (44.10; 0.58)	0.316
Indigenous, *n* (%; Std. Res)	152 (38.97; -2.15)	162 (56.25; 2.49)	0.000008
Region of residence, *n* (%; Std. Res)			0.009[2]
Beaufort Delta	42 (10.77; -0.26)	34 (11.81; 0.30)	
Dehcho	17 (4.36; -0.58)	17 (5.90; 0.67)	
Fort Smith	26 (6.67; -1.62)	36 (12.50; 1.88)	
Hay River	39 (10.00; -0.80)	38 (13.19; 0.93)	
Sahtu	21 (5.38; 0.06)	15 (5.21; -0.08)	
Tilcho	18 (4.62; -0.94)	21 (7.29; 1.09)	
Yellowknife	227 (58.20; 1.64)	127 (44.10; -1.91)	
Size of health centre, *n* (%; Std. Res)			0.0002
Yellowknife	219 (56.15; 1.94)	115(39.93; -2.26)	
Regional			
Fort Smith	26 (6.67; -1.62)	36 (12.50; 1.88)	
Hay River	36 (9.23; -0.84)	36 (12.50; 0.98)	
Inuvik	21(5.38; 0.60)	11(3.82; -0.70)	
Small community	88 (22.56; -1.42)	90 (31.25; 1.66)	
Colonoscopy, *n* (%)	333 (85.38)	212 (74.65)	0.29
Completed, *n* (%; Std. Res)	318 (95.50; 0.15)	198 (93.40; -0.19)	
Incomplete, *n* (%; Std. Res)	15 (4.50; -0.65)	14 (6.60; 0.81)	
FIT to colonoscopy, d			
Mean	159.9	183.4	0.01[1]
Median	149.0	199.5	0.02
< 60 d, *n* (%; Std. Res)	79 (23.87; 0.88)	38 (18.10; -1.10)	
60-180 d, *n* (%; Std. Res)	111 (33.53; 0.69)	59 (28.10; -0.86)	0.04
> 180 d, *n* (%; Std. Res)	141 (42.60; -1.16)	113 (53.80; 1.45)	0.01[1]
Findings, *n* (%; Std. Res)			
Low risk adenoma	99 (29.73; 1.17)	45 (21.23; -1.47)	0.03
High risk adenoma	130 (39.03; 1.41)	58 (27.36; -1.77)	0.005
Advanced adenoma	65 (19.52; 0.18)	39 (18.40; -0.23)	0.75
Cancer	13 (3.90; -1.60)	20 (9.43; 2.00)	0.008
Advanced neoplasia	78 (23.42; -0.62)	59 (27.83; 0.78)	0.25

[1]Welch two sample *t*-test.
[2]Not specific to region. Std. Res: Standardized residual; SD: Standard deviation; FIT: Fecal immunohistochemical test.

DISCUSSION

Disparities in CRC incidence and outcomes exist between populations and could be reduced through CRC screening[17]. In this retrospective cohort study of CRC screening in a remote northern population, known to experience significant disparities in CRC, FIT-based CRC screening did not facilitate earlier CRC detection. This may be due to the limited participation of only 25% of eligible individuals, and frequent participation of ineligible individuals (49.6% of FIT positive individuals who underwent

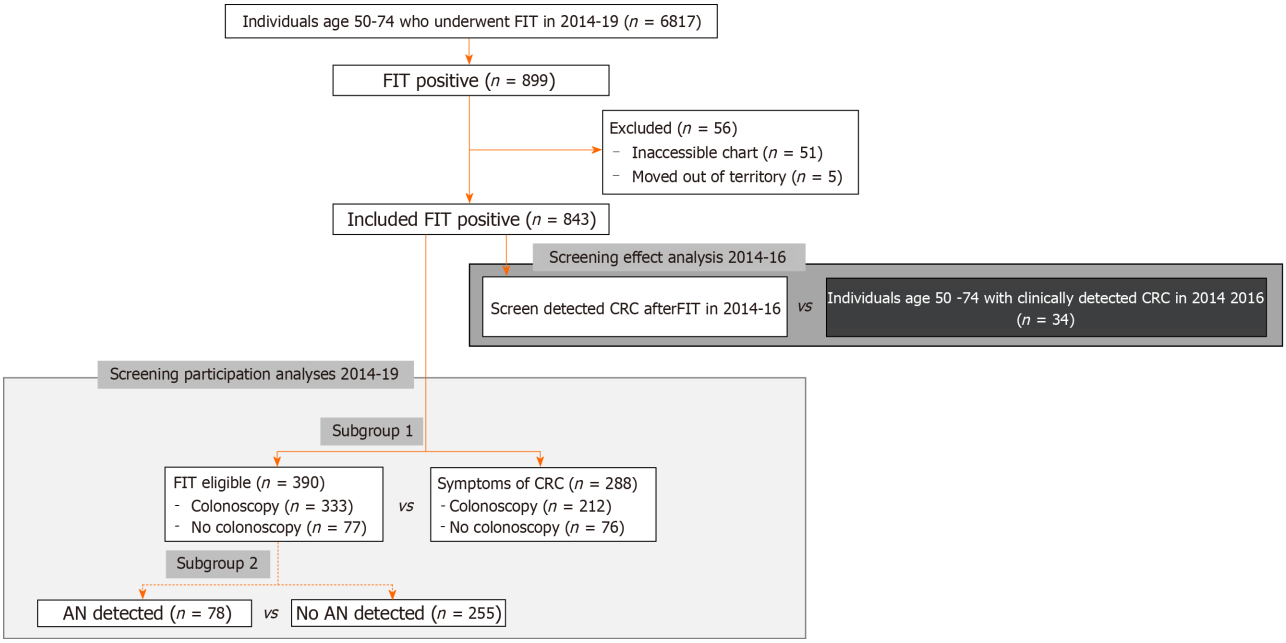

Figure 1 Inclusion of individuals in study analyses. FIT: Fecal immunohistochemical test; CRC: Colorectal cancer; AN: Advanced neoplasia.

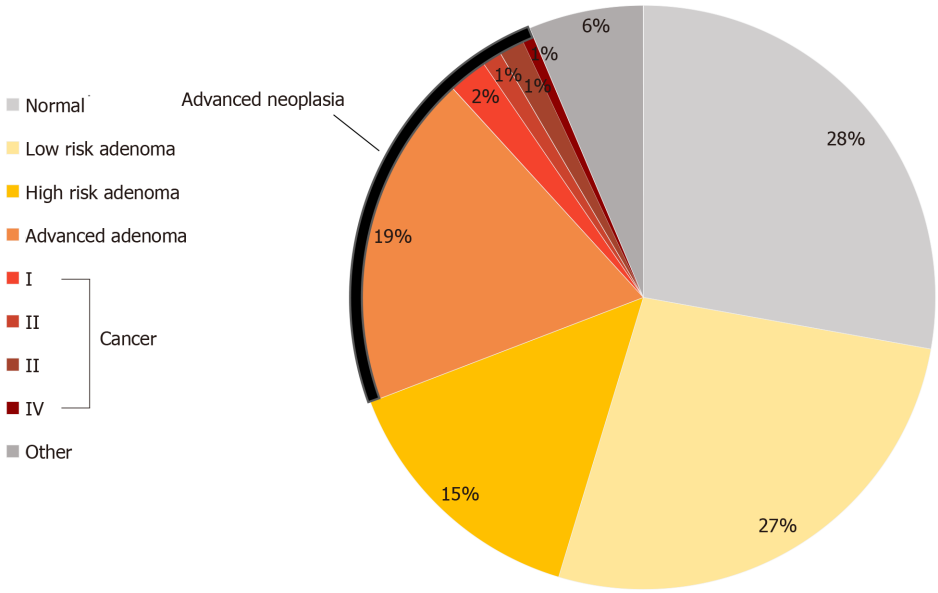

Figure 2 Colonoscopy findings after fecal immunohistochemical test positive result.

colonoscopy)[18]. Nonetheless, CRC screening appears to have facilitated effective adenoma detection, a majority of which would be amenable to removal at index colonoscopy and therefore, may reduce the risk of CRC in the long-term[19]. The positivity rate and PPV of FIT were higher than observed in a recent prospective trial by Liles *et al*[20] which employed the same brand and FIT threshold for 2761 individuals undergoing screening and observed a FIT positivity rate of 8.1%, and PPV for HRA or cancer of 21.9%-24.8% (in contrast to this study we observed a positivity rate of 12.3% and PPV of 43.8%). In reviewing CRC screening participation and outcomes, we observed three factors which appear to contribute to the relatively high rate of AN in this population which warrant further discussion: (1) Individuals with signs and symptoms of CRC frequently participated in screening, (2) Patients experienced long wait-times for colonoscopy, and (3) Indigenous individuals experienced a higher burden of CRC than non-indigenous.

Over 1 in 3 FIT positive individuals had signs or symptoms of CRC at the time of screening, despite the recommendations for their exclusion from screening. Several

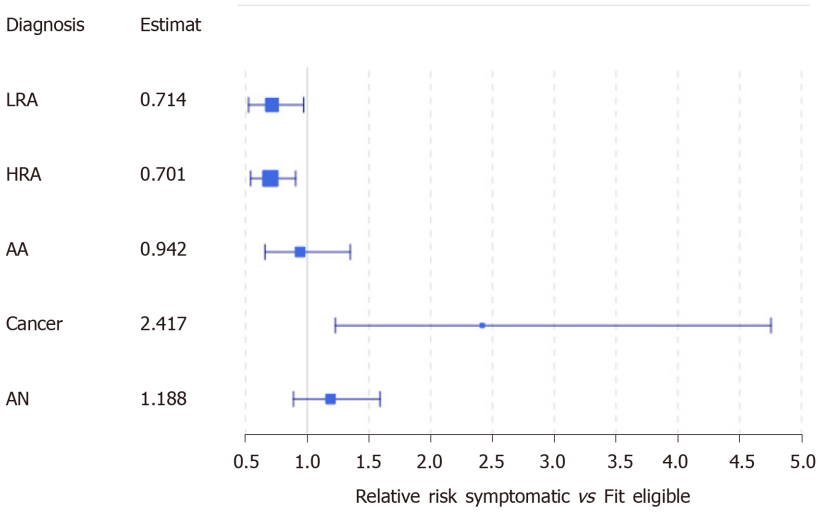

Figure 3 Relative risk of diagnosis for symptomatic patients *vs* eligible patients with 95%CI. LRA: Low-risk adenoma; HRA: High-risk adenoma; AA: Advanced adenoma; AN: Advanced neoplasia.

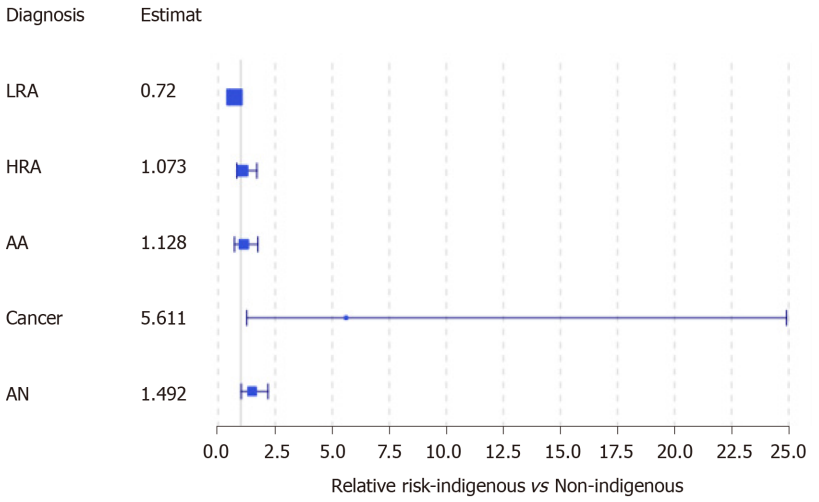

Figure 4 Relative risk of diagnosis for fecal immunohistochemical test eligible indigenous *vs* fecal immunohistochemical test eligible non-indigenous. LRA: Low-risk adenoma; HRA: High-risk adenoma; AA: Advanced adenoma; AN: Advanced neoplasia.

studies have similarly evaluated the symptoms of FIT positive individuals and observed even higher rates among participants of 47%-78%[21-23]. This may have important implications for CRC screening positivity and definition of "asymptomatic" screen-detected cancer[24]. We observed higher rates of CRC among individuals with reported signs or symptoms than FIT eligible individuals. However, the predictive value of red flag symptoms for colorectal pathology has been found to be variable[21-23]. De Klerk *et al*[23] (2018) reviewed 527 FIT positive patients and the 41% of individuals who had symptoms had a higher odds of CRC but the results were not statistically significant (OR 1.64 CI 0.86-3.13). In their analysis by individual symptoms, only a change in bowel habits or blood in the stool were associated with CRC detection at colonoscopy (OR 2.86, CI 1.23-6.62 and OR 8.65, CI 2.35-32.0). Previous systematic reviews summarizing the predictive value of red flag signs and symptoms, independent of FIT, have found rectal bleeding has diagnostic value, but other signs and symptoms only provide modest diagnostic value[25,26]. At present, guidelines clearly recommend against FIT screening of symptomatic individuals. The observed frequent use of FIT by symptomatic patients, may be partially attributed to the long wait-times for colonoscopy in the NWT. Previous interviews with clinicians in the NWT suggest that clinicians use FIT as a mechanism to accelerate a patient's access to colonoscopy[4]. This strategy does not appear to be effective, patients in this study with symptoms waited significantly longer for colonoscopy than FIT eligible individuals (P = 0.036)[13]. Further research is needed to discern the reasons individuals with red flag

symptoms to undergo screening and the impact of their participation on the diagnostic yield of FIT in order to guide screening and endoscopy protocols.

Patients in this study experienced long wait-times for colonoscopy following a positive FIT. Longer wait-times were associated with more advanced pathology at colonoscopy. National screening quality guidelines in Canada recommend a follow-up colonoscopy be completed within 60 d of a positive FIT and define a follow-up colonoscopy as one completed within 180 d of FIT[16]. In this study, only 23.87% of FIT eligible individuals met the benchmark of 60 d. Individuals who waited more than 180 d were 68.21% more likely to have AN than those waited ≤ 180 d. Other studies have similarly observed wait-times for colonoscopy after fecal screening test to be associated with more advanced pathology at colonoscopy[27,28]. Corley *et al*[27] found wait-times of 10-12 mo associated with a higher odds of CRC [OR 1.49 (95%CI 1.05-2.08)]. Flugelman *et al*[28] identified a significant relationship between increasing wait-time interval and stage of disease at presentation, as well as an association between wait-times more than 12 mo and a higher risk of CRC mortality [adjusted hazard ratio 1.53 (1.13-2.12)]. Colonoscopy wait-times could be contributing the overall CRC disparities experienced by this population and these findings reinforce the expert recommendations for prompt colonoscopy follow-up after FIT to enhance the quality of screening, and potentially, detect AN earlier. The underlying cause for long colonoscopy wait-times cannot be fully elucidated by this study but is likely multifactorial as demonstrated in our recent analysis of colonoscopy cancellations in this region[29]. Increasing colonoscopy access in this remote region is complex but could provide substantial benefit to patients.

Finally, we observed significantly higher rates of AN and cancer among indigenous individuals compared to non-indigenous. Indigenous Canadians have been found to experience important barriers to accessing cancer care and disparities in cancer outcomes in Canada[30-32]. This study provides an important contribution by reporting CRC rates among indigenous residents in northern Canada. A study of Alaskan indigenous populations observed that they experienced a higher CRC incidence than other ethnic groups in the United States. Boardman *et al*[33] assessed this further by comparing the tumour genetics among Alaskans but found no significant differences, and concluded that the cause of the higher incidence of CRC is likely multifactorial and attributable to recent diet changes, namely higher intake in fat, refined carbohydrates. Cancer is increasingly a public health concern among northern indigenous populations and our study is the first demonstrate this in CRC screening results[34]. The higher incidence of malignancy we observed advocates for further research and a potentially a re-evaluation of the current screening protocol for this cohort. Individuals with a first degree relative with a history of CRC have a 1.9-4.4 relative risk of CRC compared to average risk individual and are recommended to undergo colonoscopy screening every 5-10 years in Canada[35]. As such, indigenous individuals may benefit from enhanced screening to optimize CRC detection and control. This would require further analysis of the risks and benefits in discussion with indigenous healthcare leaders in the NWT.

Limitations

The generalizability of our results is limited to the retrospective data collected in the health records and small sample size. Cancer registry data for clinically detected CRC was only available prior to 2018 which limited the timeframe of comparing screen-detected and clinically detected CRC. FIT eligibility was derived from clinicians notes and therefore dependent on the consistency of provider documentation. We excluded 51 individuals due to inaccessible records, a higher than anticipated proportion of these individuals were from Beaufort Delta region and/or were Inuit. This is not surprising given that accessing paper records in this region was challenging. The included Inuit and Beaufort Delta residents did not experience any significant difference in colonoscopy outcomes than the remainder of the cohort. Finally, the population of the NWT is small, and factors not captured in this study such as patient comorbidities, and substance use may confound patients' cancer risk.

CONCLUSION

In this study of a northern remote population, FIT-based CRC screening did not appear to prevent CRC or provide earlier detection but did result in more frequent positive pathology results than anticipated for average risk screening. Individuals were more likely to have CRC at the time of screening if they experienced long wait-

times for colonoscopy, had clinical signs and symptoms of CRC, and/or were indigenous. Increasing access to colonoscopy, and effective triaging of FIT eligible individuals could enhance CRC screening effectiveness. Further research is needed to understand how to increase colonoscopy access in this remote region, and to discern if colonoscopy screening should be adopted among indigenous populations given their relative risk of CRC.

ARTICLE HIGHLIGHTS

Research background

Screening provides earlier colorectal cancer (CRC) detection and improves outcomes. However, it remains poorly understood if these benefits are realized with screening guidelines in remote northern populations where access to colonoscopy is limited. This study provides a critical contribution to this knowledge gap by providing an overview of the participation in, and impact of, CRC screening guidelines in a remote northern region of Canada that experiences higher rates of CRC: The Northwest Territories (NWT).

Research motivation

Previous studies suggest that remote and indigenous populations may experience significant geographic and systemic barriers to accessing CRC screening as well as a higher rate of CRC than other populations. To optimize CRC screening, a better understanding of current participation and screening outcomes in northern populations is critical.

Research objectives

This study aimed to evaluate the participation and outcomes of CRC screening in a remote northern population. In particular, we sought to understand the effectiveness of screening in the NWT and identify factors which may contribute to the likelihood of advanced neoplasia being detected among participants. Realizing these objectives will help inform future CRC screening in the NWT and our understanding of CRC screening access and effectiveness for northern populations.

Research methods

A population-based, retrospective cohort study was conducted of individuals who underwent CRC screening in the NWT in the last 5 years. This is the first study to review the results of CRC screening in a remote northern population.

Research results

Screen-detected cancer cases were not detected earlier than clinically-detected cases which suggests screening was not particularly effective and warrants further research. Potentially contributing to this trend were the findings that individuals experienced a higher incidence of CRC if they had signs and symptoms of CRC at screening, experienced long colonoscopy wait-times, or were indigenous. Further research is needed to further characterize the risk of CRC among indigenous individuals and inform strategies to improve colonoscopy access in the NWT.

Research conclusions

These findings suggest that critical gaps in colonoscopy access, triaging of eligible individuals, and knowledge of CRC risk among indigenous individuals may be impairing the CRC screening effectiveness for this remote northern population. This highlights the importance of pragmatic evaluation of CRC screening in remote and indigenous populations.

Research perspectives

Further research is needed to inform colonoscopy access for remote populations and to optimize screening for indigenous populations. Research in other northern regions is crucial to inform the generalizability of our findings.

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ORIGINAL ARTICLE

Retrospective Study

Prostate-specific membrane antigen expression in hepatocellular carcinoma, cholangiocarcinoma, and liver cirrhosis

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Author contributions: Chen LX and Zhu XH conceived and designed the study; Zou SJ, Li D, Zhou JY, and Cheng ZT collected the clinical data; Zhu YL and Kuang D contributed to the analysis; Chen LX drafted the manuscript; Zhu XH, Zou SJ, Li D, Kuang D, and Zhu YL made the comments; Zhu XH critically reviewed and revised the manuscript; Zhao J polished the manuscript; All authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Primary liver cancer includes three subtypes: Hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (CCA), and combined hepatocellular carcinoma. Patients with primary liver cancer experienced poor prognosis and high mortality, so early detection of liver cancer and improved management of metastases are both key strategies to reduce the death toll from liver cancer. Prostate-specific membrane antigen (PSMA) expression in the tumor-associated neovasculature of nonprostate malignancies including liver cancer has been reported recently, but conclusive evidence of PSMA expression based on the pathological type of liver cancer remains limited.

AIM

To study the expression of PSMA in HCC, CCA, and liver cirrhosis.

METHODS

A total of 446 formalin-fixed paraffin-embedded (FFPE) liver tumor and liver cirrhosis tissue samples were obtained retrospectively from the Pathology Department of Tongji Hospital. Immunohistochemistry was used to detect PSMA expression in these 446 FFPE liver biopsy specimens (213 HCC, 203 CCA, and 30 liver cirrhosis). The tumor compartment and the associated neovascular endothelium were separately analyzed. PSMA expression was examined by two certified pathologists, and the final results were presented in a 4-point scoring

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system (0-3 points). Correlation between PSMA expression and clinicopathological information was also assessed.

RESULTS

PSMA was expressed primarily in the neovascular endothelium associated with tumors. The positive rate of PSMA staining in HCC was significantly higher than that in CCA (86.8% *vs* 79.3%; $P = 0.001$) but was only 6.6% in liver cirrhosis ($P = 0.000$). HCC cases had more 3-score PSMA staining than CCA had (89/213, 41.8% *vs* 35/203, 17.2%; $P = 0.001$). PSMA expression correlated positively with the stage and grade of HCC and CCA. In both liver cancer subtypes, there were more PSMA$^+$ cases in stages III–V diseases than in stages I and II. High staining intensity of PSMA was more frequently observed in liver cancers at high grade and advanced stage. There was no significant association of PSMA expression with sex, age, region, α-fetoprotein, hepatitis B surface antigen, or tumor size in both tumor subtypes.

CONCLUSION

Neovascular PSMA may be a promising marker to differentiate HCC from liver cirrhosis and a prognostic marker for anti-tumor angiogenesis therapy for HCC.

Key Words: Prostate-specific membrane antigen; Hepatocellular carcinoma; Cholangiocarcinoma; Liver cirrhosis; Neovasculature; Immunohistochemistry

Core Tip: Immunohistochemistry was used to detect prostate-specific membrane antigen (PSMA) expression in hepatocellular carcinoma (HCC), cholangiocellular carcinoma (CCA), and liver cirrhosis. PSMA is specifically expressed in tumor-associated vasculature in HCC and CCA. The positive rate of PSMA staining in HCC was significantly higher than that in CCA (86.8% *vs* 79.3%), meanwhile, it was only 6.6% in liver cirrhosis, thus the potential of using PSMA-targeted imaging to distinguish HCC from liver cirrhosis may be true. PSMA expression correlated positively with stage and grade both in HCC and CCA; high staining intensity of PSMA was more frequently observed in liver cancers at high grade and advanced stage.

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INTRODUCTION

Primary liver cancer can be categorized according to its pathological characteristics into hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (CCA), and combined hepatocellular carcinoma (CHC)[1]. HCC accounts for 85%–90% cases of primary liver cancer, which is highly prevalent in China due to the epidemic of chronic hepatitis B. Most patients with primary liver cancer are diagnosed at advanced stages when treatment options are limited and subsequently experience poor prognosis and high mortality[1]. Therefore, early detection of liver cancer as well as improved management of metastases are both critical approaches to reducing the death toll from liver cancer.

Prostate-specific membrane antigen (PSMA), also known as folate hydrolase I or glutamate carboxypeptidase II, is a new biomarker that was initially defined by 7E11 immunoglobulin G monoclonal antibody[2]. PSMA is a 100 kDa transmembrane glycoprotein that can transduce extracellular signals into cytoplasm[3-6]. Originally found to be highly expressed in prostate cancer and high-grade intraepithelial neoplasia of prostate, PSMA has been extensively studied in recent decades for

prostate cancer imaging and theranostic applications[7]. For example, a large number of clinical trials have underpinned the advantage of PSMA–targeted radionuclide therapy for metastatic prostate cancer[8].

Despite its nomenclature, PSMA expression is also observed in the neovasculature of a wide range of nonprostate cancers, including glioblastoma multiforme; esophageal, gastric, breast, ovarian, colorectal, lung, adrenal, hepatocellular, pancreatic, renal cell, bladder, and testicular germ cell carcinoma; malignant melanoma; mesothelioma tumor and malignant neoplasms of the thyroid[9-26]. Several case reports have shown that HCC, CCA, and CHC have high uptake of radiotracer in PSMA-targeted positron emission tomography (PET) imaging[20-23]. A recent prospective pilot study in seven HCC patients demonstrated that the HCC lesions are hypervascular with ^{68}Ga-PSMA-positive microvessels, suggesting that ^{68}Ga-PSMA PET is more suitable for imaging HCC patients than the conventional ^{18}F-fluorodeoxyglucose (FDG)-PET[24]. We recently compared PSMA-PET with FDG-PET in HCC imaging and found that PSMA-PET exhibited higher standardized uptake value in the tumor region and higher tumor-to-background ratios (Figure 1). In addition to the findings from noninvasive imaging, a pathological evaluation of 103 HCC specimens confirmed that PSMA was expressed on 74% of tumor-associated blood vessels. PSMA expression has oncogenic consequences, including an association with tumor stage, differentiation, lymph node metastasis, and Ki67 index[25]. High vascular expression of PSMA is correlated with poor prognosis, indicating that it is an independent prognostic factor for liver cancer and subsequently a target for antiangiogenic therapy[25].

However, HCC is often accompanied with cirrhosis, which may acquire a nodular architecture with altered vascularity that resembles the regenerated nodules of early-stage HCC. As a result, the correlation between PSMA expression and the pathological classification of liver cancers remain elusive. In this retrospective study, we examined PSMA expression in 446 liver specimens (213 HCC, 203 CCA, and 30 cirrhosis) by immunohistochemistry (IHC), investigated the relationship between PSMA expression and clinicopathological findings, and discussed the potential of using PSMA-targeted imaging to distinguish HCC from liver cirrhosis.

MATERIALS AND METHODS

Specimen collection, tumor grading, and patient information

This study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (No. 2019-S951). Formalin-fixed paraffin-embedded liver tumor and liver cirrhosis tissue samples from hospitalized patients were obtained retrospectively from the Pathology Department of Tongji Hospital from January 2013 to December 2017. All samples were deidentified before analysis. A total of 446 liver specimens, including 213 HCC, 203 CCA, and 30 cirrhosis specimens, were studied. HCC and CCA were classified according to the World Health Organization and Edmondson pathological classification criteria as grade I (low), grade II (intermediate), and grade III (high)[1,26,27]. Patient characteristics and pathological features are summarized in Table 1.

IHC procedure

IHC was performed as previously described[11]. PSMA was stained with an anti-PSMA rabbit monoclonal antibody (ab133579; Abcam, Cambridge, MA, United States; 1:250 dilution) on a Leica Bond-Max autostainer and visualized with the Bond Polymer Refine Detection System (Leica Biosystems Newcastle, Newcastle upon Tyne, United Kingdom). Vascular structures were confirmed by staining with an anti-CD31 rabbit polyclonal (ab28264; Abcam; 1:100 dilution). Primary antibody-null staining was used as a negative control. Prostatic adenocarcinoma specimens with confirmed PSMA expression and tonsil specimens were used as the positive controls for PSMA and CD31 staining, respectively (Figure 2). All specimens were routinely stained with hematoxylin and eosin to verify tumor morphology prior to IHC.

IHC evaluation

The tumor compartment and the associated neovascular endothelium (ANVE) were separately analyzed on a minimum of three randomly chosen sections and observed at three different magnifications (40 ×, 100 ×, and 400 ×) *per* section. Protein expression was examined by two certified pathologists who were blinded to all the clinical data. Each pathologist assigned a score of 0 (no staining on any tumor cells or neovascular

Table 1 Clinicopathological features of liver tissues

Clinicopathological parameters		No. of cases (%)	
		HCC	CCA
Total		213	203
Gender	Male	185 (86.9)	112 (55.2)
	Female	28 (13.1)	91 (44.8)
Age of diagnosis	< 50	106 (49.8)	49 (24.1)
	≥ 50	107 (50.2)	154 (75.9)
Mean (range)		50 (19-85)	57 (41-78)
Region	Country	82 (38.5)	112 (55.2)
	Urban	131 (61.5)	91 (44.8)
AFP	< 400	134 (62.9)	-
	≥ 400	79 (37.1)	-
HBsAg	+	166 (77.9)	140 (69.0)
	-	47 (22.1)	63 (31.0)
Tumor size	< 5 cm	92 (43.2)	98 (48.3)
	≥ 5 cm	121 (56.8)	105 (51.7)
Stage	pT1	9 (4.2)	14 (6.9)
	pT2	73 (34.3)	105 (51.7)
	pT3	24 (11.3)	21 (10.3)
	pT4	107 (50.2)	63 (31.0)
Nodal status	N0	190 (89.2)	182 (89.7)
	N1	23 (10.8)	21 (10.3)
Metastasis	M0	188 (88.2)	182 (89.7)
	M1	25 (11.8)	21 (10.3)
UICC stage at diagnosis	I	16 (7.5)	14 (6.9)
	II	106 (49.8)	98 (48.3)
	III	28 (13.6)	21 (10.3)
	IV	63 (29.6)	70 (34.5)
Tumor grading	I	79 (37.1)	75 (36.9)
	II	76 (35.7)	72 (35.7)
	III	58 (27.2)	56 (27.6)

Data in parenthesis are percentages except the line of "mean". AFP: α-fetoprotein; CCA: Cholangiocellular carcinoma; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular carcinoma; UICC: Union for International Cancer Control.

endothelium); 1 (low staining intensity in < 10% of tumor cells or ANVE); 2 (low staining intensity in 10%–50% of tumor cells or ANVE, or high staining intensity in ≤ 25% of tumor cells or ANVE); and 3 (low staining intensity in > 50% of tumor cells or ANVE, or high staining intensity in > 25% of tumor cells or ANVE) (Table 2)[11]. The two scores for each section were then averaged to give the final score. A consensus review was performed in case where there was substantial disagreement between the two pathologists.

Statistical analysis

Data were analyzed using SPSS version 25.0 (SPSS, Armonk, NY, United States). $P < 0.05$ was considered statistically significant. Quantitative data were expressed as mean ± standard deviation. The χ^2 test was used to compare categorical variables.

Table 2 Standard for evaluation

Score	Stain intensity	Percent of vessels staining
0	None	0
1	Low	≤ 10%
2 (type 1)	Low	10%-50%
2 (type 2)	High	≤ 25%
3 (type 1)	Low	≥ 50%
3 (type 2)	High	> 25%

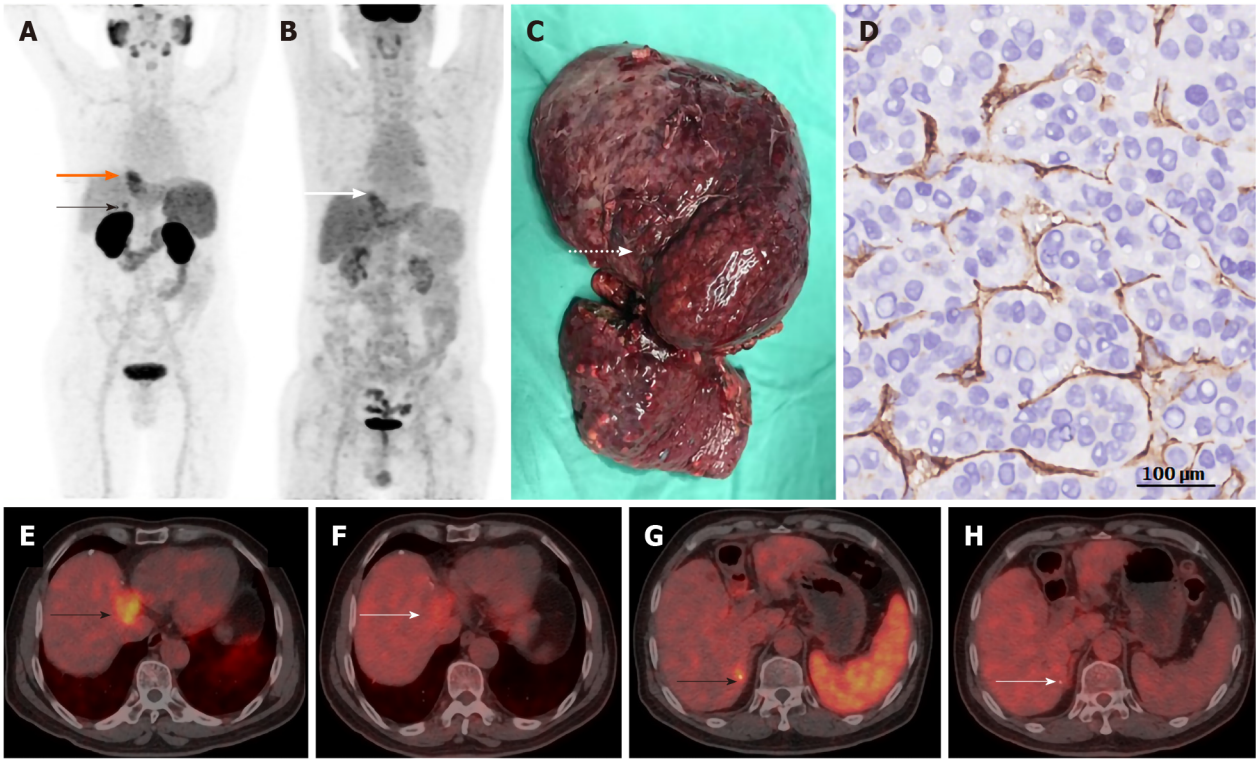

Figure 1 Positron emission tomography imaging study on a 75-year-old man with hepatocellular carcinoma. ^{18}F-Fludeoxyglucose (FDG) and ^{68}Ga-prostate-specific membrane antigen (PSMA) positron emission tomography/computed tomography imaging was performed. A: Maximal intensity projection, ^{68}Ga-PSMA revealed focal uptake [bold orange arrow, standardized uptake value (SUV)max: 7.6; black arrow, SUVmax: 5.7]; B: Maximal intensity projection, ^{18}F-FDG revealed focal uptake (bold white arrow, SUVmax: 4.6), no uptake in right lesion (white arrow); C: Gross section displayed a nodule histologically classified as hepatocellular carcinoma; D: Strong PSMA expression (400 ×, immunohistochemistry, scale bar = 100 μm) was shown in the tumor-associated vascular; E and G: Transaxial fused, ^{68}Ga-PSMA revealed focal uptake (bold black arrow, SUVmax: 7.6; black arrow, SUVmax: 5.7); F and H: Transaxial fused, ^{18}F-FDG revealed focal uptake (bold white arrow, SUVmax: 4.6), no uptake in right lesion (white arrow).

Spearman's correlation coefficient (nonparametric) was used to determine the correlation between IHC scores and clinical variables.

RESULTS

PSMA was expressed in the tumor-associated neovascular endothelium that was also positively stained with the pan-endothelial marker CD31 (Figures 3 and 4). In contrast, blood vessels in the peritumoral normal tissues were exclusively CD31$^+$, indicating that PSMA is a specific marker for the tumor-associated neovasculature. The percentage of PSMA$^+$ cases in HCC (185/213, 86.8%) and CCA (161/203, 79.3%) was 13- and 12-fold higher, respectively, than that in liver cirrhosis (2/30, 6.6%) ($P < 0.0001$, Table 3), while the percentage of PSMA$^+$ cases in HCC was significantly higher than that in CCA (86.8% *vs* 79.3%, $P = 0.001$). There were more sections with a score of 3 for PSMA expression in HCC (89/213, 41.8%) than in CCA (35/203, 17.2%, $P = 0.001$).

Table 3 Cells and tumor-associated neovascular endothelial cells of liver cancers compared with liver cirrhosis

		Number	PSMA expression score, *n*				Positive staining, *n* (%)
			0	1	2	3	
HCC	Cells	213	187	8	16	2	26 (12.2)
	NECs	213	29	31	64	89	184 (86.4)
	Total	213	28	32	64	89	185 (86.8)
CCA	Cells	203	196	0	7	0	7 (3.4)
	NECs	203	42	42	84	35	161 (79.3)
	Total	203	42	42	84	35	161 (79.3)
Cirrhosis	Cells	30	28	2	0	0	2 (6.6)

CCA: Cholangiocellular carcinoma; HCC: Hepatocellular carcinoma; NECs: Neovascular endothelial cells.

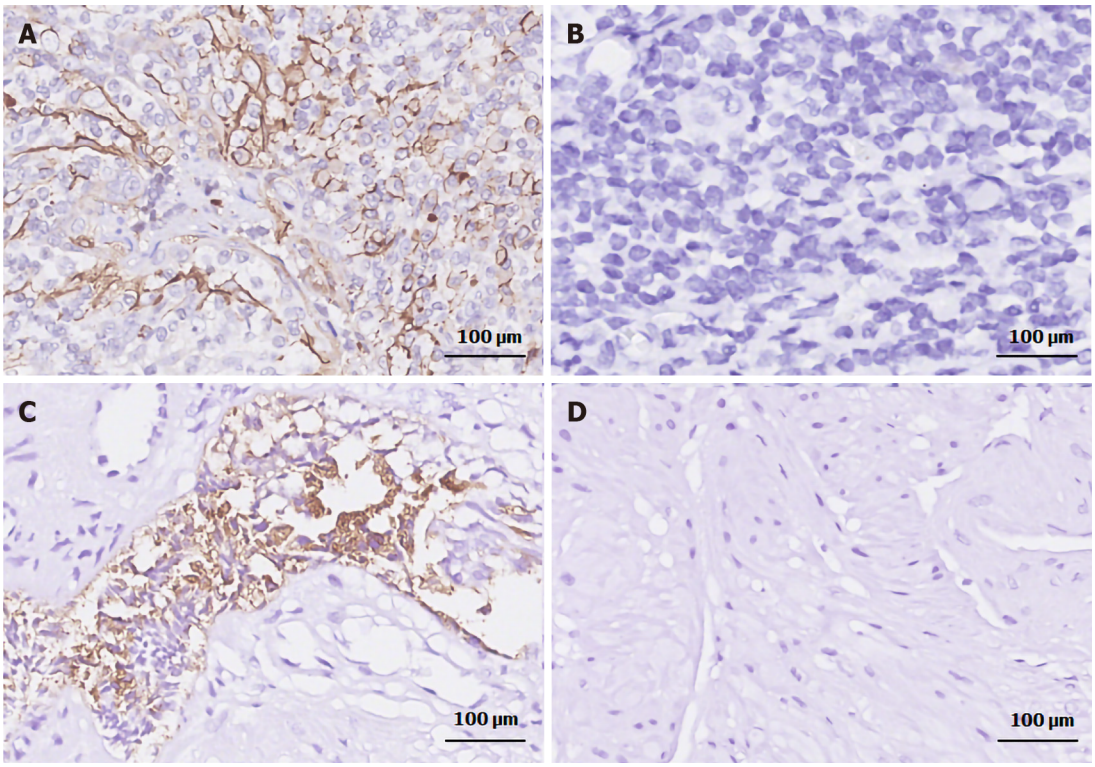

Figure 2 CD31 staining and prostate-specific membrane antigen staining. A: Positive control, CD31 staining in human tonsils (400 ×, scale bar = 100 μm); B: Negative control, CD31 staining in human tonsils (400 ×, scale bar = 100 μm); C: Anti-prostate-specific membrane antigen (PSMA) positive control, PSMA staining in human prostate cancer tissues (400 ×); D: Anti-PSMA negative control, PSMA staining in human prostate cancer tissues (400 ×).

PSMA expression correlated positively with the stage and grade of HCC and CCA. In both liver cancer subtypes, stages III–V disease had more PSMA$^+$ cases than stage I and II had, while high staining intensity of PSMA was more frequently observed in liver cancers of high grade and advanced stage. There was no significant association of PSMA expression with sex, age, region, AFP, hepatitis B surface antigen (HBsAg), or tumor size.

IHC of PSMA expression in HCC

Neovascular expression of PSMA was observed in 184/213 (86.4%) HCC cases, while no PSMA staining was found in normal vascular endothelial cells or peritumoral normal tissues. Among the 184 cases with PSMA$^+$ neovasculature, 31 (14.6%) had an expression score of 1, 64 (30.0%) a score of 2, and 89 (41.8%) a score of 3. In comparison, only 26/213 HCC cases had PSMA$^+$ tumor cells, with most of the staining in the cytoplasm and cell membrane. The PSMA staining score was 1 in eight (3.7%)

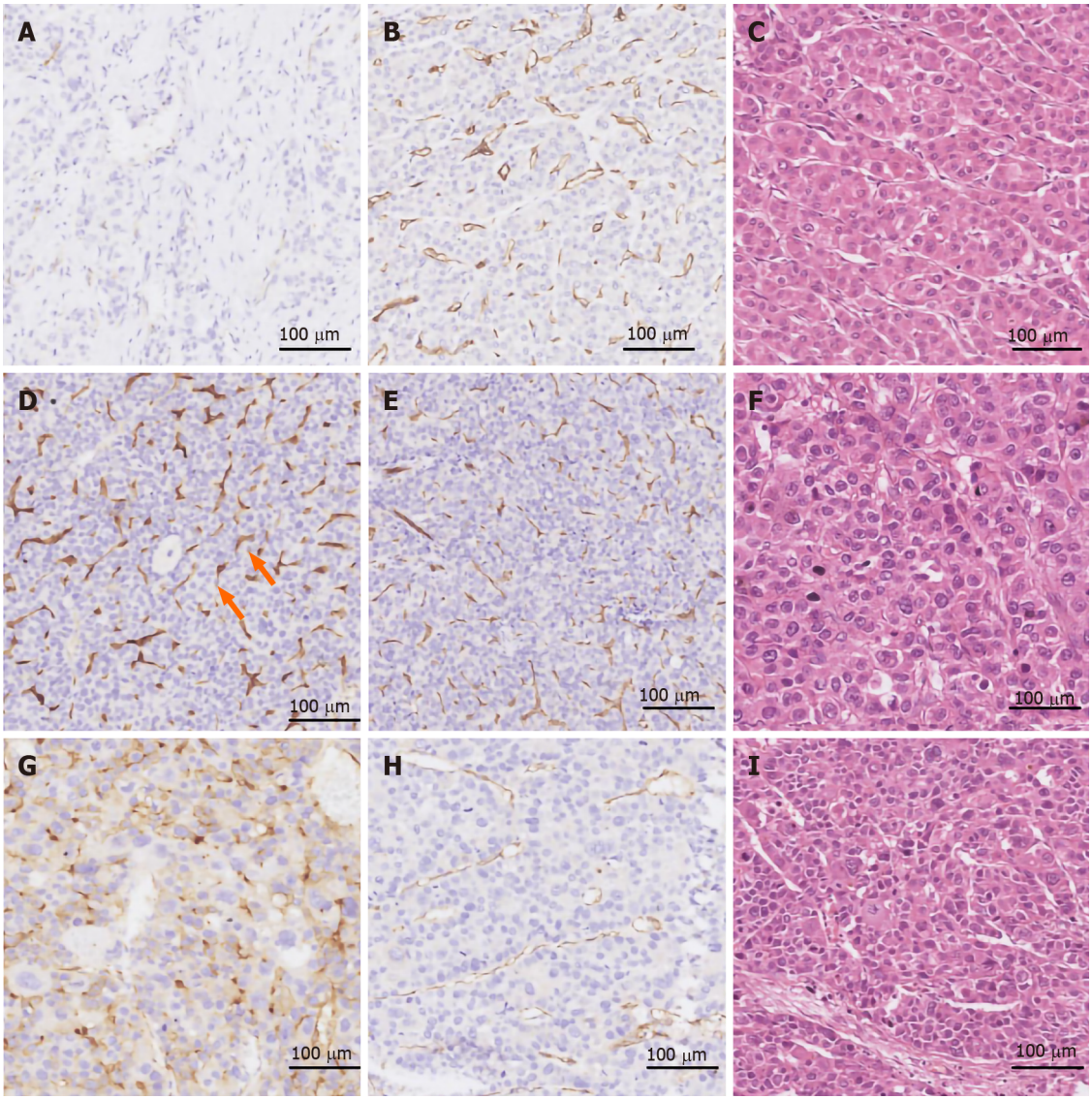

Figure 3 Prostate-specific membrane antigen staining in representative tissues samples of hepatocellular carcinoma with magnification of 400 ×, scale bar = 100 μm. A: Weak prostate-specific membrane antigen (PSMA) staining (score = 1); B, E and H: The corresponding CD31 staining; C, F and I: The corresponding hematoxylin and eosin staining; D: Strong staining (score = 3); D and E: Vessel-like structures within the tumor (bold orange arrow) showed only PSMA staining but no CD31, D and E were from adjacent slides; G: Blood vessel staining and weak staining of cellular elements (score = 3).

cases, 2 in 16 (7.5%) cases, and 3 in two (0.9%) cases (Table 3 and Figure 3). Among these 26 cases, one case showed PSMA staining exclusively in tumor cells, while the remaining 25 cases had PSMA staining in both tumor cells and neovasculature. Furthermore, in 3/213 (1.4%) cases, positive PSMA staining of tumor cells was not accompanied by nearby CD31 expression, which may be attributed to tumor necrosis. In 2/213 cases, the vessel-like structures within the tumor compartment were exclusively stained with PSMA rather than CD31 (score of 3, Figure 3D and E).

PSMA expression correlated positively with stage (Spearman r = 0.226, P = 0.001) and grade (Spearman r = 0.224, P = 0.004) of HCC. Eighty-seven of 91 (95.5%) stage III and IV HCC cases were PSMA$^+$, which was significantly higher than stage I and II HCC (97/122, 79.5%, P = 0.001). There was a higher positive rate for PSMA expression in the neovasculature of grade III (high) HCCs (57/58, 98.2%) than in those of grade II (intermediate, 65/76, 86.5%) or grade I (low, 62/79, 78.4%, P = 0.004) HCC cases. There was no significant association of PSMA expression with sex, age, region, alpha fetoprotein (AFP), HBsAg, or tumor size (Table 4).

PSMA expression by IHC in CCA

Variable levels of PSMA expression were found in tumor neovasculature but in neither normal liver tissue nor peritumoral tissue (Table 3 and Figure 4). One hundred and sixty-one (79.3%) of 203 primary CCA cases were PSMA$^+$ in the tumor neovasculature,

Table 4 Expression of prostate-specific membrane antigen in neovascularization of hepatocellular carcinoma and its relationship with clinicopathological parameters

Clinicopathological parameters	No. of cases	Tumor PSMA-positive, *n*				*P* value
		0	1	2	3	
Gender						
Male	185	25	27	52	81	0.912
Female	28	4	4	12	8	
Age of diagnosis						
< 50	106	12	14	29	51	0.331
≥ 50	107	17	17	35	38	
Mean (range)	50 (19-85)					
Region						
Urban	131	15	16	44	56	0.080
Country	82	14	15	20	33	
AFP						
< 400	134	21	18	38	57	0.254
≥ 400	79	8	13	26	32	
HBsAg						
+	166	24	23	49	70	0.990
-	47	5	8	15	19	
Tumor size						
< 5 cm	92	14	10	23	45	0.552
≥ 5 cm	121	15	21	41	44	
Stage						
pT1	9	1	2	4	2	0.812
pT2	73	19	15	18	54	
pT3	24	2	4	6	12	
pT4	107	7	10	36	21	
Nodal status						
N0	190	27	24	58	82	0.466
N1	23	2	7	6	7	
Metastasis						
M0	188	23	31	57	77	0.136
M1	25	6	0	7	12	
UICC stage at diagnosis						
I-II	122	25	17	37	43	0.001[a],*r* = 0.226
III-IV	91	4	14	27	46	
Tumor grading						
I	79	17	8	17	37	0.004[a],*r* = 0.224
II	76	11	7	25	33	
III	58	1	16	22	19	
All case	213	29	31	64	89	

[a]*P* < 0.01. AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; PSMA: Prostate-specific membrane antigen; *r*: Spearman r; UICC: Union for International Cancer Control.

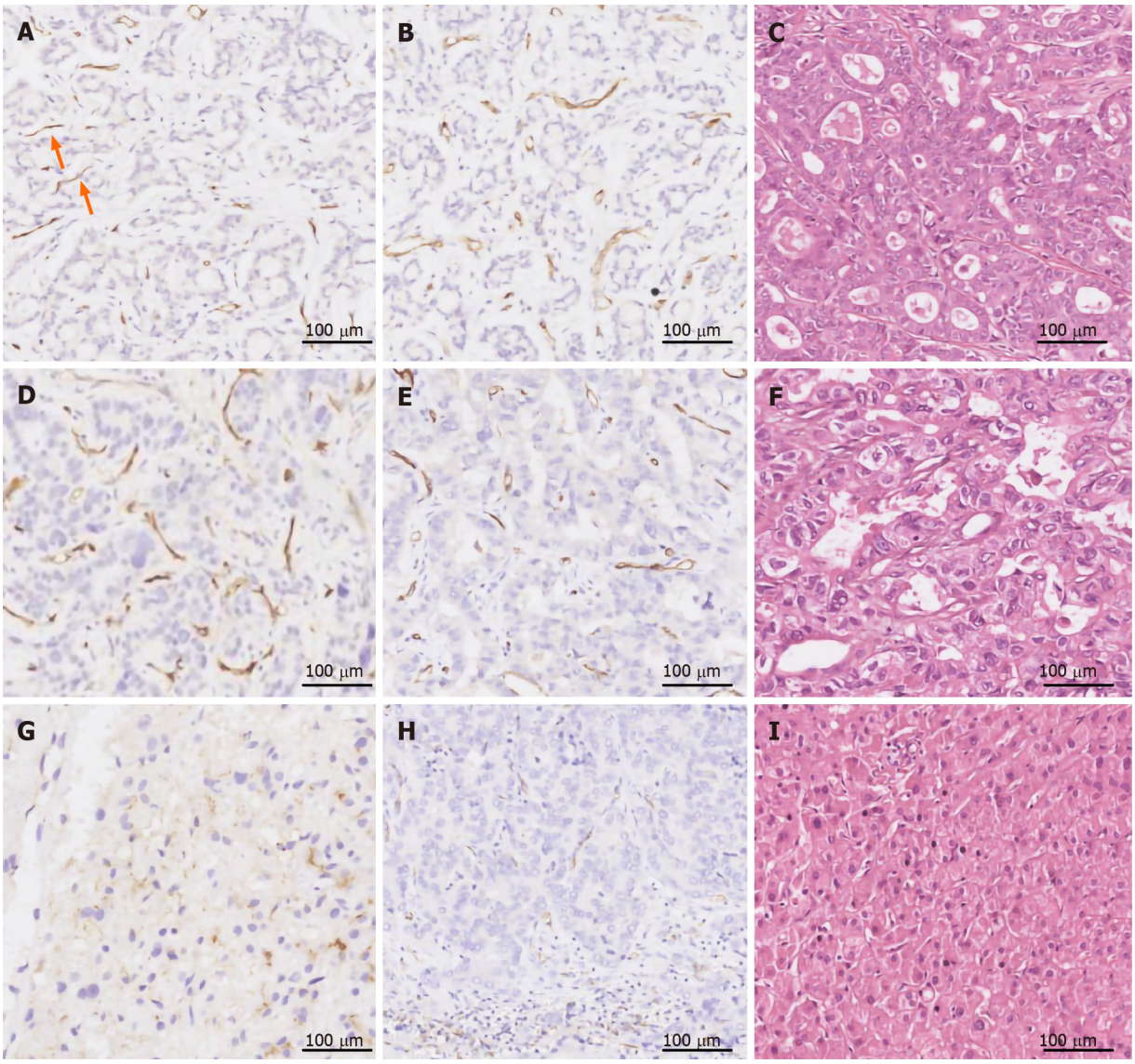

Figure 4 Prostate-specific membrane antigen staining in representative tissues samples of cholangiocarcinoma with magnification of 400 ×, scale bar = 100 μm. A: Weak prostate-specific membrane antigen (PSMA) staining (score = 1); A and B: Vessel-like structures within the tumor (bold orange arrow) showed staining exclusively for PSMA with no CD31 staining, A and B were from adjacent slides; B, E and H: The corresponding CD31 staining; C, F, and I: The corresponding hematoxylin and eosin staining; D: Strong staining (score = 3); G: Blood vessel staining and weak staining of cellular elements (score = 1).

among which, 42 cases (20.7%) had an expression score of 1, 84 (41.4%) a score of 2, and 35 (17.2%) a score of 3 (Table 3 and Figure 4). Seven (3.4%) cases had PSMA staining in both tumor cells (cytoplasm and cell membrane) and tumor-associated neovasculature endothelium, with an expression score of 2. Like HCC, one CCA case exhibited vessel-like structures within the tumor compartment that was weakly stained with PSMA (score = 1) but negative with CD31 staining (Figure 4A and B).

PSMA expression correlated positively with the stage (Spearman r = 0.211, P = 0.002) and grade (Spearman r = 0.253, P = 0.001) of CCA. Positive staining of PSMA was more frequent in stage III and IV CCAs (81/91, 89.0%) than in stage I and II CCA (80/112, 71.4%, P = 0.002). There was a higher rate of positive staining for PMSA in the tumor neovasculature of grade III (high) CCA cases (53/56, 94.6) compared to that of grade II (intermediated, 57/72, 79.0%) or grade I (low, 51/75, 68.0, P = 0.001). There was no significant correlation between PSMA expression and other clinicopathological features of CCA patients (Table 5).

Table 5 Expression of prostate-specific membrane antigen in neovascularization of cholangiocellular carcinoma and its relationship with clinicopathological parameters

Clinicopathological parameters	No. of cases	Tumor PSMA-positive, *n*				*P* value
		0	1	2	3	
Gender						
Male	112	24	13	46	29	0.773
Female	91	18	29	38	6	
Age of diagnosis						
< 50	49	8	22	6	13	0.387
≥ 50	154	34	20	78	22	
Mean (range)	57 (41-78)					
Region						
Urban	91	16	23	47	5	0.325
Country	112	26	19	37	30	
HBsAg						
+	140	29	26	69	16	0.990
-	63	13	16	15	19	
Tumor size						
< 5 cm	98	23	27	30	18	0.178
≥ 5 cm	105	19	15	54	17	
Stage						
pT1	14	5	3	4	2	0.293
pT2	105	23	21	49	12	
pT3	21	5	5	7	4	
pT4	63	9	13	24	17	
Nodal status						
N0	182	36	39	74	33	0.346
N1	21	6	3	10	2	
Metastasis						
M0	182	37	40	76	29	0.709
M1	21	5	2	8	6	
UICC stage at diagnosis						
I-II	112	32	26	38	16	0.002[a], r = 0.211
III-IV	91	10	16	46	19	
Tumor grading						
I	75	24	18	28	5	0.001[a], r = 0.253
II	72	15	18	35	6	
III	56	3	6	21	5	
All case	203	42	42	84	35	

[a]P < 0.01. HBsAg: Hepatitis B surface antigen; PSMA: Prostate-specific membrane antigen; r: Spearman r; UICC: Union for International Cancer Control.

PSMA expression by IHC in liver cirrhosis

$CD31^+$ blood vessels were observed in all 30 liver cirrhosis specimens (Figure 5). However, only two of 30 specimens showed weak PSMA staining in the cytoplasm and cell membrane of liver cells (score = 1). The remaining 28 specimens were $PSMA^-$ in either hepatocytes or vascular endothelium.

DISCUSSION

HCC is the fourth most common malignancy and the third leading cause of tumor-related death in China, accounting for 85%-90% of all primary liver cancer cases[1]. Early radical intervention or effective management at late stage are both important strategies to reduce the death toll from HCC.

PSMA is a type II transmembrane glycoprotein that has attracted extensive attention due to its specific and high expression in prostate cancer cells. PSMA was first identified by Holmes *et al*[7] from a crude membrane extract of an androgen-dependent prostate cancer cell line LNCaP[7]. Other than tumor tissue, PSMA is also highly expressed in pancreatic islets and skeletal muscle, moderately expressed in brain and ganglia of gastrointestinal tract, and weakly expressed in prostate, endometrial glands, kidney tubules, and urinary bladder. No PSMA expression was observed in the liver, spleen, or other tissues[12]. In addition to prostate cancer cells, PSMA has previously been detected in the tumor-associated neovasculature of solid tumors including HCC[9-26]. Notably, PSMA is absent in blood vessels of normal tissue due to the lack of PSMA transcription enhancement regions[28,29].

HCC is a highly vascularized tumor that is characterized by early angiogenesis. The hepatic artery is the main route to supply oxygen and nutrients to HCC, therefore making antiangiogenic therapy promising for HCC. In contrast, PSMA facilitates the invasion of endothelial cells during angiogenic sprouting and thereby supports tumor growth through provision of oxygen and nutrients[29,30]. As a result, targeted therapy against PSMA-expressing neovasculature represents a feasible option in treating rapidly growing solid tumors. Recently, several PSMA-targeted PET imaging studies reported high uptake of radiotracers in the tumor region of HCC, CCA, and CHC[20-22]. Kuyumcu *et al*[31] studied ^{68}Ga-PSMA PET imaging in 19 patients with liver cancer and found tumor uptake of radiotracers in 16 patients[31]. A multi-center phase II trial found that a PSMA-targeted therapy using an antiangiogenic drug mipsagargin led to long-term stable disease in patients with advanced liver cancer[32]. Magnetic resonance imaging after the mipsagargin treatment revealed a decrease in blood flow in liver lesions, confirming that PSMA plays an important role in liver cancer progression[32]. Jiao *et al*[25] found that PSMA was specifically expressed in the vasculature in 76 of 103 (74%) HCC specimens[25]. However, PSMA expression in liver cancer subtypes other than HCC remains to be elucidated.

Here, for the first time, we demonstrated that PSMA was expressed in the tumor-associated neovasculature of most HCC (86.8%) and CCA (79.3%) cases in a large sample set. PSMA expression was restricted to the neovasculature of HCC and CCA, while normal liver and peritumoral tissues were largely $PSMA^-$. A few vessel-like structures in the tumor compartment was $PSMA^+$ but $CD31^-$, suggesting that PSMA is a useful biomarker for early-stage tumor-associated angiogenesis. This temporal mismatch between PSMA and CD31 underscores the role of PSMA in the invasion of endothelial cells. It is worth mentioning that HCC (86.8%) exhibited a higher positive rate of PSMA staining than CCA (79.3%) did and that the HCC cases had more 3-score PSMA staining than CCA had (89/213, 41.7% *vs* 35/203, 17.2%). Therefore, PSMA could provide better diagnostic power in HCC than in CCA and functions as a valuable therapeutic target in HCC.

In some HCC and CCA cases, PSMA staining was observed in the cytoplasm and cell membrane of tumor cells, albeit with lower staining intensity than in tumor-associated neovasculature. Similarly, Nomura *et al*[10] found that < 2% of tumor cells were stained with PSMA in grade II and III glioma[10]. In contrast, Kesler *et al*[24] recently reported that three out of five HCC specimens had intense PSMA staining in intratumoral microvessels[24]. However, they did not observe any PSMA staining in the epithelial tumor cells. Such discrepancies in terms of PSMA expression can be attributed to the difference in sample size and biopsy locations.

Cirrhosis caused by viral hepatitis, especially type B and C, is the leading risk factor for HCC. The regenerated nodules of early-stage HCC are often indistinguishable from the accompanying cirrhosis, which makes ablative therapy more challenging. In our study, only two (6.7%) cases of liver cirrhosis showed weak PSMA staining in tumor

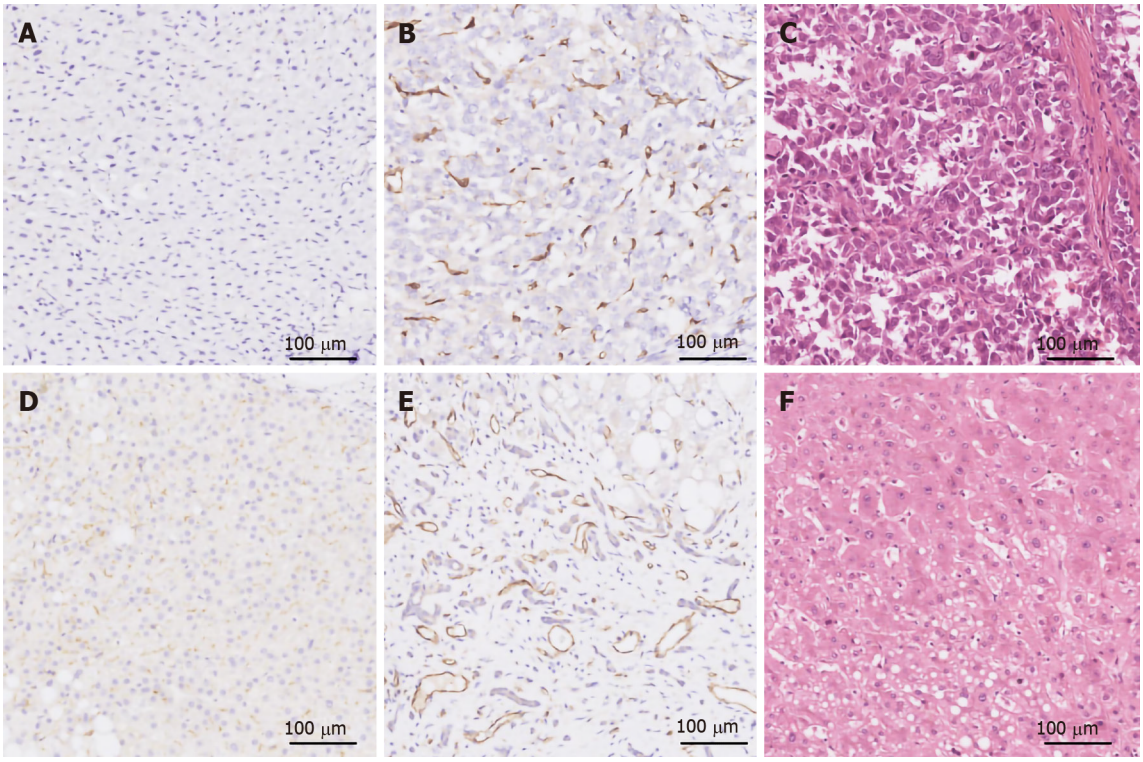

Figure 5 Prostate-specific membrane antigen staining in liver cirrhosis with magnification of 400 ×, scale bar = 100 μm. A: Liver cirrhosis showing no prostate-specific membrane antigen (PSMA) staining in blood vessels and hepatocytes (0 point); B and E: The corresponding CD31 staining; C and F: The corresponding hematoxylin and eosin staining; D: Liver cirrhosis showing no PSMA blood vessel staining with a score of 0 and light staining of cellular elements with a score of 1.

cell cytoplasm and cell membrane, with an expression score of 1. In contrast, the positive staining of PSMA was more frequent and with higher intensity in HCC and CCA. Therefore, our study proves that PSMA could be a useful biomarker to distinguish HCC from liver cirrhosis. Accordingly, PSMA-targeted PET imaging can potentially pinpoint the regenerated nodules of HCC.

In this study, PSMA expression correlated positively with the stage and grade of HCC and CCA, and stage III and IV disease tended to have higher positive rate of PSMA than stage I and II diseases. High PSMA expression was more likely to be found in the neovasculature of HCC and CCA with high grade or stage III or IV. There was no significant association of PSMA expression with sex, age, region, AFP, HBsAg, or tumor size in HCC and CCA. Jiao *et al*[25] reported that vascular PSMA expression correlated with tumor stage, tumor differentiation, lymph node metastasis, and Ki67 index[25]. They did not find any significant association between the vascular PSMA expression and age or sex, which was in accordance with our results.

CONCLUSION

PSMA was expressed primarily in the tumor-associated neovascular endothelium of liver cancer. We discovered a potential role of PSMA-targeted imaging in the detection and staging of liver cancer patients, especially those with HCC. The PSMA-targeted imaging may also be useful to distinguish liver cancer from cirrhosis. As a result, PSMA-targeted approaches represent a feasible alternative to current antiangiogenic cancer therapy.

ARTICLE HIGHLIGHTS

Research background

Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein expressed in the neovasculature of various nonprostate malignancies.

Research motivation
PSMA expression in the tumor-associated neovasculature of nonprostate malignancies including liver cancer has been reported, but conclusive evidence of PSMA expression based on the pathological type of liver cancers remains limited.

Research objectives
This retrospective study was performed to study the expression of PSMA in hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and liver cirrhosis.

Research methods
Immunohistochemistry was used to detect PSMA expression in 446 formalin-fixed paraffin-embedded liver biopsy specimens (213 HCC, 203 CCA, and 30 liver cirrhosis).

Research results
PSMA was expressed primarily in the neovascular endothelium associated with tumors. The positive rate of PSMA staining in HCC was significantly higher than that in CCA.

Research conclusions
Neovascular PSMA may be used as a promising marker to differentiate HCC from liver cirrhosis and a prognostic marker for antitumor angiogenesis for HCC.

Research perspectives
Vascular PSMA may be used as a prognostic marker for anti-tumor angiogenesis therapy for HCC.

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ORIGINAL ARTICLE

Observational Study

Oral microbiome and pancreatic cancer

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Abstract

BACKGROUND

Microbiota profiles differ between patients with pancreatic cancer and healthy people, and understanding these differences may help in early detection of pancreatic cancer. Saliva sampling is an easy and cost-effective way to determine microbiota profiles compared to fecal and tissue sample collection.

AIM

To investigate the saliva microbiome distribution in patients with pancreatic adenocarcinoma (PDAC) and the role of oral microbiota profiles in detection and risk prediction of pancreatic cancer.

METHODS

We conducted a prospective study of patients with pancreatic cancer (n = 41) and healthy individuals (n = 69). Bacterial taxa were identified by 16S ribosomal ribonucleic acid gene sequencing, and a linear discriminant analysis effect size algorithm was used to identify differences in taxa. Operational taxonomic unit values of all selected taxa were converted into a normalized Z-score, and logistic

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regressions were used to calculate risk prediction of pancreatic cancer.

RESULTS

Compared with the healthy control group, carriage of *Streptococcus* and *Leptotrichina* (z-score) was associated with a higher risk of PDAC [odds ratio (OR) = 5.344, 95% confidence interval (CI): 1.282-22.282, P = 0.021 and OR = 6.886, 95%CI: 1.423-33.337, P = 0.016, respectively]. *Veillonella* and *Neisseria* (z-score) were considered a protective microbe that decreased the risk of PDAC (OR = 0.187, 95%CI: 0.055-0.631, P = 0.007 and OR = 0.309, 95%CI: 0.100-0.952, P = 0.041, respectively). Among the patients with PDAC, patients reporting bloating have a higher abundance of *Porphyromonas* (P = 0.039), *Fusobacterium* (P = 0.024), and *Alloprevotella* (P = 0.041); while patients reporting jaundice had a higher amount of *Prevotella* (P = 0.008); patients reporting dark brown urine had a higher amount of *Veillonella* (P = 0.035). Patients reporting diarrhea had a lower amount of *Neisseria* and *Campylobacter* (P = 0.024 and P = 0.034), and patients reporting vomiting had decreased *Alloprevotella* (P = 0.036).

CONCLUSION

Saliva microbiome was able to distinguish patients with pancreatic cancer and healthy individuals. *Leptotrichia* may be specific for patients living in Sichuan Province, southwest China. Symptomatic patients had different bacteria profiles than asymptomatic patients. Combined symptom and microbiome evaluation may help in the early detection of pancreatic cancer.

Key Words: Oral microbiota; Dysbiosis; Pancreatic cancer; Cancer detection; 16s rRNA; High-throughput sequencing

Core Tip: Pancreatic adenocarcinoma (PDAC) patients benefit from early detection. This study analyzed the composition and diversity of saliva microbiota in PDAC patients through 16S ribosomal ribonucleic acid sequencing. Normalized z-score of bacteria abundance associated clinical data were analyzed for PDAC risk prediction. Microbiome abundance differences were found between PDAC patients with symptoms and patients without symptoms. Combined symptom and microbiome evaluation may help in early detection and risk prediction of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer or pancreatic adenocarcinoma (PDAC) is a lethal disease with a 5-year survival rate of about 6%[1,2]. Early detection and diagnosis are essential for effective surgery treatment that improves cancer survival[3,4], yet these remain a great challenge. A variety of diagnostic methods are available. For example, deoxyribonucleic acid (DNA) sequencing for detecting and diagnosing pancreatic cancer are limited in clinical use due to the need for fresh, high-quality specimens, tumor content, and tumor heterogeneity[5,6]. Molecular markers, such as mutant DNA or DNA methylomes, are also limited in clinical use to enhance diagnostic sensitivity or early detection of pancreatic cancer recurrence[7,8]. Biomarker Ca19-9 has been commonly used for diagnosis and prognosis of pancreatic cancer with diagnostic sensitivity of 0.78 and specificity of 0.77, but this biomarker test has limited sensitivity among patients with jaundice, pancreatitis, enteritis, and elevated blood glucose, since such patients usually have elevated Ca19-9 concentrations[9-11]. In addition, 7%-10% Lewis (a-/b-) populations could not express Ca19-9[12].

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The oral or fecal microbiota profile of gastrointestinal and colorectal cancer, oropharyngeal cancer, liver cancer, and lung cancer may be a novel and potential diagnostic biomarker[13-19]. Accumulated studies have revealed that oral and gastrointestinal microbiomes differ in abundance in patients with pancreatic cancer compared with healthy individuals[20-23]. Cancer risk increases with carriage of *Porphyromonas gingivalis*[21], *Actinobacillus actinomycetemcomitans*[21], and *Alloprevotella*[21], while *Fusobacterium*[21,24], *Leptotrichia*[21,25,26], *Neisseria elongate*[21,23], and *Streptococcus mitis*[21,23] might be a protective factor for having pancreatic cancer. However, Olson *et al*[22] did not find significant differences in the diversity of the oral microbiome among PDAC patients (n = 40), intraductal papillary mucinous neoplasms (IPMNs) (n = 39), and healthy participants (n = 58) in the United States[22]. The conflicting findings in the prior studies may be due to the differences in methodological approach and sample collection. For example, some studies performed real-time quantitative polymerase chain reaction (PCR) for validation of bacterial candidates[23], and some sequenced the microbiota profile in samples of tongue coating[20] or oral wash samples[21]. Tongue coating change is a major often-used approach of tongue diagnosis in traditional Chinese medicine, but tongue coating can only capture partial oral microbiota[27,28]. The oral wash method is more complicated and relatively expensive.

Oral cavity contains nearly 619 taxa in 13 phyla (Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Euryarchaeota, Spirochaetes, SR1, Synergistes, Tenericutes, and TM7), and 68% of these bacteria are uncultivated phylotypes[29,30]. Advanced genomic sequencing for human oral microbiome distribution makes it possible to measure the proportions of bacterial species without relying on traditional culture methods[31-33]. Saliva has been found to contain broad spectrum of bacteria with easy sampling method and is relatively cost-effective. Although there are some studies on oral flora and pancreatic cancer in non-Chinese population, the impact of geographical and medical factors, such as race and ethnicity, different dietary habits, antibiotic use, and cancer, may make the oral microbial profile differ among people from different geographic locations. In addition, there are few studies on oral saliva flora and pancreatic cancer in China. Thus, the purpose of our study was to: (1) Determine the saliva microbiome distribution of pancreatic cancer (including resectable PDAC and unresectable PDAC) among Chinese population using 16S rRNA sequencing; and (2) Select proper and specific microbiota for PDAC detecting.

MATERIALS AND METHODS

Ethical consideration

The Institutional Review Board of the West China Hospital, Sichuan University approved this prospective study. All participants signed written informed consent.

Research design and participants

This was a prospective study. We consecutively recruited 80 patients who were over age 18 years and suspected to have pancreatic tumor prior to biopsy or surgery. Histopathological results confirmed 45 patients with primary PDAC and 35 patients with non-cancer pancreatic tumors, including 9 IPMN, 11 pancreatic serous cystadenoma, 5 solid pseudopapillary neoplasm, and 10 neuroendocrine tumors. We also recruited 69 healthy participants from the community as a comparison group. Healthy adults had normal liver and renal function, normal cardio-pulmonary function, no history of cancer, and no viral infection. Participants were excluded if they had: (1) A history of prior malignancy and chemotherapy or radiotherapy; (2) Metastatic PDAC or PDAC with other cancer; (3) A history of viral infection (*i.e.* hepatitis B virus, hepatitis C virus, human immunodeficiency virus); (4) Use of antibiotics (including oral, intravenous, or intramuscular) and probiotics within 4 wk prior to enrollment; and (5) Use of corticosteroids (nasal or inhaled) or other immunosuppressants. In addition, we excluded participants with insufficient saliva sample (n = 12) for sequencing analysis and patients with non-cancer pancreatic tumors (n = 35).

Demographic and clinical phenotype

The demographic information collected included age, gender, body mass index (BMI), smoking history, alcohol consumption, dietary habit, and chronic diseases (hypertension and type II diabetes). Clinical information was also collected to include cancer site, surgery type, and cancer stages using the American Joint Commission on

Cancer, seventh edition staging manual[34].

Symptom phenotype

Since there is no measure or checklist for symptoms specific to pancreatic cancer, we developed a checklist based on literature review to assess symptoms specific to pancreatic cancer, such as bloating, jaundice, nausea, vomiting, dark brown urine, diarrhea, constipation, pale stools, pruritus, lack of appetite, pain, fatigue, and disturbed sleeping. Patients reported the presence and absence of symptoms by checking "Yes" or "No."

Saliva sample collection

Before the patients had surgery to confirm pancreatic cancer diagnosis, saliva samples were collected by trained professionals (Wang X and Li GQ). All the participants were instructed to not eat and drink for 0.5 h prior to saliva sample collection. Participants were also instructed not to brush their teeth at least 8 h prior to saliva sample collection, since brushing teeth may remove part of the oral flora. Participants were asked to rinse their mouths to remove debris from the oral cavity before saliva collection. To ensure all sample collection was at a similar time period in a day, we collected patient samples around 4:00 pm on the day of admission prior to biopsy or surgery for cancer diagnosis. Healthy subjects' saliva samples were also collected around 4:00 pm in the afternoon. About 3 mL saliva was collected in a sterile tube after it accumulated on the mouth floor. The fresh samples were placed on ice and transported to the laboratory. Samples were divided into 1.5 mL aliquots and stored immediately at -80 °C.

Genome DNA extraction

We used the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, United States) to extract bacterial genomic DNA from saliva samples. DNA concentration and purity was quantified by Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Wilmington, DE, United States) and agarose gel electrophoresis. Genome DNA with strong smear or with concentration lower than 5 μg/mL (by Qubit) was excluded for library construction.

16S rRNA gene sequencing

The third and fourth hypervariable regions (V3-V4) of the 16S rRNA gene of bacteria were amplified by PCR with a domain-specific primer: 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'- GACTACHVGGGTATCTA ATCC-3'). PCR reactions were performed with a 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, United States), 0.2 μmol/L of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s. Finally, samples were incubated at 72 °C for 5 min. The library quality was assessed by Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States). Sequencing was performed on an Illumina Novaseq6000 sequencing platform (Illumina, San Diego, CA, United States), and 250 bp paired-end reads were generated.

Statistical analysis

Phenotype data analysis: Statistical analyses were performed using SPSS (v23.0, SAGE IBM, Armonk, NY, United States). Continuous variables (age and BMI) were estimated as average ± standard error, and categorical variables were analyzed in terms of frequencies and percentages. Chi square analysis and Fisher's exact tests were used for categorical variables; *t*-test and Mann-Whitney *U* test were used for continuous variables. All tests were two-sided, and P values < 0.05 were considered statistically significant with 95% confidence interval (CI).

Microbiome data analysis

Profile and quality assurance: Raw sequences were denoised *via* FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/)[35]. Quality filtering was performed on raw sequences using QIIME quality control process (v1.9.1_http://qiime.org/index.html) and then high quality clean tags were obtained[36]. Tags were compared with gold database (http://drive5.com/uchime/uchime_download.html), and chimeras were removed with the UCHIME algorithm (v11.0, http://www.drive5.com/usearch/manual/uchime_algo.html)[37]. Effective Tags were finally obtained. All effective sequence analysis was performed by Uparse software (v7.0.1001,

http://drive5.com/uparse/)[38]. The optimized, high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity.

Microbiome diversity: According to the results of OTUs clustering analysis and the research requirements, the Venn diagram was constructed to illustrate the number of unique and shared species in saliva samples between PDAC and healthy groups. The Venn diagram was made using R program (Package_VennDiagram). We applied alpha diversity to analyze complexity of species diversity for a sample. Four indices were used: "Chao1" and "Abundance-based coverage estimator (ACE)" estimate the species abundance; "Shannon index " and "Simpson" account for the richness and evenness. The value of Simpson index was calculated as Simpson's index of diversity 1-D. Thus, higher Shannon and Simpson indices mean higher species diversity. All indices were calculated with QIIME (v1.9.1) and R software (V2.15.3, Auckland, New Zealand). We compared four indices between PDAC and healthy control group using Mann-Whitney U test. Mann-Whitney U test was used to compare the alpha diversity indices between groups of resectable PDAC (rPDAC) and unresectable PDAC (unrPDAC). The bacterial taxonomic compositions were evaluated with a linear discriminant analysis effect size algorithm (https://huttenhower.sph.harvard.edu/). $P < 0.05$ and an LDA score ≥ 2.0 were recognized as significant in Kruskal–Wallis and pairwise Wilcoxon evaluation, respectively.

Abundance of bacteria and symptom: We used Wilcoxon rank-sum test to compare the abundance of bacteria (top 10 positively expressed flora) in PDAC patients with and without typical symptoms of PDAC, including bloating, jaundice, nausea, vomiting, dark brown urine, diarrhea, constipation, pale stools, pruritus, lack of appetite, pain, fatigue, and disturbed sleeping.

Risk prediction for PDAC

Logistic regressions were used to explore the association of significant taxa with clinical covariates (age, BMI, smoking status, alcohol consumption status, history of blood hypertension, and eating habits). To avoid the occurrence of false negative diagnosis, we focused on the top 20 species (OTUs abundance) and the flora associated with PDAC that has been reported[20-23]. Finally, *Streptococcus, Prevotella, Porphyromonas, Neisseria, Veillonella, Leptotrichia, Lactobacillus, Actinomyces, Haemophilus, Rothia*, and *Fusobacterium* were selected for analysis. To make the values comparable, we converted the OTU values of all selected taxa into a normalized z-score. The tetranucleotide-derived z-score, superior to (G + C) content differences, was calculated according to the previous methods[39,40]. Odds ratio with 95%CIs were calculated.

RESULTS

Phenotypic characteristics

Between November 2017 and December 2018, a total of 157 participants were enrolled in this study; four PDAC patients and eight healthy participants were eventually excluded due to the insufficient saliva sample for sequencing analysis. A final sample of 110 included patients in PDAC ($n = 41$) and healthy individuals ($n = 69$). Table 1 shows the demographic characteristics of PDAC patients and healthy participants. Compared with the healthy group, the PDAC had lower BMIs (22.76 *vs* 24.44, $P < 0.0001$). As for eating habits, more PDAC patients (61%) preferred oily and fatty foods compared to the healthy control group ($P = 0.002$). More healthy control participants had hypertension ($P = 0.006$). Among the 41 patients with PDAC, 31 (76%) had head pancreatic cancer, and 20 (49%) patients had resectable pancreatic cancer.

Bacteria profile

Alpha-diversity analysis of the study participant groups: From 110 samples, we filtered 6356399 qualified reads. We randomly chose 2235200 reads (110 samples multiplied by 20320 reads/sample, the minimum number of reads/sample). Finally, we obtained 1975 OTUs for further analysis. A Venn diagram (Figure 1) shows the details of the OTUs at 97% identity for PDAC patients and healthy participants. The two groups had 690 shared species, 231 unique species for PDAC patient, and 389 for healthy control group. As Table 2 shows, compared with the healthy group, the PDAC group had significantly increased microbial abundance estimated by the Chao1 index and ACE index while decreased microbial diversity estimated by Shannon and Simpson indices ($P < 0.0001$). Patients with rPDAC had lower bacteria abundance and

Table 1 Demographic characteristics of participants

Variables	PDAC group, *n* = 41	Healthy control group, *n* = 69	*P* value
Age, average ± standard error	61.17 ± 1.79	64.64 ± 1.04	0.098
Gender, *n* (%)			
Male	24 (59)	50 (72)	0.132
Female	17 (41)	19 (28)	
BMI, average ± standard error	22.76 ± 0.94	24.44 ± 0.39	< 0.0001
Smoking history, *n* (%)	17 (41)	37 (54)	0.217
Alcohol consumption, *n* (%)	16 (39)	30 (43)	0.647
Dietary habit, *n* (%)			
Oily and fatty food	25 (61)	21 (31)	0.002
Salty food	6 (15)	8 (11)	0.664
Light diet	10 (24)	40 (58)	0.001
Chronic disease, *n* (%)			
Hypertension	1 (2)	15 (22)	0.006
Type II diabetes	2 (5)	6 (9)	0.714
Both	3 (7)	5 (7)	1.000
Loss of weight, *n* (%)	23 (56)	3 (4)	0.0001
Primary cancer site, *n* (%)			
Head	31 (76)	NA	NA
Body and tail	10		
Surgery, *n* (%)			
Pancreaticoduodenectomy	14 (34)	NA	NA
Distal pancreatectomy	6 (15)		
Palliative intervention techniques	21 (51)		
AJCC staging		NA	NA
I-IIB	20 (49)		
III-IV	21		

AJCC: American Joint Commission on Cancer; BMI: Body mass index; PDAC: Pancreatic adenocarcinoma.

Table 2 α-diversity indices of two groups

	PDAC group, *n* = 41	Healthy control group, *n* = 69	*P* value
Shannon	5.14 ± 0.67	5.67 ± 0.51	0.0001
Simpson	0.90 ± 0.08	0.95 ± 0.02	0.0001
Chao1	423.48 ± 55.69	295.00 ± 54.05	0.0001
ACE	424.00 ± 55.72	293.97 ± 50.09	0.0001

PDAC: Pancreatic adenocarcinoma.

diversity than patients with unrPDAC estimated by Chao1, ACE, Shannon indices, and Simpson indices. However, Shannon (P = 0.273), Simpson (P = 0.715), Chao1 (P = 0.159), and ACE (P = 0.137) were not able to distinguish rPDAC and unrPDAC.

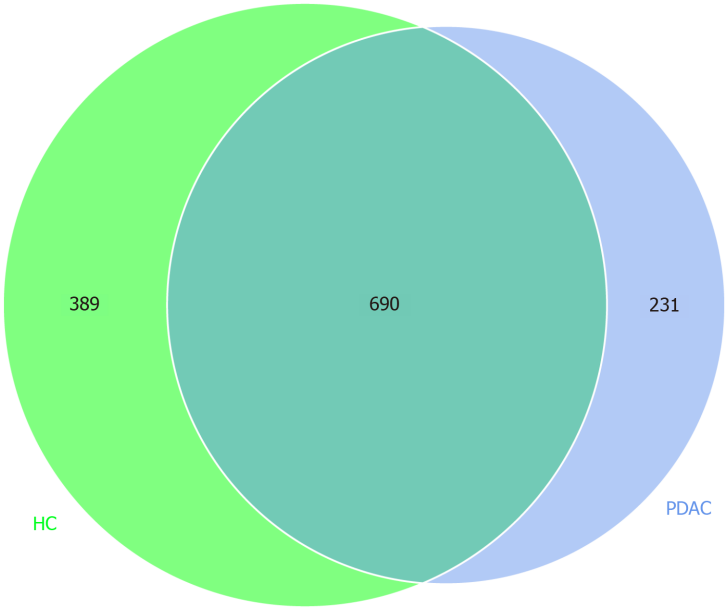

Figure 1 Microbial profiles of two groups. Venn diagram showing shared and unique operational taxonomic units (OTUs) at 97% identity among pancreatic adenocarcinoma (PDAC) group (*n* = 41) and healthy controls (HC) group (*n* = 69). PDAC group is blue; HC group is green. OTUs (690) are shared by two groups. Unique OTUs of 231 and 389 were found in PDAC and HC, respectively.

Bacterial taxonomic alterations in PDAC

We used a linear discriminant analysis effect size algorithm to assess the bacterial taxonomic compositions and differences between PDAC group and healthy control subjects. Compared with the healthy group, PDAC patients were significantly enriched in order_Lactobacillales, class_Bacilli, genus_*Streptococcus*, phylum_Firmicutes, genus_*Actinomyces*, genus_*Rothia*, genus_*Leptotrichia*, genus_*Lactobacillus*, species_*Escherichia_coli*, and order_Enterobacteriales (Figure 2A). Conversely, PDAC patients had significantly reduced abundances of *Selenomonas, Porphyromnas, Prevotella, Capnocytophaga, Alloprevotella, Tannerella*, and *Neisseria* at genus level. We also compared the bacterial distributions between rPDAC and unrPDAC patients. Figure 2B shows that species_*Escherichia coli*, genus_*Peptostreptococcus*, genus_*Asteroleplasma*, and species_*Tannerella forstythia* were more prevalent in the unrPDAC group, whereas we found reduced occurrence of species_*Bacteroides stercoris*, genus_*Megasphaera*, and genus_*Veillonella* (Figure 2).

Microbiome profile and symptoms

Table 3 presented flora abundance differences between the PDAC patients with symptoms and without symptoms. Patient reporting bloating had greater abundance of *Porphyromonas* (660.4 ± 461.0, P = 0.039), *Fusobacteria* (490.0 ± 186.6, P = 0.024), and *Alloprevotella* (155.4 ± 124.1, P = 0.041) compared to those without bloating (412.0 ± 394.3, 361.8 ± 184.4 and 99.3 ± 81.9, respectively). *Prevotella* presented greater abundance in patients without jaundice (669.4 ± 384.3, P = 0.008) compared to those with jaundice (403.2 ± 310.8). *Veillonella* presented greater abundance in patients without dark brown urine (1863.8 ± 1449.2, P = 0.035) compared to those with dark brown urine (1018.6 ± 766.7). *Alloprevotella* presented greater abundance in patients without vomiting (130.3 ± 100.9, P = 0.036) compared to those with vomiting (91.8 ± 134.4), while *Neisseria* presented greater abundance in patients with vomiting (3343.3 ± 1829.9, P = 0.024) compared to those without vomiting (1360.3 ± 1256.6). *Campylobacter* presented greater abundance in patients with diarrhea (130.5 ± 59.7, P = 0.034) compared to those without diarrhea (74.9 ± 87.2).

Logistic regression for microbiota profile

We explored the PDAC risk in relation to selected bacteria abundances (normalized z-score). As shown in Table 4, compared with healthy control group, carriage of *Streptococcus* (OR = 5.344, 95%CI: 1.282-22.282, P = 0.021) and *Leptotrichina* (OR = 6.886, 95%CI: 1.423-33.337, P = 0.016) were associated with a higher risk of PDAC. With each increase of z-score of *Streptococcus* and *Leptotrichina* in PDAC patients, the risk of pancreatic cancer increased by 5.344 odds and 6.886 odds, respectively. Carriage of

Table 3 Flora abundance differences in pancreatic adenocarcinoma patients with symptomatic phenotype

Symptoms	Microbiome	Without symptoms	With symptoms	*P* value
Bloating	*Porphyromonas*	412.0 ± 394.3	660.4 ± 461.0	0.039
	Fusobacteria	361.8 ± 184.4	490.0 ± 186.6	0.024
	Alloprevotella	99.3 ± 81.9	155.4 ± 124.1	0.041
Jaundice	*Prevotella*	669.4 ± 384.3	403.2 ± 310.8	0.008
Dark brown urine	*Veillonella*	1863.8 ± 1449.2	1018.6 ± 766.7	0.035
Vomiting	*Alloprevotella*	130.3 ± 100.9	91.8 ± 134.4	0.036
Diarrhea	*Neisseria*	1360.3 ± 1256.6	3343.3± 1829.9	0.024
	Campylobacter	74.9 ± 87.2	130.5 ± 59.7	0.034

Table 4 Oral bacteria distribution and risk of pancreatic adenocarcinoma

	Odds ratio	95%CI		*P* value
Healthy control group	Base outcome			
PDAC group				
Age	0.956	0.875	1.046	0.327
BMI	0.973	0.708	1.338	0.866
Oily and fatty food	0.759	0.122	4.730	0.768
Streptococcus	5.344	1.282	22.282	0.021
Veillonella	0.187	0.055	0.631	0.007
Neisseria	0.309	0.100	0.952	0.041
Lactobacillus	0.713	0.357	1.425	0.338
Leptotrichia	6.886	1.423	33.337	0.016
Actinomyces	4.515	0.444	45.887	0.203
Haemophilus	1.185	0.513	2.738	0.691
Prevotella	0.673	0.298	1.519	0.341
Porphyromonas	0.294	0.084	1.033	0.056
Rothia	1.257	0.467	3.384	0.650
Fusobacterium	1.006	0.335	3.017	0.576

BMI: Body mass index; CI: Confidence interval; PDAC: Pancreatic adenocarcinoma.

Veillonella and *Neisseria* were protective factors of having PDAC (OR = 0.187, 95%CI: 0.055-0.631, *P* = 0.007 and OR = 0.309, 95%CI: 0.100-0.952, *P* = 0.041, respectively). With each decrease of z-score of *Veillonella* and *Neisseria* in PDAC patients, the risk of pancreatic cancer decreased by 0.187 odds and 0.309 odds, respectively.

DISCUSSION

This prospective study found dysbacteriosis of the oral microbiota existed in patients with PDAC. Fecal bacteria flora has been the main sample method for research on pancreatic cancer[41,42]. Our study used saliva sample method, which is convenient and the quality of sample is easy to control during sample collection. When comparing bacteria profiles from our saliva samples and fecal samples from other research on Chinese PDAC patients[20,42], salivary and intestinal bacteria flora consistently had low Shannon index and high Chao1 index, and *Lactobacillus, Enterobacter*, and *Leptotrichia* at the genus level was significantly increased. This provides supporting evidence that

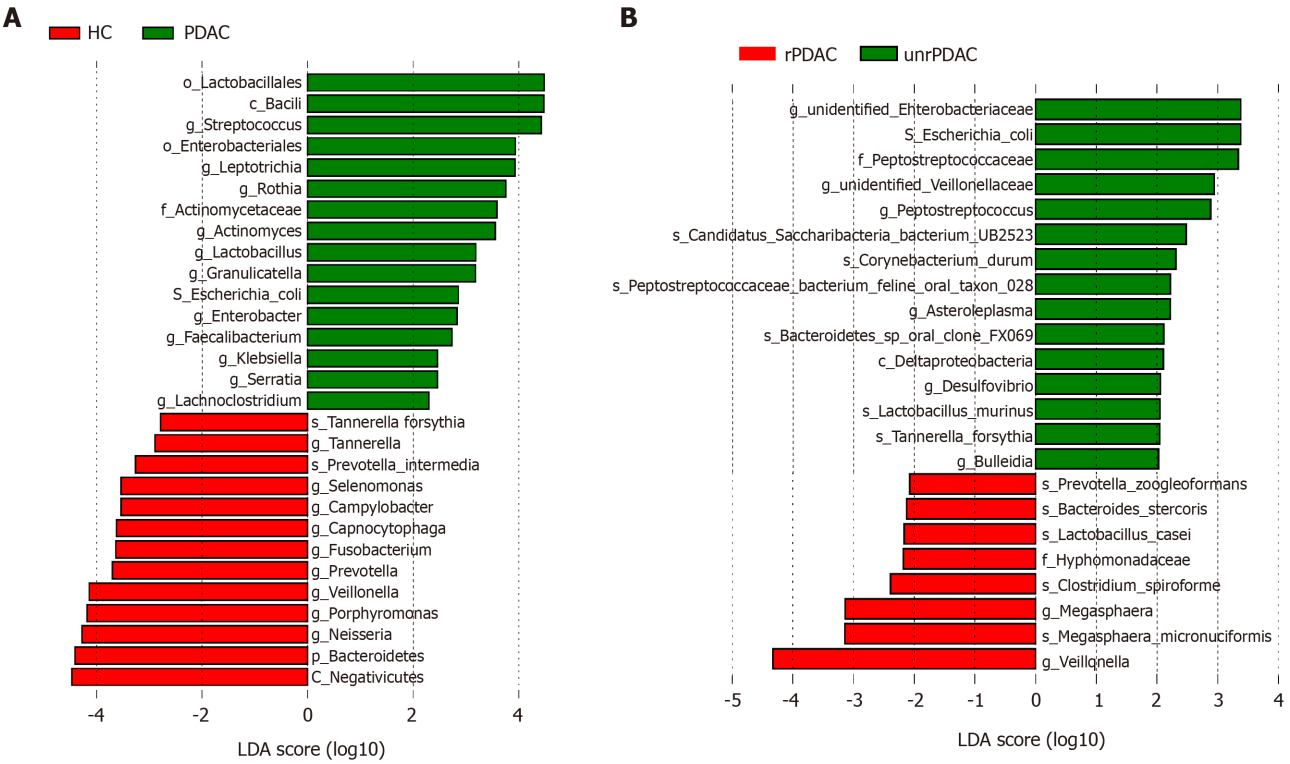

Figure 2 Linear discriminant analysis effect size and latent dirichlet allocation analysis based on operational taxonomic units. A: Shows a list of specific oral bacteria that enable discrimination between pancreatic adenocarcinoma (PDAC) patients and healthy controls (HC). Differences in oral microbial communities between PDAC patients and HC. The horizontal line with red and green denotes the means of the HC and PDAC groups, respectively; B: Resectable PDAC (rPDAC) and unresectable PDAC (unrPDAC). Differences in oral microbial communities between rPDAC group and unrPDAC. The horizontal line with red and green denotes the means of the rPDAC and unrPDAC groups, respectively.

saliva sample method yields similar bacteria flora profiles compared to the fecal sample method, which is very often difficult to collect the samples. Findings of our study also provided additional evidence to confirm that *Neisseria* and *Streptococcaceae* are risk factors for pancreatic cancer[21,23]. Currently, no studies have focused on comparing the advantages and disadvantages of using different sample collection techniques, and studies are necessary to compare the effectiveness of using different sample collection techniques, such as saliva, tongue coating, and oral wash, on sample quality for microbiota profiles and preference of patients.

In terms of microbiota abundance and species diversity, our study found that the PDAC group had significantly increased microbial abundance as estimated by the Chao1 and ACE indices and decreased microbial diversity as estimated by Shannon and Simpson indices. Lu *et al*[20] also had similar findings from a study on Chinese pancreatic cancer patients using tongue coating samples[20]. However, studies of non-Chinese population did not find any differences of alpha diversity indices of oral microbiota composition between pancreatic cancer patients and healthy individuals[22,23]. Findings of our study and Lu *et al*[20] demonstrated that seven of fourteen bacterial families (*Leptotrichiaceae, Actinomycetaceae, Lachnospiraceae, Micrococcaceae, Erysipelotrichaceae, Coriobacteriaceae, Moraxellaceae*) were consistently significantly increased, and *Porphyromonadaceae* was significantly decreased in Chinese PDAC patients. However, our study found that the abundance of three of fourteen bacterial families (*Fusobacteriaceae, Campylobacteraceae, Spirochaetaceae*) were significantly decreased in PDAC patients, while Lu *et al*[20] found significantly more abundance[20]. Both our study and the study by Lu *et al*[20] found significant increase in the genus of *Leptotrichia, Actinomyces, Rothia, Rothia, Solobacterium, Peptostreptococcus*, and *Oribacterium*. Yet, decreased abundance in *Selenomona, Tannerella*, and *Campylobacter* was found in our study using saliva sample method but was increased in the study by Lu *et al*[20] using tongue coating sample method[20]. There are four known main periodontal disease contributors: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia*, and *Prevotella intermedia* were more prevalent in PDAC patients in Fan *et al*[21]. However, except for *Actinomyces, Porphyromonas gingivalis, Tannerella forsythia*, and *Prevotella intermedia* were significantly reduced in our study. *Leptotrichia* also showed different distribution in

our study comparing to Fan *et al*[21]. Torres *et al*[43] found higher *Leptotrichia* and lower *Porphyromonas* in the saliva of patients with pancreatic cancer, but no significant differences were found in the expression of *Streptococcus mitis* and *Granulicatella adiacens*. The conflicting findings between our study and other studies may due to different sample collection methods, *e.g.*, saliva *vs* tongue coating method. Future research should compare different sample collection methods for microbiome research, *e.g.*, saliva *vs* tongue coating method. The other factor for the conflicting findings may geographic food preferences of Chinese population. For example, subjects in Lu *et al*[20]'s study were enrolled from Zhejiang University, which is located in Hangzhou (southeast of China). Generally, people in Hangzhou have different diet preferences, such as preferences for milder taste and more sugar. Subjects in our study from Sichuan Province preferred adding a large amount of different herbs and spices and more fat and salt in food, which may lead to a high incidence of digestive system tumors[44,45]. Future research should focus on the effects of geographical location, race, diet, antibiotic usage (including consuming meat products containing antibiotics), injury, illness, and hormonal change on flora analysis[46].

One important finding of our study was that bacteria flora was able to differentiate patients with rPDAC and unrPDAC. This is important because patients with rPDAC usually have better prognosis with timely surgical treatment. We found that species_*Escherichia coli* and species_*Tannerella forstythia* were increased significantly in unrPDAC, and these bacteria may be able to predict a tumor that is already advanced. In contrast, the expression of *Veillonella* demonstrated a gradual decline in saliva samples from healthy people, rPDAC, and advanced PDAC (Figure 3), which indicates that *Veillonella* may be protective bacteria for PDAC development.

Our study is the first to investigate the associations between bacteria profiles and symptoms related to pancreatic cancer. Symptomatic patients had different bacteria profiles than asymptomatic patients in our study. For examples, PDAC with bloating have a higher abundance of *Porphyromonas*, *Fusobacterium*, and *Alloprevotella*, and *Alloprevotella* is decreased in patients with vomiting. In addition, PDAC with jaundice had a higher amount of *Prevotella* compared with the PDAC without jaundice. There was a higher amount of *Veillonella* in patients with dark brown urine. PDAC with diarrhea had a lower amount of *Neisseria* and *Campylobacter* compared with PDAC without diarrhea. One benefit of having symptoms is that patients will seek medical help earlier, leading to the diagnosis of early PDAC and improved survival. The exact microbiome mechanism for symptoms is unknown, and more studies are needed to investigate the associations between microbiota and symptoms. Perhaps, combined symptom and microbiome evaluation may help in early detection of pancreatic cancer.

Our study had limitations. We did not include the data of other pancreatic diseases because the sample size was very small. Second, we used only 16S rRNA sequencing to analyze bacterial distributions; future research should include metagenomic sequencing to enhance accuracy of bacterial distributions. It should be noted that the rapid, inexpensive tests of 16S rRNA sequencing can have advantages for clinical implementation by using bacterial distribution test for early detection or prevention of PDAC. Some studies found the association between microbiome profile and dental disease[47]. Another limitation of our study was that we were not able to exclude participants with dental disease since our participants were not able to provide accurate history of dental disease, and there were no medical record regarding dental disease for us to verify participant dental disease status. In the future, it may be beneficial to have a professional dentist examine participant's oral health status so as to ascertain the potential impact of oral health status on microbiome flora profile among patients with pancreatic cancer. One strength of the study is that we compared the bacteria abundances in patients with positive symptoms to find the relative association between the occurrence of symptoms and potential functions of flora.

CONCLUSION

Saliva microbiome are able to distinguish PDAC and healthy individuals. Higher *Streptococcus* and *Leptotrichia* abundances were associated with increased risk of PDAC. *Veillonella* and *Neisseria* were protective factors for detecting PDAC. *Neisseria* was recognized by all studies to reduce the risk of pancreatic cancer while *Leptotrichia* was identified in our study as a potential specific detector of PDAC in patients living in Sichuan Province, southwest China. Symptomatic patients had different bacteria profiles than asymptomatic patients. As symptoms of PDAC are usually nonspecific, combined symptom and microbiome evaluation may help in early detection of

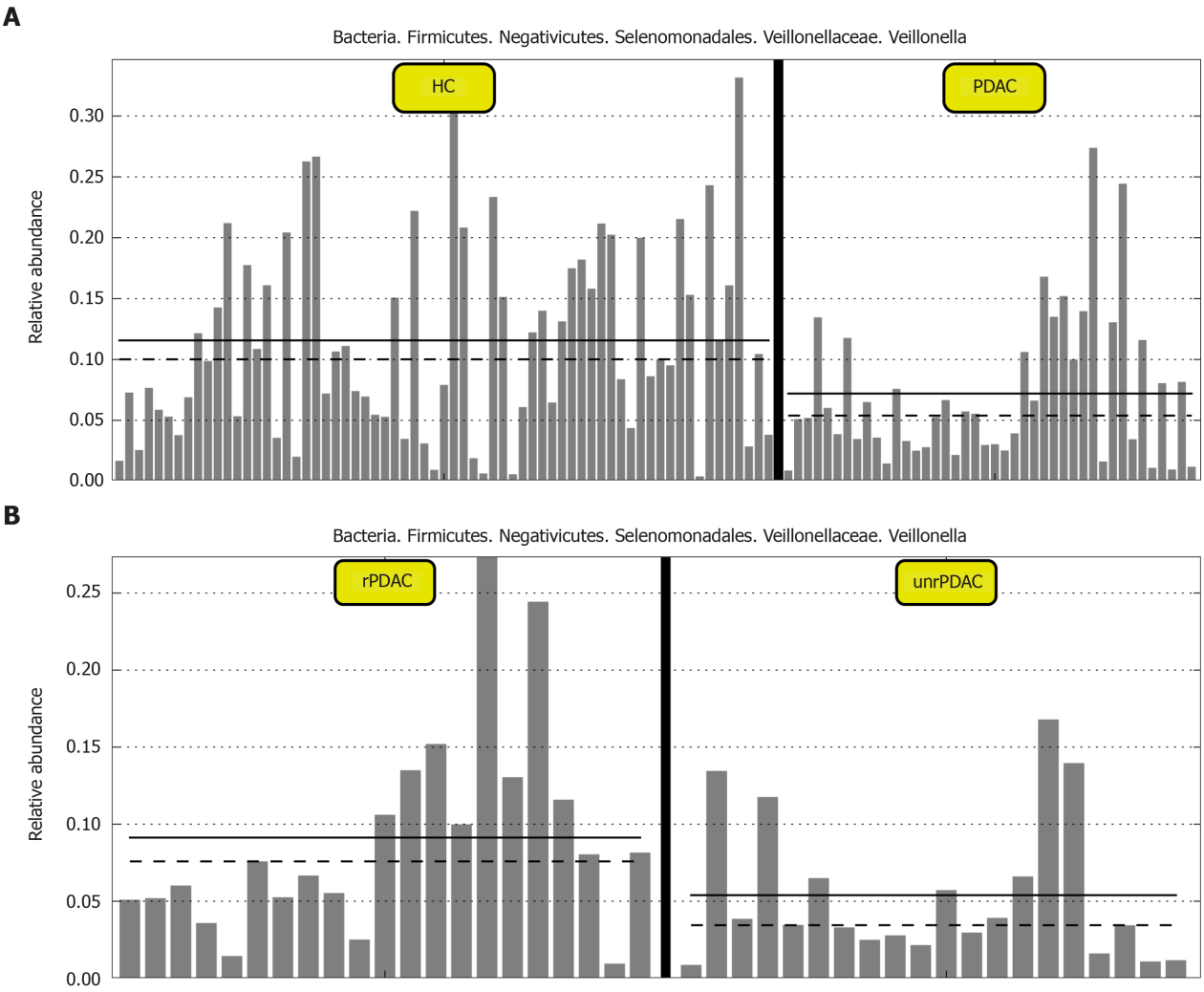

Figure 3 The abundance of *Veillonella* in different groups. The relative abundance of *Veillonella* in pancreatic adenocarcinoma (PDAC) patients is shown by the straight, and dotted lines plot the means and medians of the relative abundance. The abundance of *Veillonella* showed a gradual decline in saliva samples from healthy people, resectable PDAC (rPDAC), and unresectable PDAC (unrPDAC). HC: Healthy controls.

pancreatic cancer. Understanding the distribution of bacteria flora is essential step for developing probiotic treatment plans for reducing the risk of pancreatic cancer.

ARTICLE HIGHLIGHTS

Research background

Understanding the distribution of bacteria flora is essential step for developing probiotic treatment plans for reducing the risk of pancreatic cancer.

Research motivation

The impact of geographical and medical factors, such as race and ethnicity, different dietary habits, antibiotic use, and cancer, may make the oral microbial profile differ among people from different geographic locations.

Research objectives

To investigate the saliva microbiome distribution in patients with pancreatic adenocarcinoma and the role of oral microbiota profiles in detection and risk prediction of pancreatic cancer.

Research methods

A prospective design was utilized with 16S ribosomal ribonucleic acid gene sequencing to identify differences in bacterial taxa using a linear discriminant analysis

effect size algorithm. Operational taxonomic unit values of all selected taxa were converted into a normalized Z-score, and logistic regressions were used to calculate risk prediction of pancreatic cancer.

Research results

Saliva microbiome was able to distinguish patients with pancreatic cancer and healthy individuals. Symptomatic patients had different bacteria profiles than asymptomatic patients.

Research conclusions

Combined symptom and microbiome evaluation may help in early detection of pancreatic cancer.

Research perspectives

Further work may focus on specific microbiota verification and diagnostic ability *via* large sample studies.

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SYSTEMATIC REVIEWS

High prevalence of hepatic steatosis and vascular thrombosis in COVID-19: A systematic review and meta-analysis of autopsy data

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) disease can frequently affect the liver. Data on hepatic histopathological findings in COVID-19 is scarce.

AIM

To characterize hepatic pathological findings in patients with COVID-19.

METHODS

We conducted a systematic review with meta-analysis registered on PROSPERO (CRD42020192813), following PRISMA guidelines. Eligible trials were those including patients of any age and COVID-19 diagnosis based on a molecular test. Histopathological reports from deceased COVID-19 patients undergoing autopsy

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or liver biopsy were reviewed. Articles including less than ten patients were excluded. Proportions were pooled using random-effects models. *Q* statistic and I^2 were used to assess heterogeneity and levels of evidence, respectively.

RESULTS

We identified 18 studies from 7 countries; all were case reports and case series from autopsies. All the patients were over 15 years old, and 67.2% were male. We performed a meta-analysis of 5 studies, including 116 patients. Pooled prevalence estimates of liver histopathological findings were hepatic steatosis 55.1% [95% confidence interval (CI): 46.2-63.8], congestion of hepatic sinuses 34.7% (95%CI: 7.9-68.4), vascular thrombosis 29.4% (95%CI: 0.4-87.2), fibrosis 20.5% (95%CI: 0.6-57.9), Kupffer cell hyperplasia 13.5% (95%CI: 0.6-54.3), portal inflammation 13.2% (95%CI: 0.1-48.8), and lobular inflammation 11.6% (95%CI: 0.3-35.7). We also identified the presence of venous outflow obstruction, phlebosclerosis of the portal vein, herniated portal vein, periportal abnormal vessels, hemophagocytosis, and necrosis.

CONCLUSION

We found a high prevalence of hepatic steatosis and vascular thrombosis as major histological liver features. Other frequent findings included portal and lobular inflammation and Kupffer cell hyperplasia or proliferation. Further studies are needed to establish the mechanisms and implications of these findings.

Key Words: Pathology; SARS-CoV-2; COVID-19; Autopsies; Liver; Liver biopsies

Core Tip: In this systematic review of 18 studies, we identified that hepatic steatosis, congestion of hepatic sinuses, and vascular thrombosis are the main histological findings in deceased coronavirus disease 2019 (COVID-19) patients. Fibrosis is a common histological finding in deceased COVID-19 patients. Kupffer cell hyperplasia, portal inflammation, and lobular inflammation are related to the severe acute respiratory syndrome coronavirus 2 inflammatory process and are frequent histological findings. Other vascular abnormalities (such as venous outflow obstruction, phlebosclerosis of the portal vein, herniated portal vein, periportal abnormal vessels) are frequent histological finding in deceased COVID-19 patients.

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INTRODUCTION

The pandemic of the novel coronavirus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (also known as coronavirus disease 2019, COVID-19) has infected more than 59 million individuals worldwide and caused more than 1.4 million deaths. Although the main manifestations of COVID-19 are related to respiratory symptoms, the compromise of multiple organs has been described in the literature, including the digestive system[1]. There are several gastrointestinal manifestations, including anorexia, diarrhea, nausea/vomiting, and abdominal pain. Those symptoms have a pooled prevalence of 17.6% and are frequently observed in hospitalized patients[1,2].

The liver is also one of the most common organs affected in COVID-19. In fact, between 2%-11% of affected patients have liver comorbidities and 16%-53% of cases reported abnormal liver tests[3,4]. The most frequent abnormalities are mildly elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and are more

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common in hospitalized patients[4,5]. Elevation of alkaline phosphatase and gamma-glutamyl transferase (GGT) has also been reported[6]. Those alterations have been related to expression of angiotensin-converting enzyme 2 (ACE-2), the putative receptor of SARS-CoV-2, in endothelial cells of the liver and the biliary epithelium[7]. In addition, since the frequency of liver dysfunction increases as COVID-19 is more severe, liver damage might be directly caused by the infection of liver. Abnormal liver tests can also be partially explained by drug-induced liver injury (DILI), cytokine storm, and/or pneumonia-associated hypoxia[4].

Pathological studies in patients with SARS-CoV-2 infection have confirmed the presence of the virus in liver tissue[8]. This finding was also described in SARS and MERS infection[3]. Although histopathological information is scarce, previous reports from SARS and MERS showed steatosis, mild portal tract and lobular lymphocytic inflammation, as well as mild cellular hydropic degeneration in hepatic parenchyma[7,9-11]. Regarding SARS-CoV-2 infection, initial reports of autopsies performed in COVID-19 patients have described steatosis, mild lobular and portal activity, lymphocytic endotheliitis, and necrosis[12-14]. However, data about the main histopathological findings in COVID-19 is still scarce. Thus, aiming to provide a comprehensive synthesis of the pathological findings in liver injury due to SARS-CoV-2 reported so far, we performed a systematic review and meta-analysis of each histopathological finding in liver samples from autopsies and biopsies of COVID-19 patients.

MATERIALS AND METHODS

Overview

This systematic review with meta-analysis was registered on PROSPERO (ID: CRD42020192813) and followed a prespecified analysis plan. This study is reported in accordance with the Preferred Reporting Items for a Review and Meta-analysis (PRISMA) guidelines[15].

Eligibility criteria

Eligible trials had to include patients diagnosed with COVID-19, regardless of age and gender. The diagnosis of COVID-19 had to be based on a compatible clinical history and molecular evidence with a quantitative real-time polymerase chain reaction (qRT-PCR) for SARS-CoV-2. We included liver histopathological reports from deceased COVID-19 patients who subsequently were studied with autopsy or liver biopsies performed in alive COVID-19 patients.

We planned to include all the studies that report liver histopathological data, regardless of the design (case-reports, case-series, descriptive cases, cross-sectional studies, cohort studies, and randomized controlled trials). We excluded studies performed in vitro, animal models, or lacking evidence of SARS-CoV-2 infection from this systematic review. We did not include manuscripts performed before December 1, 2019.

Search strategy and selection process

We performed an electronic search from December 1, 2019, to June 3, 2020, in MEDLINE (*via* PubMed) and Embase databases. We used keywords and free-text words related to SARS-CoV-2 infection, autopsies, and liver biopsies. We reported the search strategy used in PubMed and Embase databases in the appendix. We hand searched (up to June 3, 2020) preprint servers (bioRxiv, medRxiv, and SSRN) and coronavirus resource centers of The Lancet, JAMA, and New England Journal of Medicine. We did not limit our search by language. Two investigators (Díaz LA and Idalsoaga F) independently screened the titles and abstracts to ascertain whether each study met the eligibility criteria. The full texts of the identified eligible articles were then evaluated to determine whether they should be included in the analysis. Disagreements between the two reviewers were resolved by consensus. In case of persistent disagreement, arbitration by a third reviewer (Arab JP) settled the discrepancy.

Data collection and risk of bias assessment

Two authors (Díaz LA and Cannistra M) independently extracted data from included studies using forms specially designed for this purpose. The following data were extracted describing the study, participants, source of sample (liver biopsy or

autopsy), and the main liver histopathological findings. Discrepancies were resolved by a third reviewer (Arab JP). Two investigators (Díaz LA and Idalsoaga F) independently assessed the risk of bias of each included trial with the Appraisal tool for Cross-Sectional Studies (AXIS) checklist for cross-sectional studies, the Institute of Health Economics (IHE) checklist tool for Case Series, the Newcastle-Ottawa Scale (NOS) for case-control studies and cohort studies, and the Cochrane Risk of Bias Tool for randomized trials and quasi-experimental studies.

Outcomes

The outcomes were defined by consensus with an expert pathologist (Graham R). The main outcomes were the frequency of vascular thrombosis (presence or absence), venous outflow obstruction (presence or absence), frequency of portal and lobular Inflammation and severity (mild, moderate, or severe), Kupffer cell hyperplasia (presence or absence), steatosis (presence and fat percentage), fibrosis (based on METAVIR score, scores range from F0 to F4, with higher numbers reflecting greater fibrosis), and ductopenia (presence or absence). Secondary outcomes were the frequency phlebosclerosis of the portal vein, herniated portal vein, congestion of hepatic sinuses, and necrosis.

Data analysis

Data were synthesized per each histopathological finding of SARS-CoV-2 infection. We estimated the prevalence of event rates in the form of a proportion (with a confidence interval of 95%). Proportions were pooled using random-effects models. Only those studies with a sample size of at least ten patients were included in the meta-analysis. We used Q statistic and I^2 to quantify heterogeneity. We planned a subgroup analysis according to the liver sample source (biopsy in alive patients or autopsy), ethnicity, and gender. We planned sensitivity analysis excluding the high risk of bias studies. A small study effect was evaluated with a funnel plot. As such, subgroup and sensitivity analyses should be considered exploratory. All statistical analyses were performed using MedCalc Statistical Software version 19.3.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020).

Grading of evidence

The quality of evidence for the outcomes was graded with the GRADE framework.

Role of the funding source

The funding source only provided support for the financing of paid manuscripts during the review process. The researchers did not receive payment or other incentives.

RESULTS

We identified 3060 records and 905 were duplicates. After a screening process against title and abstract, we obtained 58 full-text articles that were assessed for eligibility. We finally selected 18 studies for our systematic review from 7 countries (Austria, Belgium, China, Germany, Italy, Switzerland, and the United States). The selection process is described in Figure 1. All studies were observational in design; no randomized trials were identified. Among the 18 studies, 4 were case reports and 14 case series. The study with the largest sample size included a total of 48 cases[16]. Fifteen studies reported the proportion of each finding and the other three studies presented the pooled data without the prevalence of each finding. All the studies included autopsy data (*i.e.*, no liver biopsies from living patients were identified). The risk of bias assessment was considered high.

The pooled studies included a total of 167 patients. All the patients were over 15 years old and 67.2% were male. The grade of evidence was considered very low since all the information emerged from case reports and case series. For the meta-analysis of each finding, we included only five studies with at least ten patients. There was a maximum of 116 cases included in each meta-analysis. We did not perform subgroup analyses due to the low number of studies included. Table 1[17-30] summarizes the main baseline characteristics of each study. The main findings described in the selected studies were hepatic steatosis, congestion of hepatic sinuses, vascular thrombosis, fibrosis, Kupffer cell proliferation or hyperplasia, portal inflammation, lobular inflammation, venous outflow obstruction, phlebosclerosis of the portal vein, herniated portal vein, periportal abnormal vessels, hemophagocytosis, and necrosis.

Table 1 Main characteristics of studies included in the analysis

Ref.	Date	Country	Sources of samples	Nº of pts	Age (yr)	Gender	Ethnicity	Comorbidities
Barton *et al*[17]	April 2020	United States	Autopsy	2	77 and 42	2 Males	Not reported	Obesity (2/2), hypertension (1/2), splenectomy (1/2), myotonic dystrophy (1/2)
Bradley *et al*[18]	April 2020	United States	Autopsy	12	70.4 (42-84)	5 Males and 7 females	Not reported	Not reported
Bryce *et al*[29]	May 2020	United States	Autopsy	22	Not reported	Not reported	Not reported	Not reported
Buja *et al*[19]	May 2020	United States	Autopsy	3	62, 34 and 48	2 Males and 1 female	2 Hispanic and 1 afro-american	Obesity (3/3), hypertension (1/3), heart failure (1/3), diabetes (1/3) and microcytic anemia (1/3)
Craver *et al*[20]	April 2020	United States	Autopsy	1	17	Male	Afro-american	None
Lacy *et al*[21]	April 2020	United States	Autopsy	1	58	Female	Not reported	Diabetes, obesity, hyperlipidemia, Asthma, and chronic lower extremity swelling with ulceration
Lax *et al*[27]	May 2020	Austria	Autopsy	11	80.5 (66-91)	8 Males and 3 females	Not reported	Hypertension (9/11), diabetes mellitus (5/11), coronary artery disease (2/11), previous malignant disease (2/11), COPD (2/11), cerebrovascular disease (4/11), and dementia (4/11)
Martines *et al*[22]	May 2020	United States	Autopsy	8	73.5	4 Males and 4 females	7 Caucasian and 1 hispanic	Arterial hypertension (6/8), chronic kidney disease (6/8) cardiovascular disease (6/8), obesity (5/8), diabetes (4/8), chronic lung disease (2/8), immunocompromised condition (3/8), among others
Menter *et al*[23]	May 2020	Switzerland	Autopsy	17	Adult patients	Not specified	Not specified	Not specified
Prilutskiy *et al*[28]	March 2020	United States	Autopsy	4	64-91	3 Males and 1 female	3 Afro-american and 1 caucasian	Not reported
Remmelink *et al*[24]	May 2020	Belgium	Autopsy	17	72 [62-77]	12 Males and 5 females	Not reported	Cirrhosis (2/17), liver transplantation (1/17), hypertension (10/17), diabetes (9/17), cerebrovascular disease (4/17), coronary artery disease (4/17), and solid cancer (4/17)
Schaller *et al*[25]	May 2020	Germany	Autopsy	10	79 (64-90)	7 Males and 3 females	Not reported	Fatty liver disease (1/10), hypertension (7/10), atrial fibrillation (4/10), chronic kidney failure (3/10), COPD (2/10), heart failure (2/10), obesity (2/10), diabetes (1/10), among others
Sonzogni *et al*[16]	May 2020	Italy	Autopsy	48	71.2 (32-87)	35 Males and 13 females	Not reported	Not specified
Tian *et al*[14]	April 2020	China	Autopsy	4	78, 74, 81, 59	3 Males and 1 female	Asian	Chronic lymphocytic leukemia; Cirrhosis, DM2, HTA
Varga *et al*[13]	April 2020	Switzerland	Autopsy	1	58	Female	Not reported	Diabetes, hypertension, and obesity
Wang *et al*[26]	May 2020	China	Autopsy	2	50 and 79	1 Male and 1 female	2 Asian	Not reported
Xu *et al*[12]	February 2020	China	Autopsy	1	50	Male	Asian	Not reported
Yao *et al*[30]	May 2020	China	Autopsy	3	63, 69, and 79	2 Males and 1 female	3 Asian	Diabetes (1/3), hyperglycemia (1/3) bronchiectasis (1/3), solid cancer (1/3)

We did not find ductopenia in the selected studies. Figure 2 summarizes the major liver histological features. A detailed assessment of each feature is provided below. Funnel plot of the studies included in each meta-analysis are provided in Supplementary Figure 1 (Supplementary material).

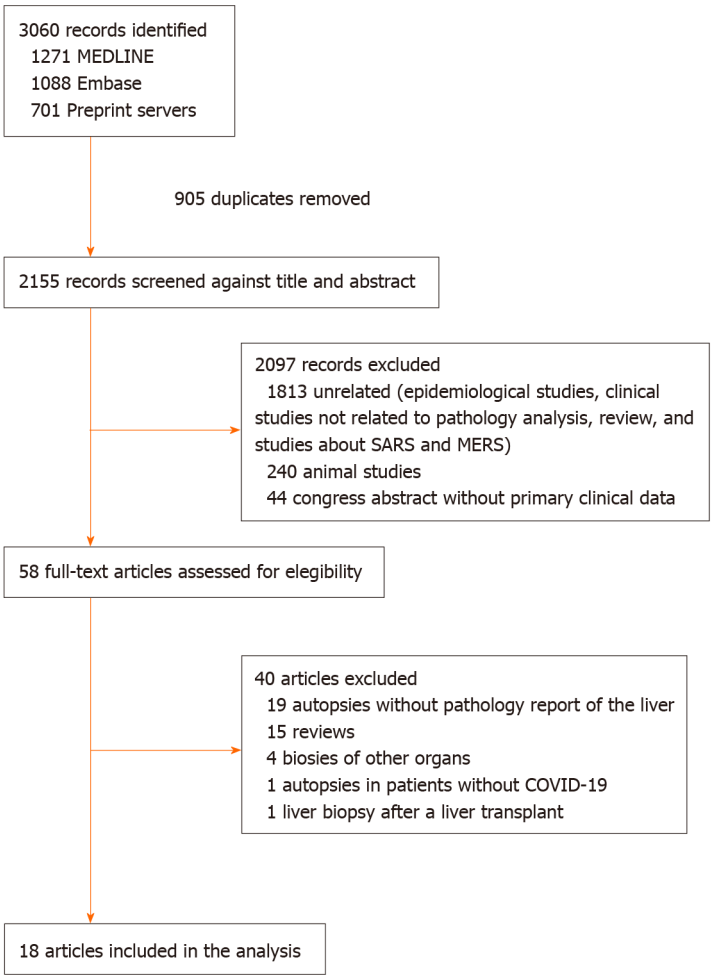

Figure 1 Study selection for the systematic review.

Hepatic steatosis

A total of 78 from 139 (65.1%) patients demonstrated hepatic steatosis. Lipid droplet size was described in only 7 cases (9%): 4 macrovesicular, 1 microvesicular, and 2 mixed (macrovesicular and microvesicular). The studies of Lax *et al*[27]and Prilutskiy *et al*[28] also described the presence of hepatic steatosis without the proportion of this finding. When meta-analyzed, data showed a pooled prevalence of hepatic steatosis of 55.1% [5 studies, 116 patients; 95% confidence interval (CI): 46.2-63.8], without significant heterogeneity among the studies (P = 0.411; I^2 = 0%) (Figure 3A).

Congestion of hepatic sinuses and necrosis

A total of 28 from 163 cases (17.2%) reported congestion of hepatic sinuses. Additionally, the study of Prilutskiy *et al*[28] described the presence of mild centrilobular congestion without the frequency of this finding. In the meta-analysis, the pooled prevalence of congestion of hepatic sinuses was 34.7% (5 studies, 79 patients; 95%CI: 7.9-68.4) (Figure 3B). The heterogeneity was significant among studies (P < 0.001; I^2 = 90.2%). Regarding necrosis, 14 of 91 patients (15.4%) presented this finding. Necrosis was also described without a proportion in the study of Yao *et al*[30]. We could not perform a meta-analysis of this finding due to the low number of patients and studies that adequately described the presence or absence of necrosis.

Vascular thrombosis and other vascular alterations

A total of 63 from 139 (45.3%) cases presented vascular thrombosis. The type of vessel thrombosed was not specified in the studies. When meta-analyzed, data showed a pooled prevalence of vascular thrombosis of 29.4% (5 studies, 116 patients; 95%CI: 0.4-87.2). The heterogeneity was significant among studies (P < 0.001; I^2 = 97.7%) (Figure 3C).

Other vascular alterations were identified in the studies. The presence of venous

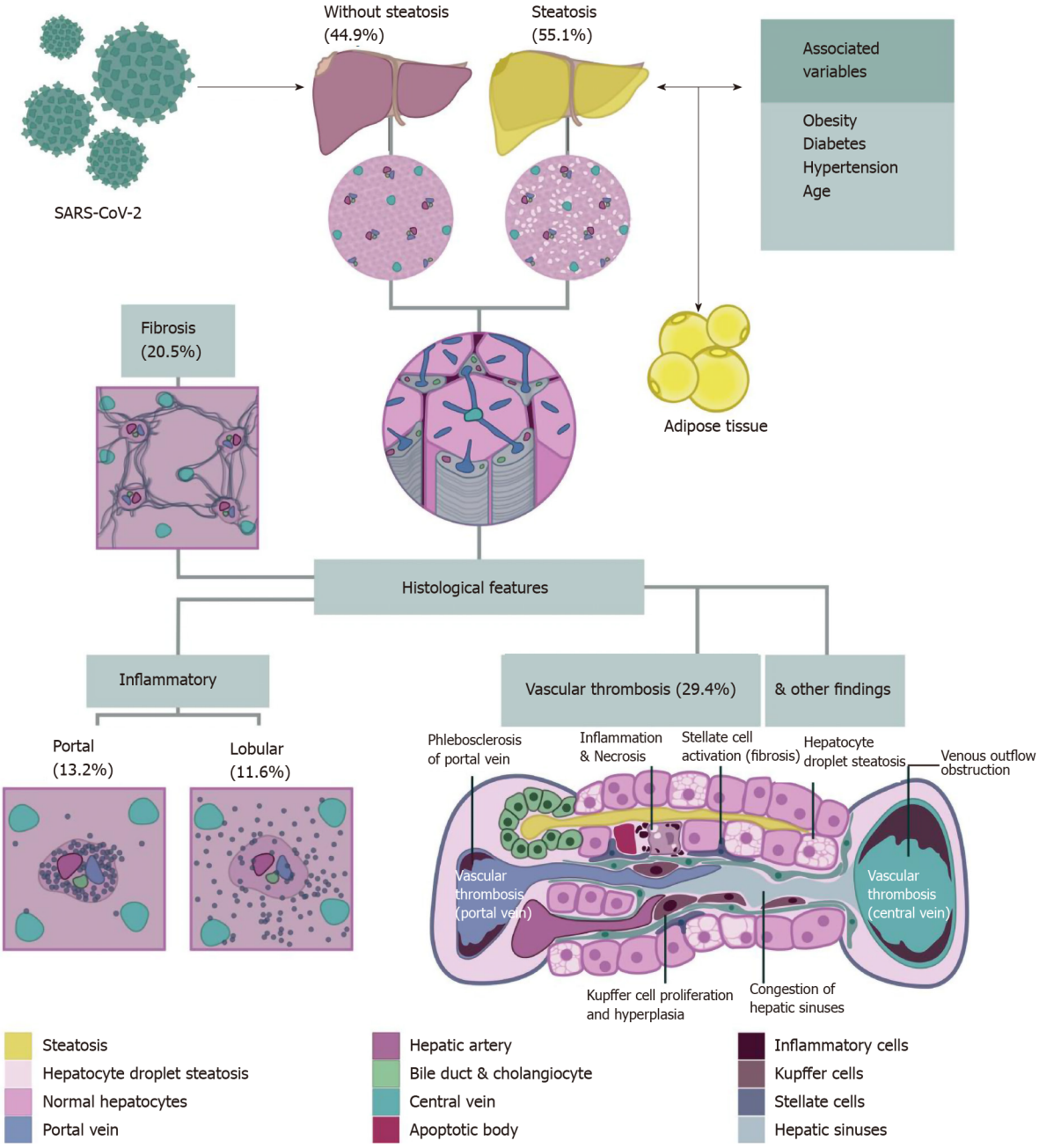

Figure 2 Major liver histological features observed in 6 studies (*n* = 116 autopsies from deceased coronavirus disease 2019 patients). Steatosis was the most frequent finding (55.1%). Studies also reported congestion of hepatic sinuses (34.6%), vascular thrombosis (29.4%), fibrosis (20.5%), Kupffer cell hyperplasia (13.5%), portal inflammation (13.2%), and lobular inflammation (11.6%), Other findings observed were venous outflow obstruction, phlebosclerosis of the portal vein, herniated portal vein, periportal abnormal vessels, hemophagocytosis, and necrosis.

outflow obstruction was described exclusively by Bryce *et al*[29], with a prevalence of 36.4% (8 of 22 cases), and 5 of these cases (62.5%) were acute. The study by Sonzogni *et al*[16] reported additional vascular alterations in a serial of 48 cases: 29 patients demonstrated phlebosclerosis of the portal vein (60.4%), 36 had a herniated portal vein (75%), and 48 (100%) had abnormal periportal vessels (27 focal, 18 multifocal, and 3 diffuse).

Hepatic fibrosis

A total of 51 from 139 (36.7%) patients had fibrosis. The grade of fibrosis was only described in 7 cases: 1 case was graded as F3 (METAVIR scale) and 6 cases were graded as F4. The study by Lax *et al*[27] and Prilutskiy *et al*[28] also described the presence of fibrosis without the proportion of this finding. In the meta-analysis, the pooled prevalence of fibrosis was 20.5% (5 studies, 116 patients; 95%CI: 0.6-57.9), with

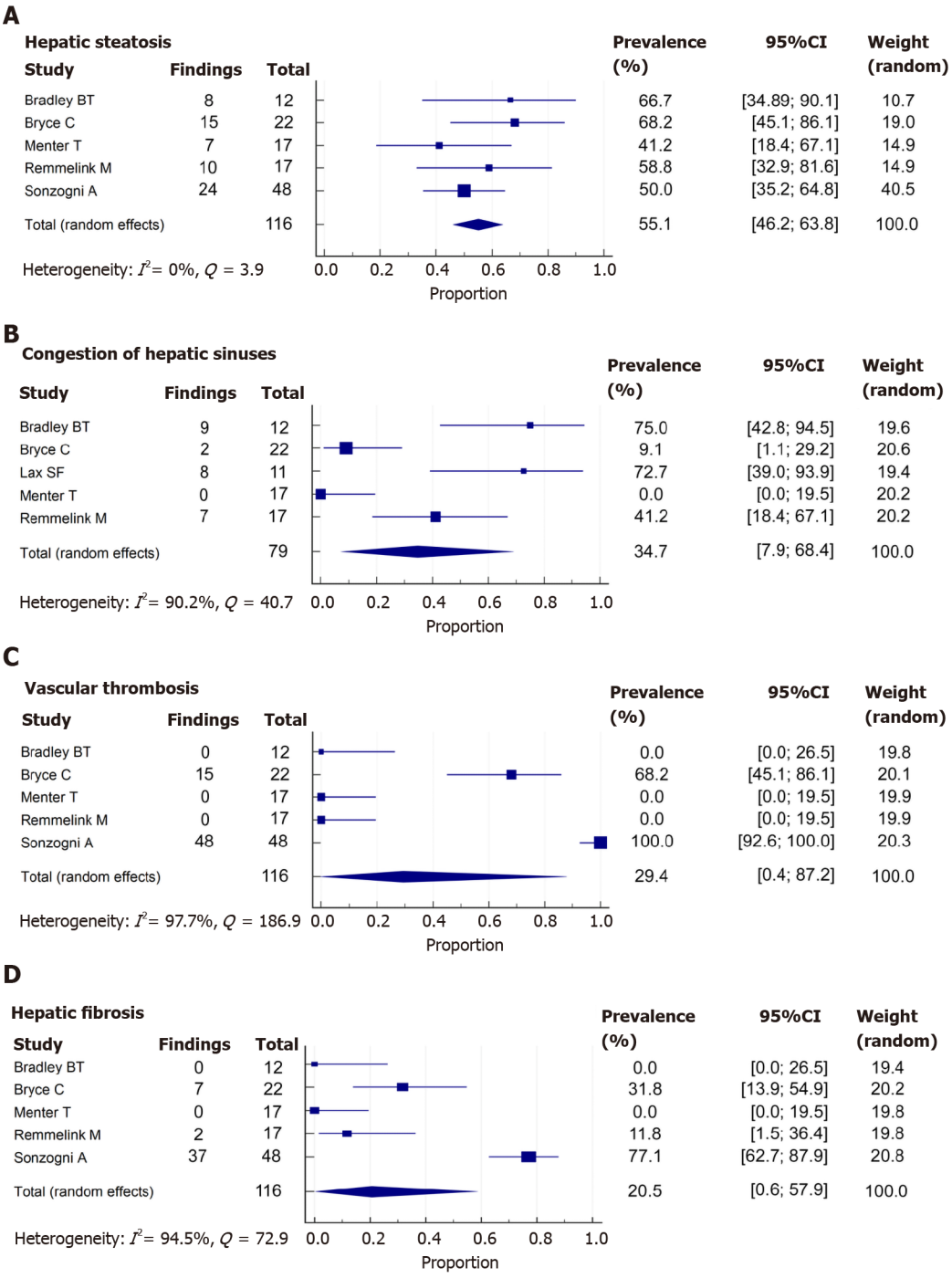

Figure 3 Forest plots of major liver histological features from deceased coronavirus disease 2019 patients. A-D: The figure includes forest plots of hepatic steatosis (A), congestion of hepatic sinuses (B), vascular thrombosis (C), and fibrosis (D). Heterogeneity was assessed using I^2 and Q statistics. CI: Confidence interval.

significant heterogeneity among the studies (P < 0.001; I^2 = 94.5%) (Figure 3D).

Kupffer cell proliferation or hyperplasia

Only 12 of 115 cases (10.4%) reported Kupffer cell proliferation or hyperplasia. The studies by Sonzogni *et al*[16] and Prilutskiy *et al*[28] also described the presence of this phenomenon, but without a prevalence. Additionally, the study of Bryce *et al*[30] described the presence of hemophagocytosis. In the meta-analysis, the pooled prevalence of Kupffer cell hyperplasia was 13.5% (5 studies, 79 patients; 95%CI: 0.6-54.3%). The heterogeneity was significant among studies (P < 0.001; I^2 = 94.3%) (Figure 4A).

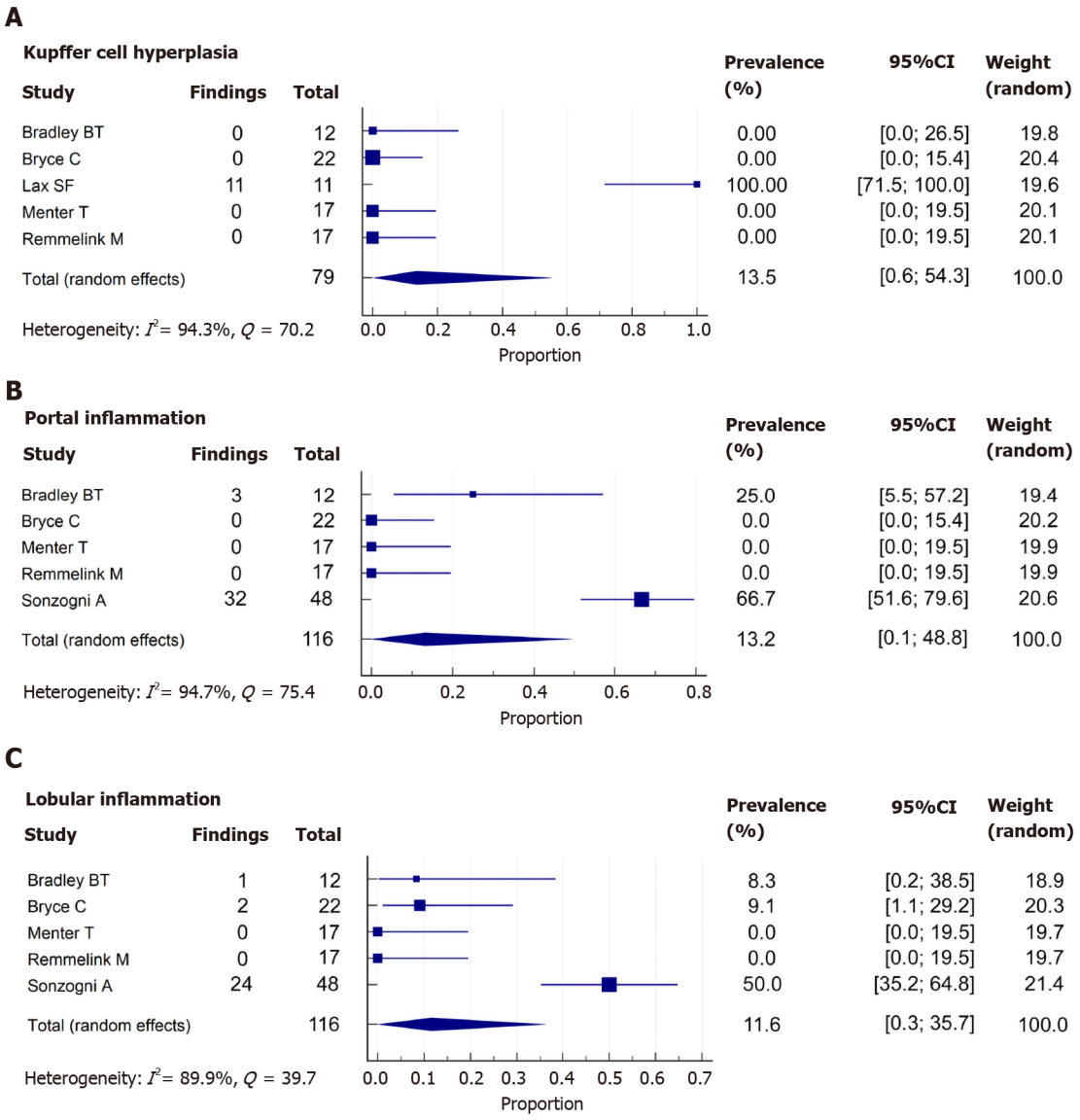

Figure 4 Forest plots of inflammatory liver histological features from deceased coronavirus disease 2019 patients. A-C: The figure includes forest plots of Kupffer cell hyperplasia (A), portal inflammation (B), and lobular inflammation (C). Heterogeneity was assessed using I^2 and Q statistics. CI: Confidence interval.

Portal and lobular inflammation

Histologic evaluation in a total of 41 out of 139 (29.5%) patients showed portal inflammation. The grade of inflammation was reported in 37 of 41 cases (90.2%); all were graded as mild, and the cells were lymphocytes and plasma cells. When meta-analyzed, data showed a pooled prevalence of portal inflammation of 13.2% (5 studies, 116 patients; 95%CI: 0.1-48.8), with significant heterogeneity noted among studies (P < 0.001; I^2 = 94.7%) (Figure 4B).

In the case of lobular inflammation, a total of 31 from 139 (22.3%) showed this finding. The grade of inflammation was reported in 28 of 31 cases (90.3%): 26 cases were mild (92.9%) and 2 were moderate (7.1%). The predominant cells were neutrophils and lymphocytes. In a meta-analysis, the pooled prevalence of lobular inflammation was 11.6% (5 studies, 116 patients; 95%CI: 0.3-35.7), with significant heterogeneity noted among studies (P < 0.001; I^2 = 89.9%) (Figure 4C).

DISCUSSION

As previously reported for patients with SARS and MERS, the liver is frequently affected during a SARS-Cov-2-related disease (COVID-19)[3,5]. In this systematic review, we identified 18 studies from 7 countries (case reports and case series) that

include data from autopsies of deceased patients with COVID-19. We identified multiple histopathological findings, including hepatic steatosis (55.1%), venous outflow obstruction (36.4%), congestion of hepatic sinuses (34.7%), vascular thrombosis (29.4%), fibrosis (20.5%), necrosis (15.4%), Kupffer cell hyperplasia (13.5%), portal inflammation (13.2%), and lobular inflammation (11.6%).

Several of the hepatic histopathological findings observed in this study have also been described in SARS and MERS patients. It may be related to the ongoing systemic inflammatory process and sepsis, affecting the liver rather than a direct manifestation of SARS-CoV-2[7,9-11]. There is still debate about whether SARS-CoV-2 directly causes liver injury or if the observed abnormalities in liver chemistries seen in COVID 19 are an indirect consequence of the disease reflecting severity, inflammation, and potentially confounding muscle injury that occurs in 19% of patients[31]. A recent study by Bloom *et al*[32] showed that AST-dominant aminotransferase elevation is common in COVID-19, is not associated with muscle damage markers and correlates with disease severity, probably reflecting true hepatic injury. In a series including 148 patients from a single-center in China, 55 (37.2%) had abnormal liver chemistries on admission. Patients with elevated admission liver chemistries were also more likely to have a high-grade fever and higher C-reactive protein[33]. In two large Chinese cohorts, 6.2% and 11.6% of patients with COVID-19 developed liver chemistries over 3 times the upper limit of normal, suggesting that a minority of patients experience significant biochemistry elevations[34,35].

The most frequent histopathological finding was steatosis. This prevalence is higher than the general population[36]. This can be partially explained by the baseline characteristics of the population. Admitted COVID-19 patients suffer from more severe disease and more frequently exhibit chronic diseases (*i.e.*, diabetes mellitus, age, hypertension, obesity, and cardiovascular disease), which are also associated with hepatic steatosis[37]. However, existing data suggest that SARS-COV-2 may affect lipid metabolism[38]. In general, all viruses alter lipid synthesis and signaling in host cells as they highjack and utilize the cellular machinery to produce lipids for their envelope. Also, it has been shown that COVID-19 patients have elevated serum levels of fatty acids and infection with other SARS viruses determine long-lasting alterations in lipid metabolism[39]. It has been shown that metabolic-dysfunction associated fatty liver (MAFLD)[40] condition is independently associated with a higher risk of severe COVID-19 (odds ratio 2.67)[41]. Also, advanced MAFLD (*i.e.*, fibrotic disease) is associated with a more severe disease irrespective of metabolic comorbidities. Since the liver hosts a significant mass of innate immune cells, hepatic release of proinflammatory cytokines may contribute to COVID-19 severity[42]. Also, some authors suggest NAFLD progression could be accelerated or exacerbated by COVID-19[42].

We observed an increased frequency of hepatic vascular alterations in patients with COVID-19. This may be due to endothelial dysfunction (endotheliitis), a pro-coagulant state, and direct vascular injury of the disease[13,43,44]. Congestion and necrosis are features of venous outflow obstruction and may also be explained by circulatory dysfunction, heart failure, and ischemia, which may complicate multiorgan failure.

Liver fibrosis was also frequently found in the published series of COVID-19 patients. Whether this is related to sustained liver injury or by prior history of chronic liver disease is unclear, though the latter is favored given the acute nature of COVID-19 disease. Of note, patients with cirrhosis have been shown to have a higher risk of mortality due to COVID-19 (relative risk of 4.6)[45].

It is important to notice that no specific histologic indicator of direct infection (*i.e.*, viral cytopathic effect) in the liver tissue. Notably, there was a report from a pediatric living donor liver allograft recipient who, on a post-operative day 4, developed respiratory distress, fever, and an approximately 5-fold elevation of liver enzymes. The patient and donor were positive for SARS-CoV-2. Liver biopsy showed moderate acute hepatitis with prominent clusters of apoptotic hepatocytes, associated cellular debris, and lobular lymphohistiocytic inflammation. Typical portal features of mild to moderate acute cellular rejection were also noted[46]. This case report raises consideration for possible direct liver injury.

The possible mechanisms by which SARS-CoV-2 exerts its pathogenetic role in the liver have been speculated in the literature. There is a known tropism of SARS-CoV-2 for ACE-2 receptors, and they are abundantly expressed in cholangiocytes. Nevertheless, a cholestatic pattern of liver injury is not the most common finding on presentation, as one might expect[47]. A second proposed mechanism is the cytokine storm, which leads to a surge in inflammatory cytokines affecting the liver[48]. SARS-CoV-2 induced acute respiratory distress syndrome and systemic inflammatory response syndrome lead to hypoxemia and shock, which can cause ischemia-reperfusion injury[49]. It has been reported that SARS-CoV-2 can infect the endothelial

cells directly and result in widespread endotheliitis[13]. Furthermore, administration of multiple drugs attempting to treat the disease have the potential to produce DILI, exacerbating the liver involvement of the disease[50].

The main limitation of our study is the inclusion of autopsies only, resulting in bias towards the most severe disease, with great influence by pre-existing co-morbid conditions. In our literature search, there were no reported liver biopsies from non-severe COVID-19 patients. A correlation between clinical condition, biochemistry, liver imaging, and autopsies would be desirable to explore. Another limitation of this systematic review is the high heterogeneity in the published articles. Finally, since this is a systematic review from autopsy data, relevant clinical information to interpret the causes of steatosis (such as alcohol consumption or body mass index) was not available.

CONCLUSION

In summary, in this systematic review and meta-analysis of autopsies from patients with COVID-19, we found a high prevalence of hepatic steatosis and the presence of vascular thrombosis as major histological liver features. Further studies are needed to establish the mechanism and implications of these findings.

ARTICLE HIGHLIGHTS

Research background

The liver is frequently involved during severe acute respiratory syndrome coronavirus 2 infection and coronavirus disease 2019 (COVID-19). However, there is no consensus about the main histopathological findings in COVID-19.

Research motivation

Identifying the main histopathological findings could help understand the mechanism of liver injury frequently observed in COVID-19 patients.

Research objectives

The characterization of the liver histopathological findings will impact the interpretation of liver chemistries and liver biopsies in COVID-19 patients.

Research methods

We conducted a systematic review and meta-analysis, including liver biopsies and autopsies of COVID-19 patients. Proportions were estimated using random-effects models.

Research results

We included 18 studies. The major histological findings are hepatic steatosis (55.1%), congestion of hepatic sinuses (34.7%), vascular thrombosis (29.4%), fibrosis (20.5%), Kupffer cell hyperplasia (13.5%), portal inflammation (13.2%), and lobular inflammation (11.6%). Other abnormalities can be identified, such as venous outflow obstruction, phlebosclerosis of the portal vein, herniated portal vein, periportal abnormal vessels, hemophagocytosis, and necrosis.

Research conclusions

Steatosis, vascular thrombosis, fibrosis, and inflammatory abnormalities are the most frequent liver histopathological findings in COVID-19 patients.

Research perspectives

The multiple liver histopathological findings observed in COVID-19 demonstrate the susceptibility to liver injury in risk populations, the inflammatory response, and thrombosis associated with this infection.

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CASE REPORT

Primary intestinal lymphangiectasia in an adult patient: A case report and review of literature

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Abstract

BACKGROUND

Primary intestinal lymphangiectasia (PIL), first described in 1961, is a rare disorder of unknown etiology resulting in protein-losing enteropathy. The disease is characterized by dilatation and leakage of intestinal lymph vessels leading to hypoalbuminemia, hypogammaglobulinemia, and lymphopenia. Since the severity and location of lymph vessels being affected can vary considerably, the range of associated symptoms is wide from mild lower-limb edema to generalized edema, abdominal and/or pleural effusion, and recurrent diarrhea, among others. Although usually developing in early childhood, we present the case of a 34-year-old woman with PIL. Moreover, we performed a literature review systematically assessing clinical presentation, and provide a practical approach to facilitate diagnosis and therapy of PIL in adults.

CASE SUMMARY

Our patient presented with unspecific symptoms of abdominal discomfort, fatigue, nausea, and recurrent edema of the lower limbs. Interestingly, a striking collinearity of clinical symptoms with female hormone status was evident. Additionally, polyglobulia, hypoalbuminemia, hypogammaglobulinemia, and transient lymphocytopenia were evident. Due to suspicion of a bone marrow disease, an extensive diagnostic investigation was carried out excluding secondary causes of polyglobulinemia and hypoalbuminemia. The diagnosis of primary intestinal lymphangiectasia was established after 22 wk by histological analysis of biopsy samples obtained *via* enteroscopy. Consecutively, the patient was put on a high-protein and low-fat diet with medium-chain triglycerides supplementation leading to significant improvement of clinical symptoms until 2 years of follow-up.

CONCLUSION

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Specialty type: Gastroenterology and hepatology

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Grade B (Very good): B, B, B
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PIL can be the reason for cryptogenic hypoalbuminemia, hypogammaglobulinemia, and lymphopenia in adulthood. Due to difficulty in correct diagnosis, treatment initiation is often delayed despite being effective and well-tolerated. This leads to a significant disease burden in affected patients. PIL is increasingly been recognized in adults since the majority of case reports were published within the last 10 years, pointing towards an underestimation of the true prevalence. The association with female hormone status warrants further investigation.

Key Words: Primary intestinal lymphangiectasia; Waldmann´s disease; Protein losing enteropathy; Hypoproteinemia; Case report

Core Tip: Although Primary intestinal lymphangiectasia usually develops in early childhood, we present the case of a 34-year-old woman. We observed a striking collinearity with female hormone status in our patient, presenting a potential area of future research. Moreover, we performed a literature review of all published case reports so far and systematically assessed clinical presentation to provide a practical approach to facilitate diagnosis and therapy of primary intestinal lymphangiectasia in adults for the first time.

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INTRODUCTION

Primary intestinal lymphangiectasia (PIL) has been first described by Waldmann *et al*[1] in 1961 as a rare disorder of intestinal lymphangiectasia that results in protein-losing enteropathy (PLE)[1]. PIL is caused by a diffuse or localized dilatation and/or rupture of intestinal lymphatic vessels in the mucosa, submucosa, or subserosa due to high pressure in lymphatic vessels[2]. Importantly, prevalence and etiology are yet unknown. However, genetic associations are been discussed since the diagnosis is generally established in childhood with very rare cases in adults[3].

Symptoms largely relate to the severity of lymph loss and consecutive loss of proteins resulting in hypoproteinemia, lymphopenia, and decreased serum levels of immunoglobulins. Among others, these symptoms include pitting edemas of the lower limbs, generalized edema, as well as pleural, epicardial, or often chylous abdominal effusion[4]. Here, we present the case of PIL in a 34-year-old female patient, together with a literature review of all case reports on PIL in adulthood (using the terms "Primary intestinal lymphangiectasia" and "Waldman's disease"), focusing on clinical presentation, and providing a diagnostic and therapeutic overview for clinicians to enhance recognition and facilitate diagnosis.

CASE PRESENTATION

Chief complaints

In October 2018, a 34-year-old woman presented with recurring nausea independent of food intake, episodes of abdominal discomfort, fatigue, occasional episodes of diarrhea as well as a feeling of increased susceptibility to opportunistic infections.

History of present illness

After the end of pregnancy earlier this year, she reported on more frequently observed limb edema. The other medical history including comorbidities and drug intake were

unremarkable.

History of past illness

Interestingly, temporary facial edema were occasionally reported already during childhood starting at the age of 12. After puberty, pronounced edema of the lower limbs, recurring nausea and fatigue were continuously reported, but vanished when using oral contraceptives or during pregnancy.

Physical examination

The physical examination at the initial contact did not reveal any abnormalities. Especially, no edema of the upper or lower limb were observed and weight was stable with a body mass index of 18.3 kg/m^2.

Laboratory examinations

During several routine blood tests after pregnancy, polyglobulia [hemoglobin 17.4 g/dL (normal range: 12.3-15.3 g/dL), blood count 5.82 (normal range: 3.60-5.00)], normal leukocyte count (4.48, normal range: 4.40-11.30) with diminished lymphocyte count (14.6%, normal range: 19.3%-51.7%) following differential blood count, and reduced total serum protein (4.9 g/dL, normal range: 6.2-8.2 g/dL) with hypoalbuminemia (2.8 g/dL, normal range: 3.4-5.0 g/dL) were observed. Additionally, quantitative immunoglobulin analysis displayed hypogammaglobulinemia of 7.3% (normal range: 11.1%-18.8%) with a pronounced deficiency of the IgG class (268 mg/dL, normal range: 700-1600 mg/dL), moderate deficiency of IgA class (54 mg/dL (normal range: 70-500 mg/dL), and reduced IgG-1 and IgG-2 subclasses (193 mg/dL, normal range: 405-1011 mg/dL; 93 mg/dL, normal range: 169-786 mg/dL). Additionally, the CD4: CD8 T-cell ratio was reduced [0.9 (normal range: 1.0–3.6) and kappa and lambda light-chains were diminished [75 mg/dL (normal range: 173-383 mg/dL), 46 mg/dL (normal range: 81-192 mg/dL)]. The urinary sediment showed no proteinuria and no signs of renal, hepatic, pancreatic, or cardiac disease. Since the laboratory constellation pointed towards a cellular and humoral immune defect, exhaustive investigations were started. Negative results for JAK2 mutations (JAK2-exon 12 sequencing and JAK2-mutation V617F) and negative BCR/ABL ratio ruled out polycythemia vera. Additionally, bone marrow analysis neither showed myeloid neoplasia nor infiltration by lymphoma, and ß2-microglobulin was within the normal range ruling out a hematogenous disease. Autoantibody screening and virus serology including hepatitis viridae, cytomegalovirus, Epstein-Bar virus, and human immunodeficiency virus were negative, and pancreatic insufficiency was excluded.

Imaging examinations

Incidentally, abdominopelvic computerized tomography (CT) showed a thickened wall of the ileum and jejunum with enlarged mesenteric lymph nodes up to 32 mm localized in the lower abdomen. Therefore, an ileocolonoscopy with exploration of > 8 cm of the ileum was performed showing a normal result. After CT-findings were confirmed on magnetic resonance imaging, a gastroduodenoscopy with standard intubation to the mid-descending duodenum revealed creamy white spots of the duodenal mucosa, suggesting lymphedema (Figure 1A). However, the histological evaluation did not show evidence for dilated lymph vessels or PIL, giardiasis, celiac disease, Whipple disease, or intestinal bowel disease, which was additionally excluded by normal calprotectin levels. Following video capsule endoscopy that showed a snowflake appearance of the mucosa (Figure 1B), double-balloon enteroscopy exploring approximately 70 cm of the jejunum verified mucosal lesions compatible with lymphangiectasia macroscopically (Figure 1C).

FINAL DIAGNOSIS

Finally, PIL was confirmed on histological and immunohistological analyses from jejunal biopsies (Figures 2 and 3).

TREATMENT

After putting the patient on a medium-chain triglyceride (MCT) diet rich in protein,

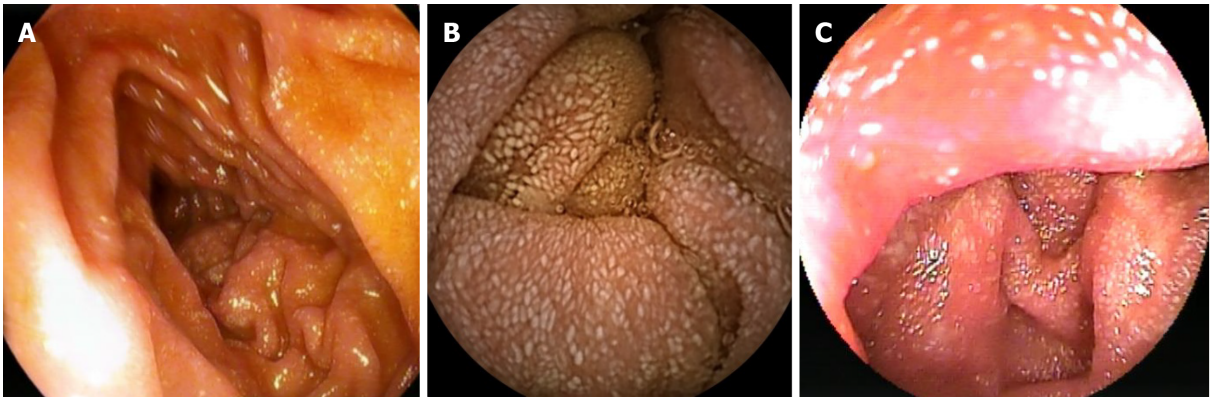

Figure 1 Imaging examinations. A: Endoscopic view of the descending part of the duodenum showing spots of lymphangiectasia suggestive for lymphedema; B: Video capsule endoscopy with a snowflake appearance of the jejunum compatible with dilated mucosal lymphatic vessels; and C: Double-balloon enteroscopy of the jejunum with an almost identical image to video capsule endoscopy.

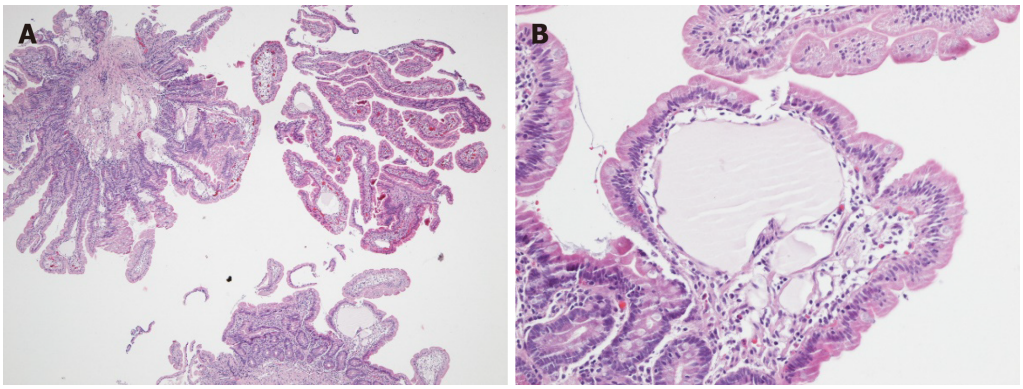

Figure 2 Histological and immunohistological analyses. A: Jejunal biopsies showing a mild and focal blunting of the villi in particular above the prominent ecstatic mucosal lymph vessels (4-fold magnification); B: Ecstatic lymph vessel without inflammatory changes or abnormalities of the epithelial intestinal cell lining (200-fold magnification).

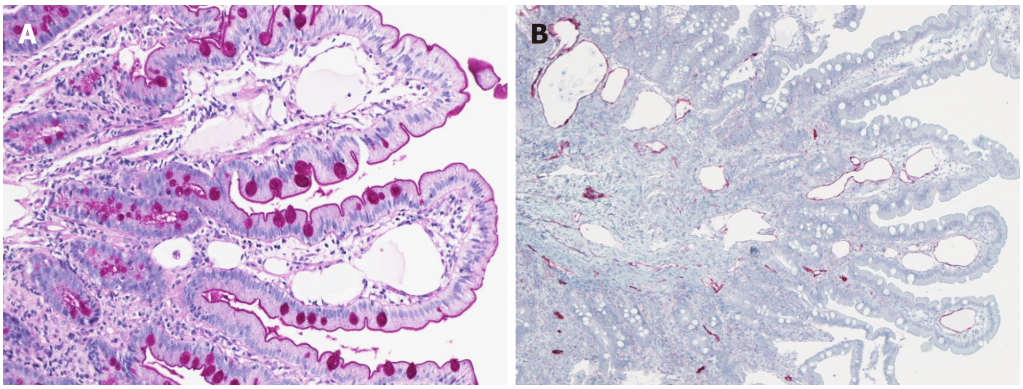

Figure 3 Histological and immunohistological analyses. A: Periodic Acid-Schiff staining did not reveal any collections of periodic acid-schiff positive histiocytes or inclusions, demonstrating the perfectly normal intestinal brush border (200-fold magnification); B: Immunohistochemistry for D2-40, a marker of lymphatic endothelial cells, confirms the endothelium to be of lymphatic origin and highlights the presence of multiple ecstatic lymph vessels in both the mucosa and submucosa (100-fold magnification).

the clinical condition of the patient significantly improved within 4 wk.

OUTCOME AND FOLLOW-UP

Until 2 years after diagnosis, mild lower limb edema were only observed between the

end of breastfeeding period and a second pregnancy, and abdominal discomfort, fatigue and nausea significantly improved. Laboratory improvement was characterized by increase in total serum protein, albumin, and quantitative immunoglobulin levels.

DISCUSSION

Due to the rarity of this disease, the worldwide incidence of PIL in humans is unknown[3]. However, a genetic predisposition is been discussed since if mostly affects children below 3 years of age[5]. This is supported by familial forms of specific syndromes that have been associated with PIL, including the yellow-nail syndrome, von Recklinghausen's disease, Turner, Noonan, Klippel-Trenaunay or Hennekam syndrome[3,6,7]. Nevertheless, cases in adult patients exist. We performed a literature review and could identify 49 cases from 46 case reports of PIL in adults in which the onset of symptoms occurred after the 18th birthday (Table 1, Supplementary Table 1[8-53]). Notably, 27/46 (58.7%) were published since 2010, indicating that this entity is increasingly been recognized and the prevalence might be underestimated. Median age at diagnosis was 43 (range: 20-83) years while median time from onset of symptoms to final diagnosis was 3 (range: 0-40) years, highlighting the difficulty in the correct diagnosis of this entity. Although a literature review of PIL cases existed reporting a mean age of 13.3 years at symptom onset and 8.5 years until diagnosis, it has to be pointed out that 75% of cases in this review included patients with symptom onset before 20 years of age[54]. In terms of gender distribution, 22 male (44.9%) and 27 female (55.1%) cases were reported.

Our case describes a 34-year-old female being diagnosed with PIL around 22 years after the first occurrence of edema, and 22 wk after the first contact at our institution. She presented with nausea, abdominal discomfort, diarrhea, and bilateral limb edemas. From 48 patients reporting on symptoms in the literature, 40 patients (83.3%) reported the presence of any peripheral/generalized edema while only 2 patients did not present with edema and 6 case reports did not report on this symptom. The majority (n = 27, 56.3%) only reported bilateral edema of the lower limb. 9/48 patients (18.8%) presented with abdominal pain, 13 patients (27.1%) presented with (chylous) ascites, 10 patients (20.8%) with pleural, and 4 patients (8.3%) with pericardial effusion. Diarrhea was present in 20/48 patients (41.7%). Other rare unspecific symptoms include changes in weight, nausea, general weakness, pallor, and gastrointestinal bleeding. These findings go in line with a former literature review of 84 PIL cases (including predominantly children), reporting limb edema, diarrhea, ascites, and lymphedema in 78%, 62%, 41%, and 22% respectively[54]. These symptoms and their varying extent can largely be explained as a consequence of lymphatic/protein and subsequently watery loss due to lower oncotic pressure in interstitial fluid.

The fact that pregnancy and oral contraception led to the vanishing of edemas in our patient is indeed surprising as this has not yet been reported. Of note, symptoms completely resolved when taking oral contraceptives and re-appeared during pill-free days in between. One may hypothesize that differences in estradiol might influence the severity of lymphedema: Morfoisse *et al*[55], who explored the role of estrogens on lymphatic endothelial cells, found that estradiol is protective of lymphedema, and blockage of the estrogen receptor is associated with stronger lymphatic leakage. However, this was only shown in an animal model of secondary lymphedema, and no other studies providing further evidence on this mechanism are available.

Among the most frequently observed laboratory findings in literature were anemia in 16 patients (33.3%), lymphocytopenia in 30 (62.5%), hypoproteinemia in 26 (54.2%), and hypoalbuminemia and hypogammaglobulinemia/reduced serum IgG level in 35 (72.9%) while no patients specifically reported the absence of the latter two laboratory findings. However, these numbers might be underestimated since not all case reports reported on these features. 12 patients (25.0%) specifically reported reduced serum levels of calcium and 5 patients (10.4%) reduced levels of magnesium, leading to occasional muscle seizures in several patients. Other findings include hypoglobulinemia with reduced numbers of IgM, IgA, and IgG, and reduced numbers of CD3+ and CD4+ cells. Profound hypoproteinemia, hypoalbuminemia, and hypogammaglobulinemia and reduced CD4:CD8 ratio could also be confirmed in our patient. However, lymphocytopenia was only transient and leucocyte count was within the normal range indicating that these parameters might be very unspecific and significantly influenced by temporary inflammatory processes in the body. This is especially true since an increased susceptibility to opportunistic infections based on

Table 1 Clinical presentation, laboratory findings and endoscopic diagnosis of all case reports describing adult patients[1] with primary intestinal lymphangiectasia

Clinical presentation		*n*
Patient characteristics	Number of adult patients with PIL	49
	Age at diagnosis, yr	43 (20-83)
	Time to final diagnosis, yr	3 (0-40)
	Male, *n* (%)	21 (42.9)
	Female, *n* (%)	28 (57.1)
Symptoms, *n* (%)	Edema	40/48 (83.3)
	Recurrent diarrhea	20/48 (41.7)
	Abdominal effusion	13/48 (27.1)
	Pleural effusion	10/48 (20.8)
	Abdominal pain	9/48 (18.8)
Laboratory findings, *n* (%)	Hypogammaglobulinemia	35/48 (72.9)
	Hypoalbuminemia	35/48 (72.9)
	Lymphocytopenia	30/48 (62.5)
	Hypoproteinemia	26/48 (54.2)
	Hypocalcemia	12/48 (25.0)
	α1-antitrypsin (stool)	10/48 (20.8)[2]
CT-scan, *n* (%)	Normal	8/27 (29.6)
	Thickened wall of small bowel	11/27 (40.7)
Diagnosis possible, *n* (%)	Gastro-duodenoscopy	21/35 (60.0)
	Ileo-colonoscopy	5/21 (23.8)
	Enteroscopy	13/13 (100)

[1]Diagnosis established after the 18th birthday.
[2]All patients (100%) in which α1-antitrypsin levels or α1-antitrypsin clearance was measured in stool samples reported elevated results. CT: Computerized tomography.

lymphocytopenia and hypogammaglobulinemia could be present. This was reported in our patient, 2 case reports of adult patients with additional 2 patients suffering from cryptococcal meningitis at initial presentation, and 3 case reports of children[5]. This humoral and cellular immunodeficiency is assumed to be due to a lymphatic loss of B- and T- lymphocytes. Interestingly, 4 patients in the literature report an extensive presence of warts, probably representing the end-stage of acquired immunodeficiency.

Notably, fecal α1-antitrypsin levels or α1-antitrypsin-clearance seem to be a good indicator for the presence of PLE/PIL in these patients with positive results in all patients who reported on this feature (10/48, 20.8%). Since α1-antitrypsin is resistant to degradation by digestive enzymes, it indicates the presence of blood proteins in the intestinal tract[56].

Because of persistently diminished IgG and IgA, an abdominal/pelvic CT scan was performed to rule out lymphoma or thymoma in our patient. This incidentally revealed a thickened wall in the jejunum and ileum with enlarged lymph nodes. Interestingly – when looking into the literature – 11/27 of patients (40.7%) undergoing a CT scan reported abnormalities in the small bowel wall while 8/27 (29.6%) had a completely normal result. However, other imaging modalities such as lymphangioscintigraphy or technetium-labeled human serum albumin (99TmTc-HSA) scintigraphy might be of higher accuracy pointing towards the diagnosis of PIL, demonstrating abnormal lymphatic, or protein leakage.

When comparing endoscopic findings in these patients, 21/35 (60.0%) gastroduodenoscopies performed in symptomatic PIL patients revealed an endoscopic view suggestive for PIL with "snowflake appearance" of the duodenal mucosa indicating lymphatic dilations while in 2 patients the diagnosis could be made

histologically despite normal macroscopic appearance. On the contrary, ileocolonoscopy was inferior with only 5/21 (23.8%) showing characteristic features in the terminal ileum that led to the diagnosis. However, it has to be mentioned that considering the improvement in technology, this rate might be underestimated due to the large number of studies performed > 10 years ago. Enteroscopy, which was performed in 14 patients including our patient to establish the diagnosis, is highly sensitive and should be regarded as the gold-standard for diagnosis. Video capsule endoscopy could be used to help with diagnosis, being similarly sensitive to detect lymphedema in the small bowel.

In our case, although a gastroduodenoscopy was performed showing an endoscopic snowflake appearance of the duodenal mucosa indicating lymphatic dilations, the histological report was normal. To our opinion, two possible explanations exist for this phenomenon: On the one hand, the dilated lymphatic endothelial cells can be distributed in different locations in the intestine to a different extent, hence the histology specimens might not show dilated lymphatic endothelial cells despite endoscopic and macroscopic appearance. On the other hand, the lack of experience of pathologists with this entity could result in an insufficient evaluation of the histological specimens and delay of diagnosis, as this was the case in our patient.

Lifelong adherence to a diet rich in protein with substitution of MCT remains the cornerstone in the therapy of PIL. Because MCTs are directly absorbed into the portal venous system bypassing the lymphatic system, they can be used to overcome chronic malnutrition. The need for dietary control in people appears to be permanent because clinical and biochemical findings seem to reappear after low-fat dietary withdrawal. 16/26 patients (61.5%) receiving MCT alone reported significant improvement in symptoms while 2 patients reported only moderate improvement. Octreotide can be regarded as the preferred treatment in patients in whom dietary changes fail to achieve significant improvement. Octreotide is a long-acting somatostatin analogue that suppresses gastrointestinal motility and hormone secretion in the pituitary gland, pancreas, and intestine. Although the mechanism of action of octreotide in diminishing protein loss through the gastrointestinal tract is unclear, theorized mechanisms of octreotide's action in PIL include decreased intestinal fat absorption, inhibition of gastrointestinal vasoactive peptides, and stimulation of the autonomic nervous system[57-59]. Octreotide is usually given at doses of 150-200 μg subcutaneously twice daily[14]. From all 29 cases that reported efficacy of therapy, octreotide was added to MCT in 6 patients and started as initial treatment in one patient, with 2 patients having an insufficient response and 2 patients report in recurrence of symptoms after discontinuation of octreotide with otherwise good response. Other medical therapeutic options include propranolol, which is thought to downregulate the RAF mitogen-activated protein kinase signaling pathway with reduced expression of VEGF, and everolimus, which is an mTOR inhibitor. mTOR is a serine/threonine kinase, representing a key enzyme for numerous cellular processes including angiogenesis and cell growth. Ozeki *et al*[60] found significant mTOR expression in tissues affected by PIL and applied everolimus (1.6 mg/m^2/day) as a treatment of PIL improving diarrhea and hypoproteinemia. However, no case report on an adult patient with PIL exists using these two substances. Surgical resection seems to be the last option both for diagnosis and therapy of PIL. In 6/49 patients, diagnosis of PIL was established after surgical resection, however in most cases without performing an enteroscopy before. All 7 patients that reported surgical resection as the form of treatment – sometimes after the failure of conservative therapy – described improvement of the clinical condition after surgery. However, long-term follow-up does not exist in these patients.

Nevertheless, long-term follow-up is needed since lymphoma have been described as long-term complications in patients with PIL. Laharie *et al*[40] reported on 12 cases of lymphoma after PIL, which was adopted for the present case report and completed by literature review of additional cases until 2020[61]. So far, 13 cases have been published with lymphoma occurring after a median of 14 years (range: 0-39) after PIL diagnosis (Supplementary Table 2).

CONCLUSION

In conclusion, PIL can be a rare cause of PLE in adults. Unspecific symptoms and a wide range of clinical manifestations can significantly hamper establishing the definite diagnosis, leading to a "diagnostic roller coaster" for the individual patient. Despite good treatment options, low recognition of this entity leads to significant morbidity in

these patients. Following review of published case reports, we present a practical overview of symptoms, laboratory findings, the accuracy of diagnostic modalities, and a potential treatment approach to facilitate diagnosis, and management of these patients. Finally, we highlight the striking collinearity with female hormone status in our patient, presenting a potential area of future research (Figure 4).

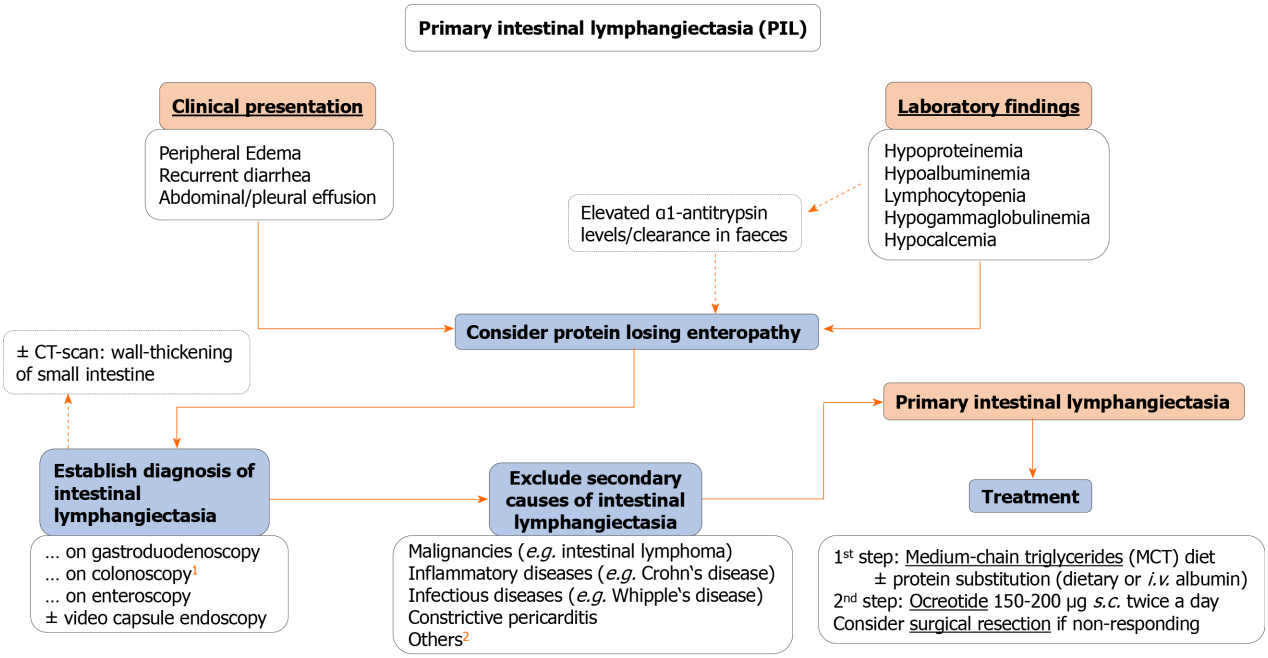

Figure 4 Proposed algorithm for diagnosis and therapy of primary intestinal lymphangiectasia in adults. [1]Including intubation of the terminal ileum. [2]Other secondary causes include: Retroperitoneal fibrosis following radio-/chemotherapy, sarcoidosis, intestinal tuberculosis, systemic sclerosis, human immunodeficiency virus-related enteropathy, Fontan-surgery.

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