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REVIEW

Immune response modulation in inflammatory bowel diseases by *Helicobacter pylori* infection

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Abstract

Many studies point to an association between *Helicobacter pylori* (*H. pylori*) infection and inflammatory bowel diseases (IBD). Although controversial, this association indicates that the presence of the bacterium somehow affects the course of IBD. It appears that *H. pylori* infection influences IBD through changes in the diversity of the gut microbiota, and hence in local chemical characteristics, and alteration in the pattern of gut immune response. The gut immune response appears to be modulated by *H. pylori* infection towards a less aggressive inflammatory response and the establishment of a targeted response to tissue repair. Therefore, a T helper 2 (Th2)/macrophage M2 response is stimulated, while the Th1/macrophage M1 response is suppressed. The immunomodulation appears to be associated with intrinsic factors of the bacteria, such as virulence factors - such oncogenic protein cytotoxin-associated antigen A, proteins such *H. pylori* neutrophil-activating protein, but also with microenvironmental changes that favor permanence of *H. pylori* in the stomach. These changes include the increase of gastric mucosal pH by urease activity, and suppression of the stomach immune response promoted by evasion mechanisms of the bacterium. Furthermore, there is a causal relationship between *H. pylori* infection and components of the innate immunity such as the NLR family pyrin domain containing 3 inflammasome that

directs IBD toward a better prognosis.

Key Words: Cytotoxin-associated antigen A oncoprotein; Gut microbiota; *Helicobacter pylori*; *Helicobacter pylori* neutrophil-activating protein; Immunological modulation; Inflammatory bowel disease; NLR family pyrin domain containing 3 inflammasome

Core Tip: *Helicobacter pylori* (*H. pylori*) infection seems to modulate the immune response triggered by inflammatory bowel disease in a way that makes it less aggressive. The virulence factors of *H. pylori*, as well as the mechanisms that allow it to remain in the stomach environment, appear to change the intestinal microenvironment and modulate the local immune response, contributing to a disease with milder symptoms and less tissue damage.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of chronic conditions affecting the gastrointestinal tract, characterized by episodes of abdominal pain, diarrhea, bloody stools, and weight loss. The two main types of IBD are Crohn's disease (CD) and ulcerative colitis (UC)[1]. In CD any area of the gastrointestinal tract can be affected, but the most affected regions are the terminal ileum, the cecum, the perianal region, and the colon, while in UC the inflammation is restricted to the colon and rectum[1]. Regarding histology, in CD the intestine presents thickened submucosa, transmural inflammation, ulceration and non-caseating granulomas, while in UC the inflammation is limited to the mucosa, with crypts and abscesses[2].

The pathogenesis of IBD is complex and involves a combination of genetic, environmental, and immunological factors. The gut microbiota is critical to homeostasis in this organ and contributes to the tolerance process, to the development and differentiation of the local and systemic immune system, and may protect the host from pathogenic enteric infections. However, in IBD, luminal bacteria trigger deregulated immune responses, acting as the main environmental factor for the development of these pathologies[3,4].

The loss of tolerance to the commensal microbiota seems to be related to the genetic susceptibility of the individual and to an imbalance in the composition of the microbiota[5]. IBD patients have changes in stool composition, with less bacterial quantity and diversity[6,7].

Genome wide association studies of IBD have identified 99 non-overlapping genetic risk loci including 28 that are shared between CD and UC[8]. These genetic alterations seem to implicate several important pathways, such as intestinal barrier function, regulation of innate and adaptive immunity, reactive oxygen species formation, autophagy, and endoplasmic reticulum stress[9]; associations were identified between polymorphisms in the genes for interleukin (IL)-10, IL-10 receptor alpha, and components of this signaling pathway - signal transducer and activator of transcription 3, tyrosine kinase 2, and JAK2, with the early development of IBD[10].

Dysregulation of the immune component in IBD is marked by abnormal mucus production, failure to repair the epithelial barrier, and excessive and persistent activation of T lymphocytes, B cells, dendritic cells, macrophages, and natural killer cells[11,12].

Naive TCD4+ cells can differentiate into T helper 1 (Th1), Th2, Th9, Th17 and regulatory T (Treg) subpopulations; the main stimulus for differentiation into Th1 cells - secreting interferon (IFN)-γ, tumor necrosis factor (TNF) and IL-2, is the expression of IL-12, while IL-4 leads to differentiation into Th2 cells, whose cytokine pattern is marked by the expression of IL-4, IL-5, IL-13 and IL-25[13]. Classically, CD and UC are described as diseases with a Th1 and Th2 immune pattern, respectively[14-16], since typically CD patients have higher IFN-γ and IL-2 expression than UC patients[17,18], that express more IL-5 and IL-13[19,20]. However, some studies contradict this information, since lower levels of IL-13 were seen in UC patients compared to CD patients[21], and high levels of IFN-γ were found in carriers of both diseases[22]. In line with this, Bernardo *et al*[23] found a mixed cytokine pattern in biopsies from UC patients, associated with low IL-13 levels.

A Th17 pattern response prevails in the gut. The differentiation into Th17 cells leads to the expression of IL-17, IL- 21, IL- 22, and IL-23, which are found in large quantities in the intestine of IBD patients[24]. A study using IL-17 receptor (IL-17R) knockout mice showed that IL-17R deficiency protected the animals from developing trinitrobenzene sulfonic acid (TNBS)-induced colitis[25-27].

Although not conclusive, there is evidence to indicate that two members of this cytokine family have different effects; IL-17A appears to have a protective effect through inhibition of the Th1 response[28], furthermore, other studies indicate that deficiency of this cytokine causes exacerbation of colitis, while IL-17F contributes to and aggravates the inflammatory process[29]. Despite the prevalence of Th17 cells in the gut in the absence of IBD, this response pattern appears to be exacerbated with disease onset leading to increased production of pattern marker cytokines. Perhaps this increase occurs relatively disproportionately, prioritizing the increase in cytokines that induce an inflammatory response, such as IL-17F.

Treg cells which is characterized by constitutive expression of forkhead/winged helix transcriptional factor P3 (FoxP3), CD25 and cytotoxic T lymphocyte-associated protein 4, play a major role in the pathogenesis of IBD[30]. In these diseases, there is a significant dysfunction in the activity of these cells, either by being in low numbers or by having their function suppressed. It has been observed that effector cells from IBD patients exhibit relative resistance to Treg-mediated suppression by expressing high levels of Smad7, an inhibitor of the transforming growth factor (TGF)-β signaling pathway[31,32]. The unbalanced and uncontrolled local immune response against the bacterial microbiota in the IBD occurs when it is poorly controlled by endogenous counter regulatory mechanisms, such as the immunosuppressive cytokine TGF-β. Studies show that the inefficiency in control occurs due to a blockade of TGF-β signaling, caused by a blockade in the phosphorylation of the signaling molecule associated with the TGF-β activated receptor, Smad3. The blockade of Smad3 activity is caused by the upregulation of the Smad7[33].

Individuals with a mutation in the *FOXP3* gene often suffer from intestinal inflammation[34]. Treg cells are able to convert to Th17 cells under inflammatory conditions, and the cytokine IL-1β is key in this process[35]. The balance between the Th17 and Treg response is important, especially in places such as the gut, where there is a commensal microbiota, to prevent deregulated immune responses. IBD patients have a reduced Treg/Th17 ratio in peripheral blood compared to healthy individuals. The installation of the inflammatory process in IBD seems to be fueled by an unbalanced increase in the production of Th17 cytokines, which promote the Th1 pattern. Pro-inflammatory cytokines produced by Th1 cells, such as IL-1β, potentiate the inflammatory process by decreasing the Treg/Th17 ratio, both by proportionally increasing the Th17 response and by reducing the suppression of the inflammatory response by Treg cells. Regarding Th9 cells - capable of exacerbating inflammatory processes by increasing epithelial permeability[36], are at high levels in CD and UC[37,38].

IMMUNOMODULATION OF THE INFLAMMATORY PROCESS AND PATHOGENESIS OF IBD BY *HELICOBACTER PYLORI*

Influence of Helicobacter pylori cytotoxin-associated antigen A+ infection on IBD prognosis

Cytotoxin-associated antigen A (*CagA*) is a gene found in the final portion of the Cag pathogenicity island of the *Helicobacter pylori* (*H. pylori*) genome and encodes the oncogenic protein CagA. Its presence is more frequent in East Asian populations than in those from the West, and determines a number of interactions between the bacterium and the host [39]. Although there is a causal relationship between *H. pylori* CagA+ and the incidence of cancers of the gastrointestinal tract[39,40], when it comes to IBD, especially CD and UC, the presence of the *CagA* gene may positively influence the prognosis of patients[41].

In a first analysis, age, genetic and environmental factors associated with innate and adaptive immunity are key factors in the development of IBD[42,43], thus the presence of *H. pylori* infection becomes yet another variable. Some studies point to several hypotheses for the improved prognosis of IBD in the presence of the *H. pylori CagA* gene, such as the conversion of the M1 macrophage lineage into M2 through modulation of the Th17/Treg immune response, in which there is a decrease in the levels of IL-17F and IL-21 with concomitant increase in the expression of IL-10 and Treg cells[44] due to increased plasma IL-13[45].

Also contributing to the process are increased anti-inflammatory responses through increased expression of CD163 and IL-10, suppression of toll like receptors (TLR) mediated signaling pathways[46], and control of the immune response through activation of pathways mediated by the basic leucine zipper transcription factor ATF-like 2 (BATF2)[47-49] (Figure 1).

The CD163 is a macrophage/monocyte scavenger receptor whose expression is positively regulated by IL-10. The upregulated expression of this receptor is one of the most marked changes in the M2/M1 phenotype switch; therefore, high expression of CD163 is characteristic in inflammatory processes[50,51]. According to studies, the number of CD163- M1 monocytes, as well as CD163+ and CD163+/IL-10+ M2 monocytes are significantly increased in individuals infected with *H. pylori*, in addition to having higher levels of IL-10. IL-10 production is significantly higher by M2 cells from individuals with *H. pylori* infection. In addition, individuals infected with CagA-positive *H. pylori* strains had a significantly higher number of CD163+ and CD163+/IL-10+ monocytes compared to those infected with CagA-negative strains[52].

The TLRs are expressed by cells of the intestinal epithelium, and by cells of the immune system present in the gut, such as leukocytes, dendritic cells, and various polymorphisms of these receptors have been associated with susceptibility to IBD[53,54] and they are involved in signaling pathways leading to the expression of several inflammatory genes[55,56]. BATF2 has unique functions in the regulation of cytokine gene expression by TLR signaling in macrophages[57].

H. pylori has metabolic adaptations for gastric colonization that, in the background, participate in modulating the inflammatory process of IBD. The urease enzyme that confers *H. pylori* resistance to stomach acidity, participates in immunomodulation as it alters particle opsonization, facilitates apoptosis, and by being presented by MHC II increases the expression of pro-inflammatory cytokines[58].

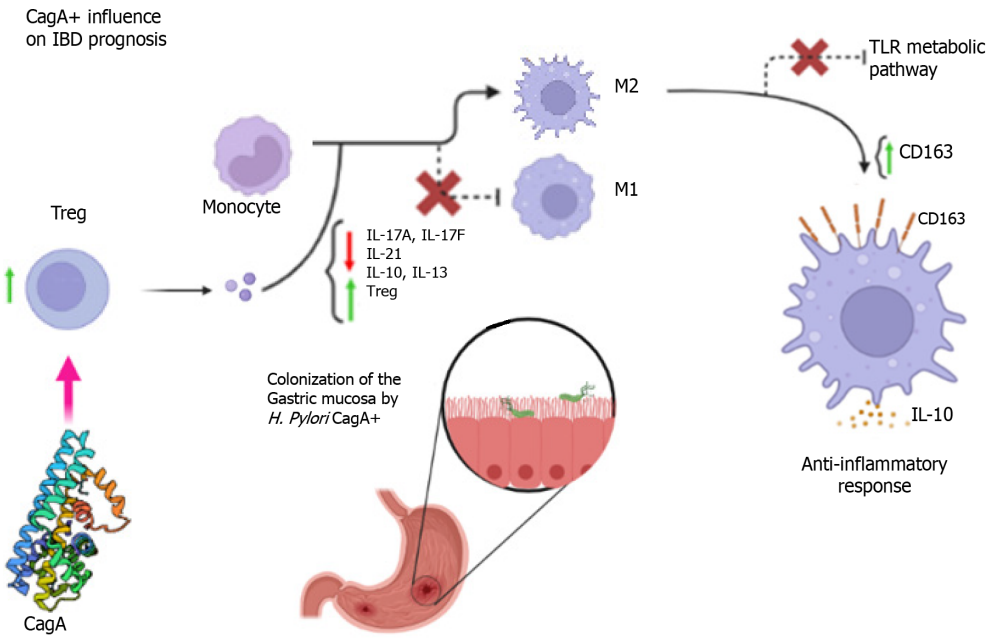

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Figure 1 Mechanisms of *Helicobacter pylori* cytotoxin-associated antigen A+ on favorable prognosis of inflammatory bowel diseases. The presence of the cytotoxin-associated antigen A gene at the Cag pathogenicity island induces the modulation of T helper 17/Treg immunological response (1), which reduces levels of interleukin (IL)-17F, IL-17A, and IL-21 and increases the expression of IL-13, IL-10, and Treg (2). These two factors are synergists in the conversion process of the M1 to M2 macrophage lineage (3). Then, M2 macrophages suppress signaling mediated by toll-like receptors (4) and activate metabolic pathways mediated by basic leucine zipper transcription factor ATF-like 2, increasing CD163, a macrophage/monocyte scavenger receptor (5). Finally, the levels of IL-10 expression also increase (6), leading to anti-inflammatory effects (7) and, therefore, to a better prognosis on inflammatory bowel diseases. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; TLR: Toll-like receptor; IBD: Inflammatory bowel disease; CagA: Cytotoxin-associated antigen A; Treg: Regulatory T.

The flagella of *H. pylori*, besides the fundamental importance in its motility for gastric colonization, also induces an inflammatory response. FlaA and FlaB flagellins can promote a humoral response by stimulating the production of specific antibodies[59,60]. Furthermore, studies report that bacteria with increased motility increase IL-8 release and suggest that genes which regulate flagellin production may alter adhesin expression[61-63] facilitating colonization. The chemoattractant effect of IL-8 is all too well known, however, data on the association of IL-8 with *H. pylori* infection are still scarce[64].

Superoxide dismutase from *H. pylori* has also been shown to have a potential immune suppressive effect by inhibiting the production of pro-inflammatory cytokines, through inhibition of pathways activated by the transcription factor nuclear factor kappa B (NF-κB), as well as macrophage inflammatory protein 1-α[65].

Modification of bacterial gut microbiota by H. pylori

The alteration in the pattern of gastric secretion by *H. pylori* is closely related to the modification of the microbiota of the gastrointestinal tract[66]. Hypochlorhydria enables colonization of the distal intestine by acidic pH-sensitive bacteria; this causes people infected with *H. pylori* to have a more diverse alpha intestinal microbiota compared to uninfected people [66-68]. These changes reflect higher percentages of acidophilic bacteria, proteobacteria, bacteria of the genera *Lactobacillus*, *Haemophilus*, *Streptococcus* and *Gemella*; in contrast, there is a decrease in the percentage of pathogenic anaerobic bacteria such as *Clostridium*[69-72].

In addition to the variation in intestinal pH being one of the factors that alter the diversity of the gut bacterial microbiota, it can also be influenced by the virulence factors CagA and VacA of *H. pylori*, which alter the immune response of the infected individual[73-75]. Hormonal factors, influenced by *H. pylori* infection such as an increase of gastrin secretion alter gut metabolism, in addition, leptin has been directly related to an increase of the amount of the probiotic bacteria *Bifidobacterium* and *Lactobacillus*[76].

Indeed, there is an altered composition of the local microbiota of IBD patients[77]. In these diseases an exacerbated immune response against the commensal microbiota occurs in genetically predisposed individuals and, studies suggest that the balance between pathogenic and beneficial bacterial species is altered in these ones[78]. Therefore, the dysbiosis promoted by *H. pylori* infection may help explain the inverse relationship between bacterial infection and IBD in individuals with the two conditions concomitantly, although the underlying molecular mechanisms are not fully understood[79].

CO-IMMUNOMODULATION OF IBD BY NLR FAMILY PYRIN DOMAIN CONTAINING 3 INFLAMMASOME AND *H. PYLORI* INFECTION

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is a multiprotein complex that plays a crucial role in the innate immune response. The complex is formed by the NLRP3 receptor, the adaptor protein caspase-recruitment domain (ASC) and the enzyme caspase-1 and, appears to play a role in the negative association between IBD and *H. pylori* infection by the IL-1β and IL-18 activity. NLRP3 recognizes a wide variety of stimuli linked to pathogen-associated molecular patterns and damage-associated molecular patterns through pattern recognition receptors such as TLRs and nucleotide-binding oligomerization domain 2. Activation of NLRP3 includes a priming process which components are expressed in greater quantities and depends on several upstream signals such as potassium and chloride efflux, calcium mobilization, lysosomal disruption, and mitochondrial dysfunction with increased reactive oxygen species[80].

Activation of the inflammasome culminates i4n expression of NF-κB and cleavage/activation of caspase-1, responsible for processing pro-IL-1β and pro-IL-18, which are cleaved into their active forms[81,82]. Furthermore, activation of NLRP3 leads to proptosis, a form of programmed cell death mediated by gasdermin D protein. Caspase-1 cleaves gasdermin D, removing its carboxy-terminal portion, which allows its insertion and polymerization into the plasma membrane forming pores. Proptosis also appears to induce the secretion of IL-1β and IL-18[83].

Caspase-1 is highly expressed in the mucosa of the patients infected with *H. pylori*. However, it is observed that together, IL-1β and IL-18 seem to play different roles in controlling the infection and the pathogenicity of the bacterium. While IL-1β presents itself as a potent pro-inflammatory agent, IL-18 has regulatory properties and controls responses mediated by TCD4+ cells. Studies have shown that mice that failed to process IL-18 in the absence of caspase-1 had less bacterial colonization, more robust Th17 responses, and more evident immune pathogenicity compared to control animals[84]. Furthermore, mesenchymal stem cells stimulated by this cytokine promote differentiation of Treg cells into virgin TCD4+ cells, limiting the Th17 response[85], promoting a tolerogenic activity and adjusting chronic inflammation in the gastric mucosa (Figure 2).

The absence of caspase-1 results in an inefficient protective immune response, with a less pronounced Th1 and Th17 pattern[86,87]. There is strong evidence of an association between IL-18 and the prevention and control of allergic diseases such as asthma and rhinitis, through pulmonary infiltration of large amounts of Treg and tolerogenic dendritic cells. This association seems especially beneficial in relation to infection with *H. pylori* CagA+ strains, as observed in a study wherein mice infected with the bacteria in the neonatal period developed specific immunological tolerance and protection against gastric immunopathology resulting from *H. pylori* CagA+ infection[88].

Studies with animal models have shown that NLRP3 activation induced by *H. pylori* infection appears to improve the prognosis of IBD. Engler *et al*[89] observed that mice exposed to the bacterium developed less severe forms of dextran sulfate sodium (DSS)-induced colitis, with significantly milder inflammation and epithelial changes. These positive effects were also observed in animals treated with *H. pylori* extracts. Such beneficial effects are accompanied by positive regulation of TGF-β and the transcription factor caudal-related homeobox transcription factor 2, which regulates the expression of mucins such as mucin 2, a fact that was associated with signaling by NLRP3 and IL-18 production by caspase-1.

Zaki *et al*[90] demonstrated that NLRP3 -/- or ASC -/- mice are more susceptible to DSS-induced colitis and caspase-1 -/- mice are more susceptible to weight loss, diarrhea, rectal bleeding and mortality during the chronic and acute phases of DSS- or TNBS-induced colitis. These findings are related to IL-18 production and its mucosal barrier repair function. Furthermore, IL-18 -/- and IL-18R -/- mice exhibit greater susceptibility to DSS-induced colitis, associated with higher mortality and more severe histopathological changes[91]. Previous studies have reported that the absence of the adaptor protein myeloid differentiation primary response 88 (MyD88), which is involved in the production of IL-18 and IL-1β, increases the severity of inflammatory disease, indicating the importance of MyD88-dependent signaling pathways, such as the TLR4-MyD88 pathway, in blocking the onset and progression of IBD[92-94]. MyD88 is the main signaling adaptor protein of the TLR family. Studies using MyD88-deficient mice suggest a dominant role for TLR/MyD88 signal transduction in preventing intestinal inflammation after acute epithelial injury by promoting epithelial repair[94,95].

Yao *et al*[96] conducted a study with NLRP3R258W mutant mice, a mutation homologous to NLRP3R260W in humans, which causes increased inflammasome activity. The mutant mice developed DSS-induced colitis with milder symptoms compared to wild-type animals, in addition to lower expression of inflammatory mediators, higher expression of IL-18 and IL-1β, and fewer colon tumors. These positive effects were associated with higher activity of Treg cells, positively regulated by IL-1β and fundamental in controlling inflammation. In this study no evidence was found that directly points to IL-18 as an effector molecule in the activation of the inflammasome, but it is believed that this cytokine may be indirectly affected by NLRP3 through secondary effects. However, IL-18 deficiency was shown to override the protective effect of the NLRP3R258W mutation.

IMMUNOMODULATION OF IBD BY NEUTROPHIL-ACTIVATING PROTEIN OF *H. PYLORI*

H. pylori neutrophil-activating protein (HP-NAP) is a virulence factor that plays an important role in immunomodulation. HP-NAP refers to *H. pylori* mini-ferritin, a protein with the ability to activate the production of oxygen radicals by neutrophils promoting their adhesion to the vascular endothelium[97]. Neutrophil adhesion occurs by the positive regulation of β-2-integrin (CD18) expression in a high-affinity state[98,99].

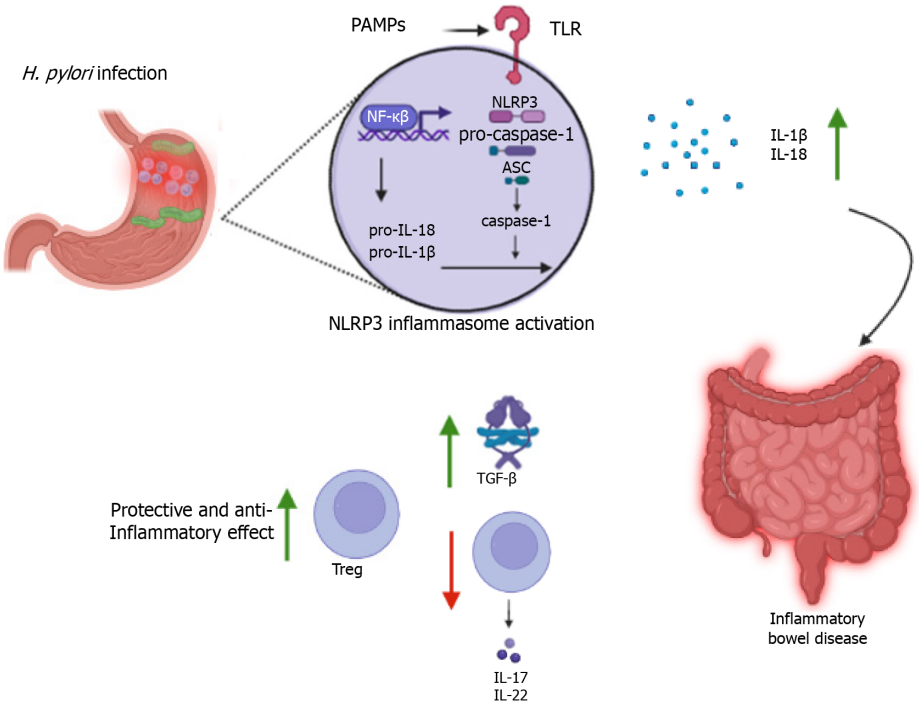

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Figure 2 Immunomodulation of the NLR family pyrin domain containing 3 inflammasome and protection against inflammatory bowel diseases. Pathogen-associated molecular patterns from *Helicobacter pylori* are recognized by the NLR family pyrin domain containing 3 (NLRP3) inflammasome toll-like receptors leading to responses that appear to be associated with improved prognosis and an anti-inflammatory effect. Activated NLRP3 is able to increase the expression of caspase-1 which activates the cytokines interleukin (IL)-18 and IL-1β. The positive regulation of these cytokines leads to extragastric immunomodulation by suppressing the T helper 17 subpopulation and increasing regulatory T, transforming growth factor-β and mucins. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; PAMPs: Pathogen-associated molecular patterns; TLR: Toll-like receptor; TGF: Transforming growth factor; NLRP3: NLR family pyrin domain containing 3; Treg: Regulatory T.

The mini-ferritins have the ability to influence host immune cells in addition to protecting bacterial DNA from oxidizing radicals[100]. HP-NAP is released by *H. pylori* near the gastric epithelial monolayer, thus activating macrophages/monocytes and mast cells, with the release of pro-inflammatory cytokines TNF-α, IL-6, IL-12 and IL-23[101,102]. Similar to the chemokine CXCL8, HP-NAP directly promotes leukocyte recruitment; after HP-NAP transcytosis by endothelial cells, part of these proteins remain bound to the luminal portion of the endothelium, increasing the expression of β-2-integrin and changing the local spatial conformation, a fact that culminates in the extravasation of immune cells[99,103].

The IL-8 secretion by neutrophils present at the site of inflammation enables the recruitment of more neutrophils and other immune cells. IL-8 secretion is promoted by HP-NAP and mediated through interactions with TLR2 receptors and pertussis toxin-sensitive G proteins[104]. HP-NAP also induces mast cells and basophils to secrete TNF-α, IL-6, IL-8, IL-12 and IL-23, and stimulates mast cells to release histamine and IL-6[101,102,105]. Besides influencing innate immunity, HP-NAP modulates adaptive immunity by promoting the release of IL-12 and IL-23 by neutrophils and monocytes, thereby causing a Th1 polarization, and directing the maturation of monocytes to mature dendritic cells[102].

The mature dendritic cells are stimulated by HP-NAP to express MHCII and release Th1 pattern cytokines such as IL-12[102,106]. The increased secretion of IL-12 in the gastrointestinal microenvironment, promoted by HP-NAP, causes gastric specific T lymphocyte subpopulations to be able to produce large amounts of IFN-γ and TNF-α and, to exhibit cytotoxic activity[102]. Increased Th1 pattern and cytotoxic response, induced by *H. pylori* infection, may be beneficial in pathologies which the Th2 response is the detrimental mechanism (Figure 3). The ability of HP-NAP to reduce Th2 activity due to the polarization towards Th1 was proven in experiments using mice with atopic dermatitis[107]. Therefore, HP-NAP may have a therapeutic effect in situations which there is a predominance of Th2 response, as was observed in HP-NAP inoculation assays in mice with allergic asthma, which significantly reduced serum immunoglobulin E levels with concomitant increase in IL-2, thus decreasing eosinophil infiltration[108].

Thus, the Th1-directed polarization promoted by HP-NAP, may be a possible explanation for the improvement of IBD symptoms, whose pathogenesis may be the result of a dysregulated Th2 response, as occurs in UC, in which there is a predominance of a Th2 response and inhibition of the Th1 response, in individuals infected with *H. pylori*[109-112].

CONCLUSION

Although significant progress has been made over the last few years in defining the mechanisms that *H. pylori* use to influence on IBD evolution, there is clearly much that remains to be elucidated and many questions persist. In this review

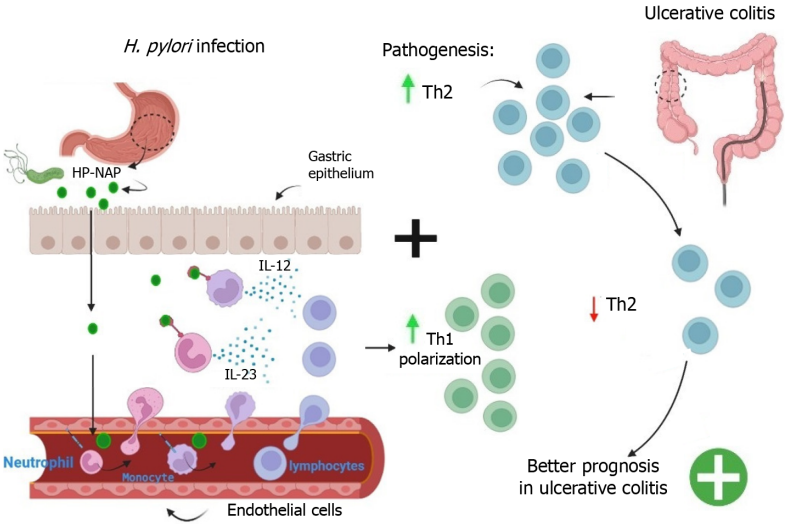

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Figure 3 Hypothesis on correlation between concomitant infection with *Helicobacter pylori* and the presence of ulcerative colitis (inflammatory bowel disease), which has a pathological pattern of exacerbated T helper 2 response. The schematic shows HP-NAP being secreted into the gastric mucosa by *Helicobacter pylori* (*H. pylori*). Then, HP-NAP undergoes a process of transcytosis by endothelial cells, binding to the luminal side of the blood vessel. With this, there is an alteration in the expression of β-2-integrin, with the recruitment of neutrophils and monocytes, which carry out diapedesis. The recruited leukocytes secrete cytokines [interleukin (IL)-12 and IL-23], which promote a polarization of the circulating lymphocytes to a T helper 1 (Th1) pattern. The Th1 polarization causes a reduction in the Th2 response, this could explain the improvement of ulcerative colitis symptoms in patients infected with *H. pylori*. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; Th: T helper; HP-NAP: *Helicobacter pylori* neutrophil-activating protein.

we emphasize the role of *H. pylori* CagA+ and HP-NAP on favorable prognosis of IBD. The pathogenesis of IBD is complex and involves a combination of genetic, environmental, and immunological factors. Classically, CD and UC are described as diseases with a Th1 and Th2 immune pattern, but the prevalence of type response remains under study.

Regarding the cellular pattern, Th17 cells have been demonstrated at the site of inflammation, but the levels of total cytokine markers of these patterns remain variable on models' diversity. Treg cells play a major role in the pathogenesis of IBD by suppression of Smad3 and consequently overexpression Smad7, an inhibitor of the TGF-β signaling pathway. Moreover, Th9 cells - capable of exacerbating inflammatory processes by increasing epithelial permeability, are at high levels in CD and UC.

In summary, targeting NLRP3 inflammasome by *H. pylori* infection allows the exacerbation of IL-1β and IL-18 that culminates in high levels of TGF-β and low levels of IL-17 and IL-22 on IBD. The patients with *CagA* gene can induce the Treg cells which contributes to the polarization of the M1 macrophage into M2 macrophage lineage through concomitant increase of the expression of IL-10 and more Treg cells. At the same time, HP-NAP has an important role in immunomodulation by reactive oxygen species production neutrophil-induced. The ability of HP-NAP to reduce Th2 activity due to the polarization towards Th1 response may be a possible explanation for the improvement of IBD symptoms, in which there is a predominance of a Th2 response and inhibition of the Th1 response like occurs in individuals infected with *H. pylori*.

Regarding the capacity of CagA+ and HP-NAP to control inflammation and autoimmunity, and their implication in preventing IBD evolution, it seems probable that a clear understanding of how CagA+ and HP-NAP work on Treg cells, cytokines and macrophages-induced will present definitive opportunities for therapeutic intervention.

FOOTNOTES

Author contributions: Feilstrecker Balani G, Picasky da Silveira Freitas JE, dos Santos Cortez M collected the data and wrote the manuscript; Freire de Melo F performed the critical analysis of the manuscript; Zarpelon-Schutz AC and Teixeira KN performed the critical analysis, corrected the manuscript and coordinated the study; and all authors approved the final version of the manuscript.

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REVIEW

Helicobacter pylori intragastric colonization and migration: Endoscopic manifestations and potential mechanisms

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Abstract

After being ingested and entering the human stomach, *Helicobacter pylori* (*H. pylori*) adopts several effective strategies to adhere to and colonize the gastric mucosa and move to different regions of the stomach to obtain more nutrients and escape from the harsher environments of the stomach, leading to acute infection and chronic gastritis, which is the basis of malignant gastric tumors. The endoscopic manifestations and pathological features of *H. pylori* infection are diverse and vary with the duration of infection. In this review, we describe the endoscopic manifestations of each stage of *H. pylori* gastritis and then reveal the potential mechanisms of bacterial intragastric colonization and migration from the perspective of endoscopists to provide direction for future research on the effective therapy and management of *H. pylori* infection.

Key Words: *Helicobacter pylori*; Colonization; Endoscopy; Gastritis; Infection

Core Tip: *Helicobacter pylori* (*H. pylori*) adopts several effective strategies to adhere to and colonize the gastric mucosa and move to different regions of the stomach, leading to acute infection and chronic gastritis that can be observed through endoscopy. Herein, we describe the endoscopic manifestations of each stage of *H. pylori* gastritis and then discuss the potential mechanisms of bacterial intragastric colonization and migration from the perspective of endoscopists to provide direction for future research on the effective therapy and management of *H. pylori* infection.

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INTRODUCTION

More than half of the world's population is estimated to be infected by the gram-negative, flagellated, spiral-shaped bacterium *Helicobacter pylori* (*H. pylori*)[1]. The bacterium has received intensive attention because *H. pylori* infection is closely associated with the development of peptic ulcers, mucosa-associated lymphoid tissue lymphoma and gastric cancer (GC), resulting in at least 500000 deaths per year[2-4]. The slow carcinogenic process is known as Correa's cascade [5]: At first, gastritis occurs in all infected individuals[2], and then a series of intermediate stages (characterized by precancerous lesions), including atrophy, intestinal metaplasia (IM) and dysplasia, may slowly develop, and eventually, 1%-3% of infected patients develop gastric malignant tumors[6].

All gastric mucosal lesions that occur after *H. pylori* infection can be observed by skilled endoscopists through upper gastrointestinal endoscopy. Based on the Kyoto classification of gastritis, endoscopic features, such as nodularity, diffuse redness, spotty redness, mucosal swelling, enlarged folds, xanthoma, atrophy and IM, are helpful in diagnosing *H. pylori* gastritis[7]. Atrophy can be endoscopically identified with high confidence by applying the Kimura-Takemoto classification[8], while IM and dysplasia can be diagnosed more accurately with advanced image-enhanced endoscopy (IEE) [9].

The highly motile pathogen *H. pylori* usually infects young children[3] and initiates acute infection that lasts for only a few weeks[10,11] and chronic inflammation that can last for the lifetime of the host[12]. Its ability to swim in the gastric mucus and colonize the stomach enables it to survive in the hostile gastric environment[13] and leads to various endoscopic and histological features as gastric mucosal lesions progress[14]. Many articles and reviews have reported the underlying mechanisms, but few have linked endoscopic features to mechanisms. Therefore, in the following sections, we describe the endoscopic manifestations of each stage of *H. pylori* gastritis and summarize the process and potential mechanisms of intragastric colonization by *H. pylori* and its migration.

ACUTE INFECTION

Acute *H. pylori* infection only lasts for a few weeks[10,11] and has been rarely observed or reported in recent decades. The endoscopic manifestation of gastric erythema and a gaping pylorus[10,11] is always featureless. Although the gastric mucosa does not appear damaged at this stage, initial colonization of the mucosa is the basis of a series of lesions, such as atrophic gastritis, peptic ulcer and even gastric carcinoma.

The prevalence of *H. pylori* infection is high, but colonization by this microbe is not easy. Multiple spontaneous eradication events may occur before colonization, leading to acute infection[15]. Sophisticated strategies have been adopted by *H. pylori* that have enabled it to adapt to and survive in the hostile gastric environment.

When *H. pylori* is ingested by adults, it is almost completely destroyed in the gastric acid, while it is easier to survive in the stomach of children younger than the age of five, both in developing and developed countries[3]. Bucker *et al*[3] simulated the pH changes of the postprandial stage in babies, young children and adults and suggested that the bacteria were easiest to reach the mucus layer in young children, whose feature of postprandial gastric condition is moderate food-induced pH elevation and slow reacidification.

During the process of slow reacidification, the urease enzyme is believed to play a key role in bacterial survival and adhesion. Urea is degraded by the urease enzyme, which buffers the cytoplasm and periplasm[16]. This confers many benefits. First, *H. pylori* prefers to live in an environment with elevated pH. A recent study[17] showed that *H. pylori* does not escape from phosphate buffer solutions of pH 6.6 and 7.0. Second, intracellular urease could also increase membrane potential, thereby allowing protein synthesis at a low pH[18]. Third, mucosal viscosity highly depends on acidity[19]. At a less acidic pH, the mucus is less gel-like, which enables *H. pylori* to more easily move through the mucus layer[20]. Fourth, trefoil factor 1 (TFF1) is a member of the trefoil peptide family of proteins and is coexpressed with Mucin-5AC (MUC5AC), a gel-forming mucin that is predominantly secreted and expressed by gastric surface epithelial cells in the stomach[21]. The optimum pH for bacterial binding to TFF1, which thereby promotes colonization, was found to be 5.0-6.0[22]. In addition, urea and bicarbonate were considered to have a chemotactic effect on *H. pylori in vitro*[23], but research by Schreiber *et al*[13] shows that neither the urea/ammonium gradient nor the bicarbonate/CO_2 gradient are essential for the orientation of *H. pylori in vivo*.

However, this does not mean that a neutral or alkaline environment is suitable for *H. pylori*. Previous studies have shown that *H. pylori* is sensitive to alkaline conditions[24], and its growth is limited at neutral pH[25]. To prevent lethal alkalinization of the cytoplasm, *H. pylori* utilizes a proton-gated channel, UreI, which regulates the uptake of urea[26] and only functions in conditions of an acidic pH; thus, the transport of urea into the bacterial cell does not occur at a neutral pH[24]. Therefore, *H. pylori* prefers a weakly acidic environment.

The epithelial surface of the stomach is covered with an approximately 300 μm thick layer of secreted mucus, which mainly consists of mucins Mucin 6 (MUC6) and MUC5AC[27]. MUC6 exists in each layer of the mucus gel, while MUC5AC is mainly present on the surface and bottom. The increase in the viscosity of gastric mucus gel is due to this natural stratification of mucins[28]. While protecting gastric epithelial cells, the mucus layer also plays an important role in the colonization process. The pH is approximately neutral at the epithelium and very acidic (pH 1-2) close to the lumen [21], resulting in a mucus pH gradient that can be used by *H. pylori* for precise spatial orientation[13]. The membrane-bound chemoreceptor TlpA of *H. pylori* detects and mediates repulsion from environments with a lower pH, and the cytoplasmic chemoreceptor TlpD mediates both attraction to higher pH environments and repulsion from lower pH environments[17,29]. Under this chemotactic effect, *H. pylori* penetrates the gastric mucus quickly and reaches the narrow region within 25 μm of the gastric epithelial surface with the help of its two to six sheathed unipolar flagella and helical shape[30,31].

After approaching the lower mucus layer, the majority of *H. pylori* swim in gastric mucus, while others directly adhere to epithelial cells[13,31]. Although it is considered a noninvasive gastric pathogen to date[19], *H. pylori* can indeed bind to, invade, be internalized into and proliferate in gastric epithelial cells[27,32]. The invasiveness of *H. pylori* may partially depend on the strain. Research by Camorlinga-Ponce *et al*[33] showed that CagA-negative bacteria adhered to the surface of the apical epithelium, while CagA-positive bacteria were identified in the intercellular spaces or the immediate vicinity of epithelial cells. Sigal *et al*[34] found a subgroup of *H. pylori* associated with cells deep in the antral glands. These microbes can promote gland hyperplasia by inducing stem cell proliferation and expansion and altering gene expression of stem cells[34].

H. pylori adheres to epithelial cells mainly by outer membrane proteins (OMPs). Blood group antigen-binding adhesion (BabA) and sialic acid-binding adhesion (SabA) are important OMPs[19,27]. Lewis antigens are common in normal, infected and inflamed gastric mucosa[35,36]. BabA can identify and bind to Lewis b antigen[35], while SabA can bind to the antigens Lewis a and Lewis X[36], and its expression can quickly respond to the changes in the stomach or different areas of the stomach, enabling the bacteria to adapt to host's immune responses and varied microenvironments to maintain long-term colonization and infection[37]. In addition to BabA and SabA, other surface proteins, such as AlpA, AlpB, DupA, outer inflammatory protein A (OipA) and HopZ, are considered related to adhesion, but none of them has been shown to be essential to adhesive mechanisms[38]. After *H. pylori* adheres to epithelial cells, the Cag type IV secretion system (T4SS) promotes CagA translocation into host cells, resulting in changes in cell shape, disruption of cell□cell junctions, altered cell polarity and cell adhesion, increased cell motility and cell migration, increased cell proliferation, β-catenin activation, and epithelial-mesenchymal transition[39]. Some bacteria are internalized into the cytoplasm of gastric epithelial cells through endocytosis within 45 minutes of bacterial attachment to the cell surface[32]. *H. pylori* can replicate and proliferate in epithelial cells[40], escape the immune response, and exit cells to colonize and infect cells again when the external environment is suitable for survival[27].

In an artificial ingestion study[10], histological examination during the acute phase of *H. pylori* infection showed many polymorphonuclear neutrophil leucocytes (PMNs) in the lamina propria and on the surface of the mucosa and an absence of intracellular mucus. Spiral bacilli adhered to the surface and glandular epithelium as well as among PMNs in the mucus[10]. Zhao *et al*[41] proposed a novel staging strategy according to the depth and degree of gastric mucosal injury induced by *H. pylori* infection and the progression of lesions. Stage I means the bacteria were present in the mucus layer, stage IIA refers to the specific adhesion to and selective destruction of gastric epithelial cells, and stage IIB refers to the degeneration and shedding of surface mucus cells[41]. It seems that stages I and II are consistent with the pathological characteristics of acute *H. pylori* infection.

CHRONIC GASTRITIS

Cases of *H. pylori* gastritis that are observed by doctors usually involve chronic gastritis that has lasted for years[42]. Chronic gastritis has various endoscopic findings, among which nodularity, diffuse redness, spotty redness, xanthoma, mucosal swelling, enlarged folds, atrophy, and IM are common in *H. pylori*-infected gastric mucosa[43,44] (Figure 1). Considering the severity and progression of chronic *H. pylori* gastritis, we discuss endoscopic manifestations and potential mechanisms from the following three aspects: (1) Early stage of *H. pylori* infection; (2) corpus inflammation; and (3) atrophy and intestinal metaplasia, which are summarized in Table 1.

Early stage of H. pylori infection

Nodular gastritis is considered a feature of an early stage of *H. pylori* infection in adults and is more common in children, with an incidence of 32.9% to 85%[45,46]. It appears more frequently in the antral mucosa than in the corpus mucosa[47]. Nodularity is characterized by a miliary pattern resembling "gooseflesh" in the gastric mucosa on endoscopy[46] and follicular lymphoid hyperplasia with intraepithelial lymphocytosis on histological examination[47]. Okamura *et al*[48] further demonstrated that superficially located, enlarged hyperplastic lymphoid follicles corresponded to nodular and/or granular lesions, and the percentage of MECA-79 high endothelial venule (HEV)-like vessels was greater in areas with gooseflesh-like lesions in nodules than in normal gastric mucosa. The pathogenesis of nodular gastritis may involve a Th2

Table 1 The mechanisms of common endoscopic features

Endoscopic features	Mechanisms
Nodularity	Follicular lymphoid hyperplasia with intraepithelial lymphocytosis[47]; Superficially located, enlarged hyperplastic lymphoid follicles [48]; Increased numbers of MECA-79 HEV-like vessels[48]; Th2 immune response[49]
Diffuse redness	Infiltration of neutrophils and monocytes[44,58]
Spotty redness	Unclear
Mucosal swelling	Infiltration by neutrophils and monocytes[44]
Enlarged folds	Tumor necrosis factor-alpha gene polymorphism[64]; Genome wide hypomethylation and regional hypermethylation[65,66]; Stimulation of epithelial cell proliferation and inhibition of acid secretion induced by interleukin 1 beta and hepatocyte growth factor [61,62]; Inhibition of acid secretion caused by morphological changes in parietal cells[63]
Xanthoma	Unclear
Atrophy	Cellular injury inflicted by *Helicobacter pylori* or mediated by inflammation or apoptosis[77]; Th1 immune response[78]; C-X-C motif chemokine receptor 2-mediated cellular senescence[79]
Intestinal metaplasia	Death of parietal cells and reprograming of chief cells[82]

immune response, which is more likely to occur in children[49,50].

Early colonization usually occurs in the gastric antrum, and early inflammation is always more serious in the gastric antrum, which is consistent with endoscopic findings. Animal research suggested that the wild-type *H. pylori* strain mostly colonized the antrum and the transition zone between the antrum and corpus rather than the corpus[31,34]. Rolig *et al*[51] demonstrated that inflammation was worse in the antrum than in the corpus in mice infected with wild-type *H. pylori* strains. This may be associated with the particularity of antral glands and chemotaxis of the bacterium.

It is well known that the corpus is populated by oxyntic glands containing many acid-secreting parietal cells that promote acidic conditions in the stomach. In contrast, the antrum, which is defined by the presence of gastrin-expressing G cells, mainly comprises the pyloric or antral glands containing MUC6-expressing deep mucous cells, G cells, D cells, enterochromaffin cells and foveolar surface mucous cells[52]. Interestingly, oxyntic glands also exist in the human gastric antrum, but the proportion of parietal cells and chief parietal cells is significantly less than that in corpus glands[53]. The effects of parietal cells in the antrum on *H. pylori* colonization remains unclear. However, generally, the weaker acidic environment of the antrum provides the bacteria with more opportunities to survive and colonize.

The chemotaxis system of *H. pylori* includes three membrane-bound chemoreceptors, including TlpA, TlpB, and TlpC; one cytoplasmic chemoreceptor, TlpD[29]; three core signaling complex proteins, including CheW, CheA and CheY[54,55]; and auxiliary chemotaxis proteins containing CheV-type coupling proteins (CheV1, CheV2, and CheV3), CheZ phosphatase and ChePep[56]. The role of pH sensing in chemotaxis has been mentioned above. In addition, a study by Rolig *et al*[51] shows that chemotaxis is required for *H. pylori* to swim to and achieve normal bacterial loads in the antrum and transition zone. The number of nonchemotactic mutant (Che-) *H. pylori* strains at this site was found to increase more slowly than that of the wild-type strains. TlpD plays a major role in this process. Therefore, chemotaxis may be necessary for *H. pylori* to locate or to maintain colonization of the antrum.

Corpus inflammation

Previous clinical studies focused on the relationship between endoscopic findings and *H. pylori* infection and demonstrated that diffuse redness, spotty redness, mucosal swelling and enlarged folds under endoscopy are associated with *H. pylori* infection[14,46]. Diffuse redness, defined as uniform redness with continuous expansion involving the nonatrophic mucosa in the region of fundic gland, and mucosal swelling, defined as swollen gastric mucosa in the region of fundic gland or thick, uneven mucosa in the region of pyloric gland, correlate predominantly with the degree of neutrophilic and mononuclear cell infiltration caused by *H. pylori* infection[44,57-59]. Spotty redness comprises multiple spotted small flat erythema, commonly observed in the upper corpus and fornix[44], but its mechanism remains unclear. An enlarged fold is defined as a fold with a width of 5 mm or more in the gastric greater curvature, which is not or only partially flattened by air insufflation[60]. Stimulation of epithelial cell proliferation, inhibition of acid secretion, tumor necrosis factor-alpha gene polymorphism, genome-wide hypomethylation and regional hypermethylation may play a role in the generation of enlarged folds caused by bacterial infection[61-66]. We describe another perspective: these endoscopic features that are mainly observed in the corpus indicate the existence of corpus inflammation, the development of gastric mucosal lesions, and a later stage of *H. pylori* infection that differs from the early stage and mainly manifests as antral inflammation.

H. pylori can survive in and colonize the harsh conditions of the corpus that are promoted by oxyntic glands. This has been indicated by previous studies. *H. pylori* was identified in the corpus in 83% of patients with a previous diagnosis of intestinal metaplasia and known *H. pylori* infection[67]. Biopsies taken from the corpus are conducive to an accurate histologic diagnosis and assessment of *H. pylori* infection[68,69]. Combined antrum and corpus biopsies can lead to a significantly better success rate of *H. pylori* culture than single antrum biopsy[4].

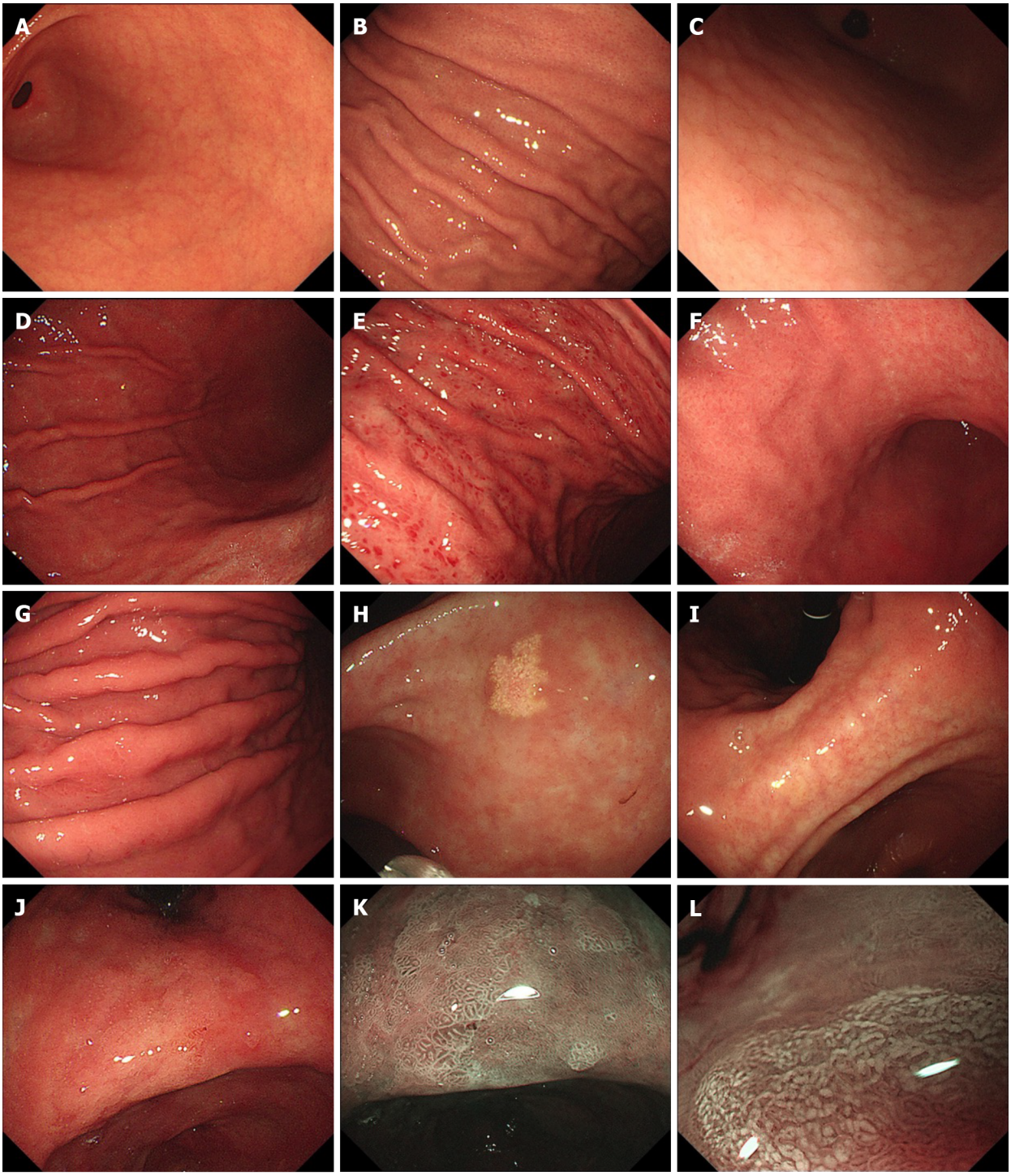

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Figure 1 Endoscopic features without and with *Helicobacter pylori* infection. A: Antrum without *Helicobacter pylori* infection; B: Corpus without *Helicobacter pylori* infection; C: Nodularity; D: Diffuse redness; E: Spotty redness; F: Mucosal swelling; G: Enlarged folds; H: Xanthoma; I: Atrophy; J: Intestinal metaplasia; K: Light-blue crest; L: White opaque substance.

H. pylori also reaches the corpus under the guidance of chemotaxis, but afterward, chemotaxis is not needed for *H. pylori* populations to increase[51]. It is likely that the spontaneous eradication of the bacteria is almost impossible at this stage. However, to live, proliferate and induce chronic infection, bacteria need to acquire nutrients and escape immune reactions in addition to adapting to acidic environments, as mentioned above. Due to the low permeability of the mucosal layer, essential nutrients (for example, Fe^{3+}) for ingested microorganisms are scarce in the stomach[70]. Following the successful colonization of gastric epithelial cells, *H. pylori* induces immune cells that cause cell damage to shed nutrients onto the surface of the gastric mucosa for survival[71]. However, *H. pylori* needs to take measures to protect itself from host immunity. Sophisticated mechanisms participate in the response to innate immunity; these mechanisms include: (1) The induction of mitochondrial-dependent apoptosis in macrophages; (2) the defense against NO products available in the gastric microniche through production of peroxiredoxin by the AhpC gene; and (3) the reduction of NO or O_2^- radicals

by arginase due to substrate competition; responses to adaptive immunity, which have been elaborated in a previous review, include: (1) The binding of the VacA toxin to an unknown surface ligand in T cells, which results in actin rearrangement and then inhibition of cell proliferation; (2) The promotion of vacuoles in host cells, which leads to apoptosis by an anion-selective channel formed by the VacA toxin; and (3) VacA binding to mitochondria, which activates the associated apoptotic pathway[19]. In addition, *H. pylori* can be internalized into epithelial cells through endocytosis[32]. Long-term exposure to VacA during chronic infection causes the formation of immature autophagosomes, resulting in a failure to clear the bacteria[72].

In the novel pathological staging strategy mentioned above[41], stage III, the laminar lesion stage, may be consistent with the early stage of gastric antrum and corpus inflammation. Stage III is subdivided into: (1) Stage IIIA: Infiltration of inflammatory cells and vacuolar-like degeneration; (2) stage IIIB: The development of mucous neck cell hyperplasia, glandular hyperplasia and heteroplasia, and serrated structures; (3) stage IIIC: Mucosal ulcers develop; and (4) stage IIID: Histologically diffuse lymphocyte proliferation occurs, and many lymphatic follicles of varying sizes are present.

Atrophy and intestinal metaplasia

In the absence of treatment, the inflammation and immune response caused by *H. pylori* infection may lead to atrophic gastritis[73], which is defined as the loss of gastric glands, with or without metaplasia[74]. This process takes several years in humans[75]. Early *H. pylori* eradication should be considered for preventing GC development prior to the appearance of atrophy or metaplasia because the benefits of *H. pylori* eradication diminish after the gastric IM stage is reached, which is referred to as the "point of no return"[76].

Gastric gland replacement by connective tissue or inflammatory cells is referred to as atrophy[73,74]. Previous studies have reported that atrophy may be related to the Th1 immune response and cellular injury, which is directly inflicted by the bacteria or mediated by inflammation or apoptosis[77,78]. A recent study showed a new mechanism of *H. pylori* –induced atrophy through C-X-C motif chemokine receptor 2 (CXCR2)-mediated cellular senescence[79]. However, in general, the pathogenetic mechanisms that trigger atrophy are still debated.

Color changes (yellowish pale) in the mucosa, mucosal thinning and visible vascular patterns are typical endoscopic atrophic features[80]. In 1966, Kimura and Takemoto described the appearance of an "atrophic transitional zone" in patients with gastritis for the first time, which was subsequently known as the endoscopic atrophic border[8]. The differences in mucosal color and the visibility of capillary networks are remarkable between the two sides of the endoscopic atrophic border[81]. The degree of atrophy can be divided into 6 types based on the location of the endoscopic atrophic border. Endoscopic atrophic findings that are only visible in the antrum are referred to as closed type C-1. In closed types C-2 and C-3, atrophy can be observed in the angulus and the lesser curvature of the corpus. In open type O-1, the atrophic border lies between the lesser curvature and the anterior wall; in type O-2, it lies within the anterior wall; and in type O-3, the endoscopic atrophic area is widely spread within the border between the anterior wall and the greater curvature[81].

When deep damage to the gastric mucosa occurs, acid-secreting parietal cells die, and pepsin-secreting chief cells are reprogrammed into mucin-secreting, wound-healing cells to reduce endogenous production of caustic substances; this response to injury is known as metaplasia[82]. Pathologically, metaplasia refers to gland replacement by a different type of epithelium in a tissue where it is not normally found[74,83]. The characteristics of mucus secretion were used to discriminate metaplastic lineages[83]. Pseudopyloric metaplasia is defined as the presence of MUC6- and trefoil factor 2 (TFF2)-expressing cells at the base of corpus glands with a morphology more characteristic of mucus-producing deep antral glands[84]. IM refers to the presence of Mucin2 (MUC2)/trefoil factor 3 (TFF3)-expressing intestinal-type goblet cells in the stomach[85]. IM can be divided into two types: (1) Incomplete IM, which may be found in either the superficial or foveolar epithelium and in the glands and is characterized by secretive columnar cells that secrete mucin into the apical cytoplasm and the presence of goblet cells; and (2) complete IM, which is characterized by columnar absorptive cells without mucin secretion and the presence of goblet cells[86]. Both incomplete and complete IM can be subdivided into small intestinal type and colonic type (Table 2).

An ash-colored flat nodular change has been considered a typical endoscopic finding of IM since the last century[80]. With the development of endoscopic technology, advanced IEE, including narrow band imaging (NBI) endoscopy, has been used as a more accurate IM diagnostic tool than traditional white light endoscopy[9]. Various markers are related to gastric IM[87]. Light-blue crest (LBC) (Figure 1K), a light blue line observed on the surface of gastric mucosal epithelium, is the earliest mentioned IEE finding[88]. Combining the findings of white opaque substance (WOS) (Figure 1L), white mucosal epithelium observed under IEE, and LBC improves the sensitivity of diagnosing IM[89]. Through systematic review and meta-analysis, the diagnostic sensitivity and specificity of LBC were found to be 0.79 [95% confidence interval (CI): 0.76-0.81] and 0.95 (95%CI: 0.94-0.96), respectively. The sensitivities of the groove type (GT) and marginal turbid band (MTB) were 0.49 (95%CI: 0.43-0.54) and 0.47 (95%CI: 0.40-0.53), respectively, and the specificities were 0.92 (95%CI, 0.89-0.94) and 0.92 (95%CI: 0.89-0.95)[87], respectively. In addition, researchers derived a classification for endoscopic grading of gastric IM (EGGIM) using IEE, which permits immediate grading of intestinal metaplasia without biopsies and is beneficial for GC risk stratification[90].

In addition, gastric xanthoma is a common endoscopic finding in patients with *H. pylori* infection and may serve as a warning endoscopic sign for advanced atrophic gastritis, intestinal metaplasia and GC[91-93]. It is a small yellowish or yellowish-white plaque-like or nodular lesion characterized by the accumulation of lipids, containing cholesterol, low-density oxidized lipoprotein, low-density lipoprotein and neutral fat, in histiocytic foam cells[93,94]. However, the etiopathogenesis is also unclear.

Table 2 Intestinal metaplasia with different mucin secretion

Cells	Incomplete intestinal metaplasia		Complete intestinal metaplasia	
	Small intestinal type	Colonic type	Small intestinal type	Colonic type
Columnar cells	Neutral and scanty sialomucins	Sulpho- and scanty sialomucins	No mucin secretion	No mucin secretion
Goblet cells	Sialomucins	sialomucins	Neutral and sialomucins	Sulpho- and sialomucins

INTRAGASTRIC MIGRATION

H. pylori has shared a coevolutionary history with humans for more than 60000 years[41,95]. Human migration has led to the global distribution of the bacterium from East Africa to other continents[19]. In addition to geographical migration, *H. pylori* has the ability to move between different regions of the stomach.

The motility of *H. pylori* provided by its flagella and helical shape is the basis of intragastric migration. The bacterium possesses two to six sheathed unipolar flagella[96]. The sheath, which consists of both proteins and lipopolysaccharide, protects the flagellar filaments from gastric acid[97]. Expression of the two major flagellar proteins, FlaA and FlaB, is required for full motility of the bacteria[21]. An efficient screw-like movement resulting from the characteristic helical shape of *H. pylori* also provides an advantage for penetrating the gastric mucus layer[98]. Any mutation in the genes associated with bacterial morphology, such as Ccrp89, Ccrp58, Ccrp1142 and Ccrp1143, can lead to a deficiency in bacterial shape and motility[99].

The chemotaxis system of *H. pylori* is necessary for intragastric migration. Chemotactic signals sensed by chemoreceptors are transmitted to the histidine kinase CheA through the coupling protein CheW or CheV1[100]. Repellents activate CheA autophosphorylation, and CheY is subsequently phosphorylated *via* histidine-to-aspartate phosphorelay [101]. Phosphorylated CheY interacts with the flagellar motor, causing it to rotate clockwise and the bacteria to reverse or change direction[56]. Alternatively, the bacteria swim straight because chemicals perceived as attractants squelch CheA autophosphorylation[56]. As described above, the ability of chemoreceptors to sense pH guides the bacteria to the surface of the gastric epithelium. It has been suggested that different regions of the stomach contain unique chemotactic signals [51]. The gastric antrum is usually the first colonized area because of its weaker acidic environment but not due to chemotaxis. The chemotactic signals produced by the antrum or transition zone play an important role in the increase in *H. pylori* numbers that occurs from 14 h to 1 wk after colonization[51]. Chemotaxis is also required when *H. pylori* migrates to the corpus from the antrum but is not needed for the increase in bacterial populations after the initial colonization of the corpus[27]. In addition, *H. pylori* can swim toward injured epithelia[102].

H. pylori can simultaneously survive in the antrum and the corpus in general. However, when atrophy occurs, an environment that is unfavorable to the growth of *H. pylori* develops, and the bacteria can only be found in a small percentage of endoscopic biopsy specimens[103]. Research has revealed that atrophy in the corpus manifests as a continuous sheet of pseudopyloric metaplasia and forms an advancing histologically atrophic front, the presence of which is similar to the spread of antral mucosa toward the corpus and is faster in the lesser curvature[104]. This pattern is the same as the endoscopic atrophic border described by Kimura and Takemoto[8]. This may indicate that the suitable region in which *H. pylori* survives shrinks as the atrophic front advances and is well discriminated by the endoscopic atrophic border.

In addition, *H. pylori* can migrate to the duodenum and colonize the duodenal gastric metaplasia (DGM) with a bacterial density 100-fold lower than that in the antrum[105,106]. DGM is characterized by the metaplastic replacement of normal duodenal epithelial cells with cells displaying a phenotype similar to that of mucus-secreting cells of the gastric mucosa[107]. It is frequently found in patients with duodenal ulcers with a prevalence of 72 to 90% and is associated with the chronicity and recurrence of duodenal ulcer disease[108-110]. The exact pathogenesis of DGM remains unclear. It is speculated that a high acid burden in the duodenum caused by increased gastrin secretion and the inflammatory damage to duodenal mucosa induced by bacterial cytotoxin may lead to the development of DGM in patients with *H. pylori* infection[109]. Liu and Wright[111] considered that metaplastic cells originate from Brunner's gland duct epithelium or basal buds growing out of the crypts of Lieberkühn and migrate in straight lines. However, Shaoul *et al*[112] suggested that DGM develops from goblet cells that simultaneously express gastric antigens, MUC5AC and TFF1, and intestinal antigen, MUC2 core antigen, migrate upward and transform to foveolar-like cells at the site of early metaplastic patches. Published results about the association between *H. pylori* infection and DGM are also conflicting. Some studies reported that *H. pylori* infection was one of the independent risk factors for DGM[113], the amount of *H. pylori* in the duodenal bulb might be related to the extent of gastric metaplasia in the duodenal bulb[114], and the presence of DGM significantly decreased after *H. pylori* eradication[109]. However, some researchers have suggested that DGM is associated with high acid output in the stomach rather than gastric *H. pylori* infection[115-117].

CARDIA

The endoscopic characteristics of the cardia have received little attention in previous studies. In recent years, cardiac nodularity, which involves the appearance of miliary nodules or scattered small whitish circular colorations within 2 cm of the esophagogastric junction, has been proposed by researchers[46,118].

Cardia glands lack chief cells and parietal cells, and have similar characteristics to the pyloric glands[53]. The cardiac and pyloric glands secrete mucus and bicarbonate and are involved in the defense of the gastric epithelium[46]. In addition, both of them secrete MUC6 and pepsinogen II rather than pepsinogen I[46]. Unlike the fundic glands, the similarity of the cardiac and pyloric glands may lead to the appearance of cardiac nodularity.

Nodularity can be observed more frequently in the stomach of children and improves gradually with age[119,120]. Reportedly, the eradication of *H. pylori* in patients with antral nodularity could effectively prevent diffuse-type GC[119]. A study by Nishikawa *et al*[119] suggested that compared with patients without cardiac nodularity, patients with cardiac nodularity were significantly younger and had lower IM scores. Therefore, cardiac nodularity may also be a feature of the early stage of *H. pylori* infection, but further research is needed to analyze its clinicopathological importance.

CONCLUSION

H. pylori infection has received worldwide attention for decades. In this review, we described the process of intragastric colonization by *H. pylori* and its migration and tried to identify a link between endoscopic manifestations and potential mechanisms. Upper gastrointestinal endoscopy and pathological examination of biopsy specimens are useful tools for diagnosing *H. pylori*-induced gastritis and estimating the risk of *H. pylori*-induced GC. In addition to animal models, exploring the mechanisms of *H. pylori* infection requires biopsy sampling. However, extensive study is needed to evaluate the association between endoscopic manifestations and mechanisms.

FOOTNOTES

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MINIREVIEWS

Systemic treatments for resectable carcinoma of the esophagus

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Abstract

One of the most prevalent malignancies in the world is esophageal cancer (EC). The 5-year survival rate of EC remains pitiful despite treatment advancements. Neoadjuvant chemoradiotherapy in conjunction with esophagectomy is the standard of care for patients with resectable disease. The pathological complete response rate, however, is not acceptable. A distant metastasis or a locoregional recurrence will occur in about half of the patients. To increase the clinical effectiveness of therapy, it is consequently vital to investigate cutting-edge and potent therapeutic modalities. The approach to the management of resectable EC using immunotherapy has been considerably altered by immune checkpoint inhibitors. Systemic immunotherapy has recently been shown to have the potential to increase the survival of patients with resectable EC, according to growing clinical data. A combination of chemotherapy, radiation, and immunotherapy may have a synergistic antitumor impact because, according to mounting evidence, these treatments can stimulate the immune system *via* a number of different pathways. In light of this, it makes sense to consider the value of neoadjuvant immunotherapy for patients with surgically treatable EC. In this review, we clarify the rationale for neoadjuvant immunotherapy in resectable EC patients, recap the clinical outcomes of these approaches, go through the upcoming and ongoing investigations, and emphasize the difficulties and unmet research requirements.

Key Words: Systemic treatment; Resectable carcinoma of the esophagus; Personalized medicine; Biomarkers; Chemotherapy; Chemoradiotherapy; Immunotherapy; Immune checkpoint inhibitors

Core Tip: Despite improvements in neoadjuvant and adjuvant treatment approaches in recent years, the average life expectancy of patients with resectable esophageal cancer (EC) still falls below 5 years. Immunotherapy has been effectively used as a first-line therapy for many oncological diseases at advanced stages for over ten years. Immunotherapy drugs are also making great progress in resectable situations, while it is still debatable whether this treatment should be limited to a certain patient subgroup based on biomarker selection. In order to treat resectable EC, immunotherapy, in particular immune checkpoint inhibitors, has made significant strides. This review also provides a brief overview of potential ongoing clinical studies.

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INTRODUCTION

With over 544000 expected deaths from esophageal cancer (EC) in 2020, EC is the sixth most common cancer-related cause of death globally. In East Asia, esophageal squamous cell carcinoma (ESCC) covers up around 90% of cases of EC, in contrast to Western nations[1-3]. Surgery continues to be the therapy of choice for early-stage EC. Unfortunately, the majority of EC patients are stage 4 or advanced stage at the time of diagnosis, and only surgery has a modest impact, hence a 5-year survival rate is only 25%. Systemic treatment, as opposed to surgery alone, may increase survival for resectable locally advanced EC. As a result, preoperative systemic treatment combined with surgery has become the norm for treating EC patients. Despite this, approximately half of these patients experience local recurrence or distant metastases following surgery[4-6]. To enhance survival, it is therefore vital to investigate innovative and effective therapies. Immune checkpoint inhibitors (ICIs) have recently made substantial advancements in a range of malignancies, including EC, with several trials demonstrating longer overall survival (OS), a higher objective response rate (ORR), and a decreased frequency of grade 3-5 adverse events (AEs) as second-line therapy. According to the most recent findings, treating resectable EC patients as first-line therapy with programmed death 1 (PD-1) inhibitors combined with chemotherapy resulted in considerably longer progression-free survival (PFS) and OS than treatment with only chemotherapy. These findings imply that ICIs have a bright future in EC management. ICI systemic treatment is now being extensively researched for EC patients and has been tested in a number of malignancies[7-10]. The rationale for ICI neoadjuvant treatment in EC, the published findings, the upcoming and ongoing studies, the unanswered questions, and the suggestions for more research will all be included in this review.

ROLE OF DIET AND MICROBIOTA IN EC

Some of the traditional risk factors for EC seem to be linked to changes in the esophagus' natural microbiome. In ESCC patients, drinking alcohol has been connected to changes in the microbiota's diversity. In animal studies, high-fat diets have been related to esophageal dysplasia and changes in the microbiome. Gram-negative organisms, which are present in lesions that are precursors to EC, were shown to be more prevalent in those who consumed less fiber. Changes in the esophagus bacterial population have been linked to smoking and medications like proton pump inhibitors and antibiotics. Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, and Fusobacteria make up the majority of the microbiota in a healthy esophagus. In comparison, reduced microbial diversity, which appears to start in the precursor stages of EC, is a characteristic of malignancy of the esophagus. The EC microbiome is distinguished at the taxonomic level by a switch from Gram-positive to Gram-negative bacteria. The genera *Fusobacterium*, *Streptococcus*, *Veilonella*, and *Prevotella* are those that are most often enriched in EC. Although particular changes in the EC microbiome can be detected, a consistent microbiota profile linked with EC has not yet been discovered. The varied technical processes, such as the sampling strategy, the types of samples examined, and the analytic approach used to profile the microbiome, as well as the relatively small number of people participated in each experiment, may be to blame for this discrepancy. Future research should take method standardization into account. To study and integrate the impacts of various exposures, including those of nutrition, with the microbiome and the changes that occur during the carcinogenesis process, multidisciplinary research efforts will be necessary. This is especially true in studies of esophageal carcinogenesis associated with the microbiome. Large, well-characterized prospective cohort studies would be beneficial for dissecting these complicated connections, and they would undoubtedly aid in the development of creative preventive and treatment plans that would lessen the incidence of EC[11].

SYSTEMIC TREATMENT OPTIONS FOR RESECTABLE EC

Squamous cell carcinoma and adenocarcinoma, the two principal histological forms of EC, have very different epidemi-

ologies. To some extent, the therapy choices for these two categories differ. The two main therapeutic options for EC are local and systemic therapies. The tumor's histological type, location, extension, and size can all contribute to guiding therapy selections. Endoscopic and surgical resection are the primary treatments for locally advanced EC. Lymphatic node metastases arise early in EC due to the extensive lymphatic drainage in the esophagus submucosa, and the cancer is frequently detected at an advanced stage[12]. For resectable EC, the main systemic treatment options include neoadjuvant chemotherapy (NCT), neoadjuvant chemoradiotherapy (NCRT), and perioperative chemotherapy (PCT). According to the most recent National Comprehensive Cancer Network guidelines for esophageal and esophagogastric junction cancers, the optimal treatment options for both localized squamous cell carcinoma (SCC) and actinic cheilitis (AC) staged as Tis-T2 (low-risk lesions: < 3 cm, well-differentiated) without lymph node metastases include esophagectomy and endoscopic treatments. Patients with unresectable EC should get definitive chemoradiation. Current clinical research focuses mostly on locally progressed EC between the preferable single surgery and the recommended final chemoradiation. The systemic therapeutic approaches listed encompass NCT, NCRT, and PCT indicating a range between the two histological groups. As an adjuvant therapy, immunotherapy is employed[13].

The most prevalent subtype of EC in the world is ESCC, which manifests in the naturally occurring esophageal epithelium. An estimated 456000 people were diagnosed with EC in 2012, of whom 398000 had ESCC. Around 80% of ESCC cases worldwide were found in Central and Southeast Asia. More than half of all cases globally originated in China alone[14]. For 366 patients with locally advanced resectable esophageal or esophagogastric junctional cancer, NCRT that included carboplatin, paclitaxel, and concurrent 41.4 Gy radiotherapy was compared to surgery alone (178 for NCRT and surgery *vs.* 188 for surgery alone). A minimum projected follow-up term of 10 years was guaranteed by the follow-up period, which ended on December 31, 2018. In the first year, patients visited the outpatient clinic once every three months. In the second year, they visited once every six months, and so on until the fifth year, when they visited once a year. After 5 years of follow-up, patients with symptoms could visit the outpatient clinic. Participants in the chemoradiotherapy-surgery group survived more than those in the surgery arm, with 10-year OS rates of 38% and 25%, respectively. The respective 10-year OS rates in the chemoradiotherapy-surgery and surgery groups for patients with SCC were 46% and 23%, respectively, and 36% and 26% for patients with AC. Participants in the chemoradiotherapy-surgical group died from EC at a lower rate than those in the surgery arm, with 10-year absolute risks of 47% and 64%, respectively. Other causes of death were comparable in the chemoradiotherapy-surgery and surgery arms, with 10-year absolute risks of 15% and 11%, respectively. They concluded that the OS advantage of preoperative NCRT for patients with locally advanced resectable esophageal or junctional carcinoma, according to the ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study (CROSS) study, lasts at least ten years[15].

NEOCRTEC 5010, a phase 3 randomized, open-label, multicenter, clinical trial, supported the findings of ESCC patients from the CROSS study. The NCRT group outperformed the surgery-only group in terms of R0 resection rate (98.4% *vs.* 91.2%), median OS (100.1 mo *vs.* 66.5 mo), and disease-free survival (DFS, 100.1 mos vs. 41.7 mos). According to the long-term efficacy of the NEOCRTEC 5010 research, the OS advantage in patients with locally advanced resectable ESCC who received the NCRT regimen was maintained for at least 10 years[16]. The CROSS trial also discovered that NCRT decreased solitary distant relapse (27% *vs.* 28%) but did not lessen isolated distant relapse (8% *vs.* 18%) or synchronous locoregional and distant relapse (13% *vs.* 22%). Relapse following NCRT is thus a critical obstacle to overcome[15].

Esophageal adenocarcinoma (EAC), also known as gastroesophageal adenocarcinoma or esophagogastric junction cancer, usually develops in the lower third of the esophagus. Obesity is its main risk factor since it mostly affects people with a history of gastroesophageal reflux disease[14]. About two-thirds of the histological categories of EC in high-income nations are AC. It is currently the most common histological type in seven high-income countries, including Canada, Denmark, Norway, Australia, New Zealand, Ireland, and the United Kingdom[17]. The CROSS-study's findings similarly positioned NCRT as the therapy of choice for resectable EAC, although superior NCRT effectiveness was shown in ESCC. As a result, depending on specific circumstances, PCT and NCT are different tactics. In order to successfully treat resectable EAC, PCT is crucial. The landmark-like phase 3 Medical Research Council Adjuvant Gastric Infusion Chemotherapy Study (MAGIC) initially showed the survival advantage of PCT in AC. This study revealed that patients with non-metastatic AC who had PCT with epirubicin, cisplatin, and fluorouracil (FU) had better PFS and OS. The MAGIC trial had more treatment-related AEs (TRAEs) than the CROSS study did[15,18]. The FU plus Leucovorin, Oxaliplatin, and Docetaxel triplet FU plus Leucovorin, Oxaliplatin, and Docetaxel (FLOT) regimen was used in a later, important phase 2/3 trial, the FLOT4 trial, which demonstrated superiority in R0 resection rate (85% *vs.* 78%) and median OS (50 mo *vs.* 35 mo). In recent score-matched research, the therapeutic effects of NCRT and PCT were contrasted. The findings demonstrated no discernible differences in tumor response or survival rates between these two common regimens. After PCT, there were more TRAEs (42/97 *vs.* 30/97)[19]. Another effective therapy for resectable EAC is NCT. Preoperative FU and cisplatin (FC) showed a survival benefit over surgery alone in the Medical Research Council OEO2 study[20,21]. The survival benefit remained at a median follow-up of 6 years [hazard ratio (HR), 0.84; 95% confidence interval (CI), 0.72-0.98; $P = 0.03$], while the cisplatin group had a better OS at 2 years (HR, 0.79; 95% CI, 0.67- 0.93; $P = 0.004$). Another sizable experiment, nevertheless, was unable to show the same result[22]. Triplet chemotherapy (epirubicin, cisplatin, and capecitabine) did not improve OS in the OE05 study. Additionally, it was connected to greater toxicity than FC[23]. NCT is therefore hardly employed.

A few noteworthy clinical studies on neoadjuvant therapy for resectable EC have recently been conducted. As a preoperative treatment for locally advanced ESCC, the NExT study seeks to demonstrate that docetaxel, cisplatin plus 5-FU (DCF), and radiotherapy with cisplatin plus 5-FU are superior than FC in terms of OS. The superior OS and manageable toxicity of DCF indicate a new standard therapeutic strategy for ESCC[24]. In 2021, Wang *et al*[25] conducted a prospective, multicenter, open-label, randomized clinical study on 264 patients with locally advanced ESCC to assess the efficacy and safety of NCRT *vs.* NCT followed by minimally invasive esophagectomy (MIE). The postoperative

morbidity rates of 264 patients, 47.4% in the NCRT group and 42.6% in the NCT group, did not significantly vary between the two groups. Based on the Clavien-Dindo classification, the degree of complications was distributed similarly across the two groups. For the NCRT group and the NCT group, the 90-day perioperative mortality rate was 3.5% and 2.8%, respectively. Patients in the NCRT group, however, showed higher rates of negative lymph nodes (66.1% *vs.* 46.2%) and pathologic complete responses (35.7% *vs.* 3.8%). They came to the conclusion that NCRT followed by MIE was safer and produced better histopathologic results than NCT followed by MIE for the treatment of locally advanced ESCC. According to a recent publication by Tang *et al*[26], among patients with cT3-4aN0-1M0 ESCC, NCRT followed by MIE was not significantly related to a better OS than NCT. The findings highlight the ongoing question of the ideal neoadjuvant therapeutic plan for locally progressing bulky ESCC. In the Neo-AEGIS study, 377 European patients were assigned at random to receive PCT, or CROSS. In the 3-year OS, the first OS analysis did not reveal any statistically significant differences (HR, 1.02; 95% CI, 0.74-1.42). However, the results must be taken seriously because the majority of the evaluable perioperative patients did not get the current standard of care FLOT (epirubicin, cisplatin and FU/epirubicin, cisplatin and capecitabine/epirubicin, oxaliplatin and FU/epirubicin, oxaliplatin and capecitabine prior to 2018, FLOT option 2019-2020, only 15% of chemotherapy arm patients received FLOT)[27].

MOLECULAR MECHANISMS OF ICIS FOR EC TREATMENT

Utilizing one's own immune system to find and eliminate tumor cells is the goal of immunotherapy. Understanding the anti-tumor response and the strategies a malignancy may use to modify or inhibit the immune response has seen significant breakthroughs. This has made it possible for a new field of medicine to flourish, one that has shown considerable promise in treating conditions like EC that previously had a low survival rate. Our knowledge of the tumor microenvironment (TME) is one of the essential components for the creation of novel therapies. The TME is a complicated environment where a variety of immune and stromal cell subtypes interact to either promote or prevent tumor growth [28,29]. A crucial component of the immune response against tumors is the effector T cell. To maintain the immune system's equilibrium, negative regulators are produced on the surface of T cells during the activation process. Immune checkpoints are what they are known as. T cell activation, function, proliferation, and survival may be diminished when these immune checkpoints and their ligands are triggered during T cell receptor (TCR) signaling, which promotes tumor immune evasion. When compared to other solid tumor forms, EC is distinguished by having a significant amount of tumor-infiltrating T cells and monocytes/macrophages. The bulk of the tumor-infiltrating T cells are worn-out cluster of differentiation (CD) 8+ T cells and regulatory T cells (Tregs). Tregs, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells, and cancer-associated fibroblasts all contribute to the immunosuppressive TME of EC through immune checkpoint-related mechanisms. The CD8+ T cells in the EC TME display high quantities of immunological checkpoint molecules such the lymphocyte activation gene-3 (LAG-3), cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death PD-1, and programmed cell death-ligand (PD-L) 1[30,31].

PD-1 and PD-L1

Activated cytotoxic T cells and their primary ligands are the best examples of how PD-1 functions. Cancer cells and antigen-presenting cells (APCs) both have PD-L1 and PD-L2 molecules on their surfaces. T cell immunological fatigue is promoted by intracellular phosphorylation events that occur as a result of PD-1 engagement with its ligands, either through processes that depend on or are independent of TCR-CD28. The phosphoinositide 3-kinase route and the mitogen-activated protein kinase pathway are the two principals downstream signaling pathways involved in the TCR-CD28-dependent process. T cell depression that is not caused by TCR-CD28 is also caused by increased expression of the B-cell activating transcriptional factor[32-34]. CD8+ T cells in EC TME consistently exhibit significant levels of PD-1 expression. The findings of several clinical studies revealed that the objective response to immunotherapy was highly correlated with the high level of PD-L1 expression in EC tumor cells[35-37]. As a result, one of the suggested biomarkers for identifying EC patients who may benefit from ICIs is the degree of PD-L1 expression. Other than tumoral or healthy epithelial cells, TAMs play a significant role in the expression of PD-1 ligands in the EC TME. M2 polarization increases PD-L2 expression in TAMs and leads to immune evasion *via* the PD-1 signaling cascade regulated by the C-C motif chemokine ligand 2-C-C motif chemokine receptor 2 axis[38]. Additionally, TAMs showed PD-1 expression, which may increase PD-L1 expression in tumor cells. Increased CD8+ T cell abundance was seen in the responders in the clinical trials of PD-1 blockade therapy for EC, suggesting that the PD-1 inhibitors were successful in saving the worn-out T cells. To increase anti-tumor activity, these T cells settle on the tumor and fill the EC TME. Additionally, the decrease in the fraction of M2-type TAMs coincided with the rise in CD8+ T cell density, demonstrating that other innate immune cells in the EC TME were highly significant to the effectiveness of PD-1 blockade therapy for EC patients. In addition to the PD-1/PD-L1 pathway, interactions between PD-L1, CD80, and PD-L2-repulsive guidance molecule family member B are necessary for ICIs that target PD-L1 to be efficacious[39,40] (Figure 1).

LAG-3

The precise signaling pathways downstream of LAG-3 are unclear, although it is known that LAG-3 has a distinct signaling route that is not shared by other immunological checkpoints. It is not unexpected that LAG-3 binds to major histocompatibility complex (MHC)-II given that their structures are comparable. However, the affinity of the interaction between LAG-3 and MHC-II is substantially stronger. LAG-3 affects CD8+ T cell activity as well, pointing to the possibility of new LAG-3 ligands. LAG-3 is one of the hallmarks of worn-out CD8+ T cells and is considerably overexpressed in ESCC. Positive LAG-3 expression strongly predicted lower recurrence-free survival and OS in ESCC patients

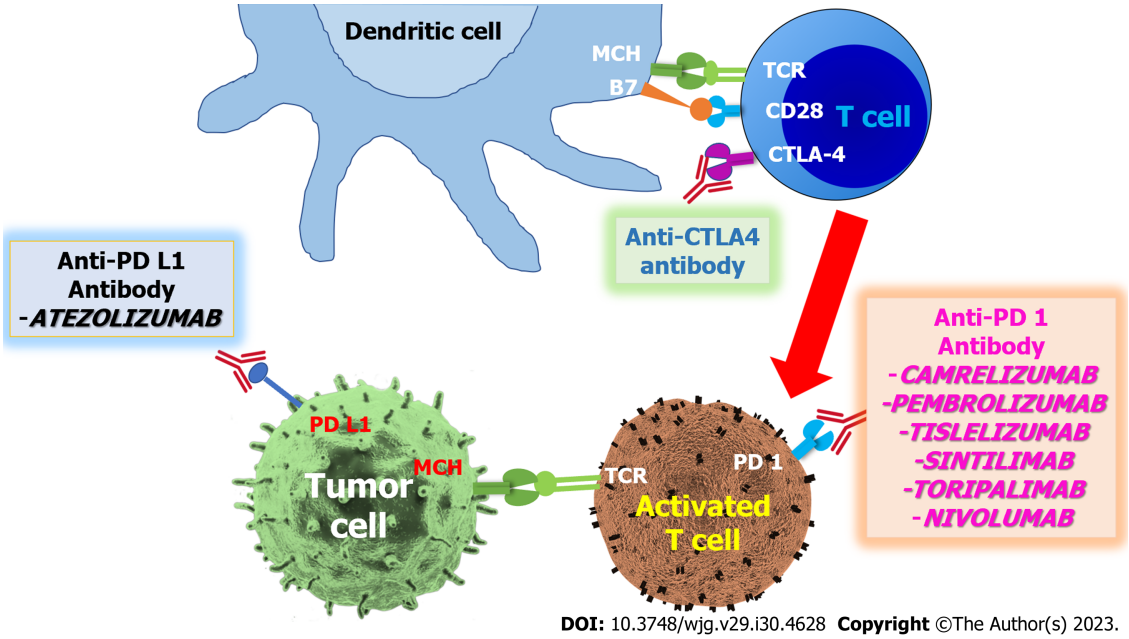

Figure 1 The mechanisms of action of immune checkpoint inhibitors in resectable esophageal cancer immunotherapy. CD28: Cluster of differentiation 28; CTLA-4: Cytotoxic T lymphocyte antigen 4; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; TCR: T cell receptor.

and was significantly correlated with CTLA-4 expression. Strong correlations were found between high levels of CD8+ tumor-infiltrating lymphocytes (TILs) in the EAC TME and high levels of LAG-3 expression. Research on individuals with unresectable EAC found connections between the LAG-3 gene and their response to the combination treatment of nivolumab and ipilimumab. Numerous LAG-3+ CD8+ T cells in the EAC TME are thought to be remnants of a robust anti-tumor immune response that was suppressed by PD-L1 and LAG-3-related mechanisms. The EAC patients who exhibit this TME trait are a good fit for ICIs. As a result, LAG-3 is a novel target for ICI's treatment of EC and is generating a lot of attention[41-44].

CTLA-4

The engagement of the TCR by peptides from the MHC and crucial positive co-stimulatory signals produced by the interaction of CD80 (B7.1) or CD86 (B7.2) on the surface of APCs and CD28 on the surface of T cells are both necessary for T cell activation. This process is damaged by CTLA-4. Intracellularly stored CTLA-4 is transferred onto the surface of T cells during the initial stages of T cell activation, competing with CD80 with a higher affinity and diminishing the signal. *Via* phosphorylation reactions, CTLA-4 attenuates signals downstream of the TCR intrinsically after interacting with CD80. The primary professional APCs whose immunological capabilities are compromised in individuals with EC, along with a reduction in CD80 and CD86 expression, are dendritic cells. The high level of CTLA-4 expression on the surface of Tregs in the EC TME significantly contributes to immunosuppression. Further weakening the anti-tumor immune response, elevated expression of CTLA-4 is found in ESCC tumor cells on both an mRNA and protein level, in addition to effector T cells and Tregs in EC patients. Previous research has shown that ESCC patients with high tumor cell CTLA-4 expression had a poor prognosis, patients with ESCC who exhibited little tumor cell CTLA-4 expression, however, had a longer OS. Additionally, it's been proven that CD80 guards against metaplasia in inflammatory esophageal carcinogenesis, which is linked to EAC. In light of these findings, scientists developed ICIs that specifically target CTLA-4 to enhance EC's anti-tumor immunity. We still don't know how CTLA-4 blocking treatment impacts EC TME and effector T cells[45-47].

ICIS FOR RESECTABLE EC TREATMENT

Neoadjuvant immunotherapy is the main subgroup of the clinical studies on ICI treatment for resectable EC. Camrelizumab, Pembrolizumab, Tremelimumab, Tislelizumab, Sintilimab, Atezolizumab, Nivolumab, Durvalumab, Relatlimab, Toripalimab, and IMC-001 were among the ICIs employed in these research. According to the CROSS-study's protocol, neoadjuvant immunotherapies are frequently used in conjunction with NCRT or NCT.

Camrelizumab

The National Medical Products Administration has authorized the PD-1 inhibitor Camrelizumab as a first-line therapy for unresectable ESCC. The neoadjuvant use of Camrelizumab for resectable locally advanced ESCC has been the focus of a significant number of clinical trials, making it one of the most popular ICIs being studied for its therapeutic efficacy. The

majority of these trials have proven the beneficial effects of combining Camrelizumab with NCT and NCRT. In research done by Wu *et al*[48], 38 patients with resectable ESCC were examined to determine the effectiveness and safety of NCT coupled with immunotherapy. Pembrolizumab (55.26%) and Camrelizumab (31.58%) were the two drugs most often taken by patients. The results of their analysis of 19 individuals revealed that 13 patients (68.42%) had a radiological partial response (PR) on computed tomography scans. 35 patients (92.11%) underwent R0 resection, and postoperative problems occurred in 10 individuals (26.32%). A substantial positive correlation between the pathological remission rate and the regression rate of the sum of lesion diameters was also found. Major pathologic response (MPR) rates were 42.11% in ESCC patients.

In a trial by Liu *et al*[49,50], the primary endpoint was used to assess the safety as well as the efficacy of Camrelizumab with NCT in 60 patients with locally advanced ESCC. Fifty-five (91.7%) of these patients successfully completed the whole two-cycle therapy. Fifty-one patients had surgery, and 50 (98.0%) of them had R0 resection. Twenty patients (39.2%) had pathological complete response (pCR) (ypT0N0), while five patients (9%; ypT0N+) had a full response to the main tumor but persistent illness in the lymph nodes alone. Leukocytopenia (86.7%) was the most prevalent of the 58 TRAEs that affected individuals (96.7%). One patient (1.7% of patients) experienced a grade 5 adverse event, while 34 patients (56.7%) experienced AEs of grade 3 or worse. They stated that chemotherapy and Camrelizumab had strong anticancer effects that had been validated and shown without any unanticipated safety effects. These results demonstrated the efficacy of Camrelizumab with chemotherapy as a neoadjuvant therapy for locally advanced ESCC. It is necessary to conduct a phase 3 randomized controlled study to further illustrate potential survival gains (Table 1).

Pembrolizumab

The United States Food and Drug Administration (FDA) has authorized the PD-1 inhibitor pembrolizumab as a first-line therapy for unresectable ESCC and EAC when combined with chemotherapy. Neoadjuvant immunotherapy has undergone clinical testing to treat both resectable ESCC and EAC. Preoperative Pembrolizumab with concurrent chemoradiotherapy for resectable locally advanced ESCC is being tested in the phase 1b, single-arm PALACE I trial, with safety as the main end measure. Of the 20 participants, 18 (90%) underwent surgery, and 19 (95%) received complete preoperative care. The patient had grade 3 lymphopenia and leukopenia, and she passed away while undergoing surgery, which is why the neoadjuvant therapy was not completed. The disease progression led to the discontinuation of surgery for one participant after full neoadjuvant therapy. All 20 patients experienced the development of TRAEs of any grade during the neoadjuvant therapy phase. The most frequent TRAEs were leukopenia (100%), lymphopenia (100%), anemia (80%), esophagitis (55%), alopecia (55%), and fatigue (55%), the majority of which were grade 1 or 2. With a pCR rate of 56% and a substantial pathological response of 89%, the R0 resection rate was 94%. All of the patients who received radical resection were free of disease recurrence at the median postoperative follow-up of 6.6 mo[51]. The PALACE II multicenter trial is still underway and has a larger sample size (143 participants), with pCR set as the major end measure[52]. Thirty-one eligible patients were included in a phase 1b/2 study to examine the effects of trimodal therapy with Pembrolizumab in the treatment of gastroesophageal junction (GEJ) adenocarcinoma. Twenty-eight individuals got R0 resection, and 29 of the 31 patients received all recommended doses of neoadjuvant Pembrolizumab. All safety criteria were satisfied. The primary efficacy goal was not attained [7/31 (22.6%)]. Individuals with high baseline expression of PD-L1 in the TME [combined positive score (CPS) > 10] had a substantially higher pCR rate than patients with low expression. Additionally, PFS and OS were longer for patients with high PD-L1 expression compared to propensity-score-matched patients. Extracellular vesicles (EV) were investigated to determine whether they may detect additional responders among trial participants with PD-L1 CPS < 10. A greater plasma level of PD-L1-expressing EVs was substantially linked with a higher pCR. They indicated that when Pembrolizumab was added to trimodal treatment, it did not achieve the intended pCR endpoint because of its satisfactory tolerability. Exploratory findings revealed that individuals who are most likely to achieve tumor response may be identified by having high levels of PD-L1 expression in the TME or on EVs[53].

Pembrolizumab in conjunction with chemotherapy and simple chemotherapy were compared for their effectiveness and safety in a trial by Huang *et al*[54], which involved 54 ESCC patients with stages II–IVa (23 in the combination group and 31 in the simple chemotherapy group). After two cycles of neoadjuvant therapy, major surgical intervention was given to all patients. They discovered that the combination group's pCR, ORR, and tumor regression 2 score were all considerably greater than those of the basic chemotherapy group (30.4% *vs.* 9.7% and 80.7% *vs.* 50.0%, respectively). Additionally, there was no statistically significant difference between the two groups' complete esophagectomy and R0/R1 resection rates. They concluded that pembrolizumab with chemotherapy demonstrated encouraging activity with a tolerable safety profile. Additionally, it might present a brand-new neoadjuvant treatment strategy for ESCC patients. In a single-arm study, Duan *et al*[55] evaluated the efficacy and safety of neoadjuvant pembrolizumab with chemotherapy in 18 patients with resectable ESCC. They found that postoperative pathology showed pCR in 6 cases (6/13, 46.2%) and MPR in 9 cases (9/13, 69.2%). Grade 3 or higher: Significant TRAEs occurred in 5 individuals (5/18, 27.8%). The amount of residual viable tumor (RVT) in pretreatment specimens was not substantially correlated with PD-L1 expression. While there was a weak association between postoperative forkhead box P3-positive+ T cells/CD4+ T cell ratios and RVT, there was a substantial correlation between changes in CD68+ macrophage counts between pre- and post-treatment specimens.

Tislelizumab

Another inhibitor of PD-1 is Tislelizumab. Following systemic therapy, ESCCs that cannot be removed, have relapsed, or have spread have been approved for consideration by the FDA. Tislelizumab was combined with NCT in the TD-NICE study, a phase 2, single-arm clinical study. The MPR served as the study's main outcome indicator. Of the 45 patients, 36 had surgery and received comprehensive neoadjuvant care. Eighty percent of R0 resections were successful, with 72% of pCR and 50% of MPR. Leukopenia (73%), anemia (51%), and thrombocytopenia (49%) were the most prevalent TRAEs.

Table 1 The immunotherapies for resectable esophageal cancer patients

Name	Ref.	Number of patients	Treatment regimens	Outcome	Conclusion
Camrelizumab	Wu *et al* [48], 2021	12	Cisplatin + paclitaxel + camrelizumab = 8; carboplatin + paclitaxel + camrelizumab = 4	MPR, 5/7 (71.43%)	Patients with ESCC had an MPR rate of 42.11%; The SLD regression rate has a certain guiding relevance for the impact of immunotherapy, and the adoption of the NACI regimen may not raise the likelihood of problems in neoadjuvant treatment and surgery
Camrelizumab	Liu *et al* [49], 2022	60	Carboplatin + paclitaxel + camrelizumab	Proceeded to surgery, 51/60 (85.00%); R0 resection, 50/51 (98.00%); pCR, 5/20 (39.20%)	Camrelizumab plus weekly chemotherapy as a promising neoadjuvant treatment for locally advanced ESCC, and further phase 3 randomized controlled trial is warranted
Camrelizumab	Liu *et al* [50], 2022	56	Cisplatin + paclitaxel + camrelizumab	Proceeded to surgery, 51/56 (77.40%); pCR, 18/56 (39.20%); MPR, 12/56 (21.43%)	Camrelizumab plus neoadjuvant chemotherapy in resectable ESCC demonstrates promising efficacy with acceptable toxicity, providing a feasible and effective option
Pembrolizumab	Li *et al* [51], 2021	20	Carboplatin + paclitaxel + pembrolizumab + radiotherapy	Proceeded to surgery, 18/20 (90.00%); pCR, 10/18 (55.60%)	PPCT was safe and did not delay surgery in resectable EC
Pembrolizumab	Zhu *et al* [53], 2022	31	Carboplatin + paclitaxel + pembrolizumab + radiotherapy	Proceeded to surgery, 28/29 (96.55%); pCR, 7/31 (22.58%)	Incorporating anti–PD-1 therapy into neoadjuvant chemoradiation and adjuvant treatment of GEJ adenocarcinoma may improve pCR and survival
Pembrolizumab	Huang *et al*[54], 2021	54	Nedaplatin + docetaxel + pembrolizumab = 23 *vs.* nedaplatin + docetaxel = 31	pCR 30.4%/9.7%; ORR 86.9%/95.7%	Pembrolizumab combined with chemotherapy showed promising activity with a manageable safety profile and it could offer a potential new neoadjuvant treatment approach for patients with ESCC
Pembrolizumab	Duan *et al*[55], 2022	18	Nedaplatin + paclitaxel + pembrolizumab = 13 or nedaplatin + docetaxel + pembrolizumab = 5	Proceeded to surgery, 13/18 (72.22%); MPR, 9/13 (69.20%); pCR, 6/13 (46.15%)	The combination of neoadjuvant immunotherapy and chemotherapy for ESCC is associated with a high pathological response and immunologic effects in the tumor microenvironment
Tislelizumab	Yan *et al* [56], 2022	45	Carboplatin + paclitaxel + tislelizumab	Proceeded to surgery, 36/45 (80.00%); MPR (69.20%); pCR (50.00%)	Tislelizumab plus chemotherapy as neoadjuvant therapy demonstrates promising antitumor activity for resectable ESCC with high rates of MPR, pCR, and R0 resection, as well as acceptable tolerability
Sintilimab	Chen *et al* [57], 2023	30	Cisplatin + S1 + paclitaxel + sintilimab	Proceeded to surgery, 30/30 (100.00%); MPR (50.00%); pCR (20.00%)	Neoadjuvant sintilimab plus platinum-based triplet chemotherapy appeared safe and feasible, did not delay surgery and induced a pCR rate of 20.0% in patients with potentially resectable ESCC
Atezolizumab	van den Ende *et al* [58], 2021	40	Carboplatin + Paclitaxel + Atezolizumab + Radiotherapy	Proceeded to surgery, 33/40 (82.50%); MPR (50.00%); pCR, 10/40 (25.00%)	The addition of atezolizumab to conventional nCRT for resectable EC was feasible without compromising surgical outcomes
Toripalimab	Xing *et al* [59], 2021	30	Cisplatin + paclitaxel + toripalimab D3 *vs.* cisplatin + paclitaxel + toripalimab D1	Proceeded to surgery, 11/15 *vs.* 13/15; pCR 4/15 *vs.* 1/15	The study showed that delaying toripalimab to day 3 in chemoimmunotherapy might achieve a higher pCR rate than that on the same day
Toripalimab	He *et al* [60], 2022	20	Carboplatin + paclitaxel + toripalimab	Proceeded to surgery, 16/20; R0 resection, 14/16; MPR, 7/16; pCR, 4/16	The combination of toripalimab plus paclitaxel and carboplatin is safe, feasible, and effective in locally advanced resectable ESCC
Toripalimab	Gao *et al* [61], 2022	20	Cisplatin + docetaxel + toripalimab	ORR, 14/20; MPR, 5/12; pCR, 2/12	Toripalimab combined with docetaxel and cisplatin as a novel neoadjuvant therapy was safe and effective in locally advanced ESCC
Toripalimab	Zhang *et al*[62], 2023	60	S1 + paclitaxel + toripalimab	R0 resection, 55/60; MPR, 27/60; pCR, 16/60	Neoadjuvant therapy with toripalimab, nab-paclitaxel and S1 was less toxic and showed promising antitumor activity in patients with resectable ESCC
Nivolumab	Kelly *et al* [63], 2021	794	Chemoradiotherapy+ nivolumab = 532 *vs.* chemoradiotherapy+ placebo = 262	DFS, 22.4/11.0 mo; distant recurrence, 29/39%; locoregional recurrence, 12/17%	Nivolumab adjuvant treatment significantly increased DFS compared to placebo in patients with resected esophagus or gastroesophageal junction cancer who had received neoadjuvant chemoradiotherapy

DFS: Disease-free survival; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; GEJ: Gastroesophageal junction; MPR: Major pathological

response; NACI: Neoadjuvant chemotherapy combined with immunotherapy; nCRT: Neoadjuvant chemoradiotherapy; ORR: Objective response rate; pCR: Pathological complete response; PD-1: Programmed cell death protein 1; SLD: Sum of lesion diameter; PPCT: Preoperative pembrolizumab with concurrent chemoradiotherapy.

Nineteen (42.2%) of the 45 patients had TRAEs in grades 3 to 4. Seventy-eight percent of the 36 individuals had postoperative problems. There were no surgical delays or deaths brought on by the therapy. They discovered that Tislelizumab in combination with chemotherapy as neoadjuvant therapy shows encouraging anticancer effectiveness for resectable ESCC with high rates of MPR, pCR, and R0 resection, as well as moderate tolerability[56].

Sintilimab

Sintilimab, another PD-1 inhibitor, has been approved by the FDA for the treatment of certain subtypes of non-small cell lung cancer (NSCLC) and Hodgkin's lymphoma. The primary use of Sintilimab is as a neoadjuvant therapy for ESCC. In 30 patients with possibly resectable ESCC, Chen *et al*[57] performed a single-arm, phase 2, open-label study to evaluate the safety and surgical viability of intravenous Sintilimab preoperatively with triplet chemotherapy (cisplatin, liposomal paclitaxel, and S-1) for a total of two cycles every 3 wk. The MPR and pCR rates were discovered to be 50.0% and 20.0%, respectively. Patients were more likely to react if they had a greater TMB and more clonal mutations. Changes in the circulating tumor DNA high-releaser status and the v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 are adversely linked with the response to neoadjuvant ICI. They discovered that neoadjuvant Sintilimab in conjunction with platinum-based triplet chemotherapy was safe and effective, did not delay surgery, and led to a 20.0% pCR rate in patients with ESCC who would be eligible for resection. A total of 36.7% (11/30) of patients experienced TRAEs of grades 3–4. Reduced neutrophil count (73.3%), anemia (76.7%), and reduced white cell count (76.7%) were the most common TRAEs. Hematological toxicities constituted all TRAEs. This confirmed that it was safe to use. To confirm the viability of the suggested approach, more investigation is needed.

Atezolizumab

A PD-L1 inhibitor called Atezolizumab has been given FDA approval for the treatment of melanoma, NSCLC, small cell lung cancer, and some types of urothelial cancer. In the phase 2 feasibility trial PD-L1 Targeting in Resectable EC (PERFECT), NCRT is coupled with Atezolizumab to treat resectable EAC. The major end measure of this trial was feasibility, which was determined as the percentage of Atezolizumab treatments that were completed. Thirty-four (85%) of the 40 patients finished the full course of atezolizumab treatment. Any missed cycles were brought on by autoimmune-related toxicity (n = 3), progression (n = 2), and death (n = 1). The R0 resection rate was 100% with 30% pCR. The PERFECT study had a median OS of 29.7 mo and a median PFS of 19.4 mo[58]. Fatigue (95%), mucositis (60%), nausea (53%), and anorexia (43%) were the most prevalent TRAEs. A grade 3–4 TRAE was encountered by 16 patients (40%). The three most prevalent symptoms were syncope (8%), nausea (8%), and anorexia (10%). The aforementioned TRAEs mostly took place during NCRT in conjunction with ICI. One patient who passed away from a pulmonary embolus had a grade 5 TRAE. Thirteen individuals (33%) had serious TRAEs that resulted in hospitalization or death.

Toripalimab

The FDA authorized Toripalimab, an anti-PD-1 monoclonal antibody, for the treatment of ESCC, nasopharyngeal carcinoma, mucosal melanoma, and sarcoma. In order to assess the efficacy of neoadjuvant chemoimmunotherapy (Toripalimab) in 30 patients with locally advanced ESCC, Xing *et al*[59] conducted an open-label, randomized phase 2 investigation. The patients were divided into two groups: The experimental group, which got chemotherapy on day 1 and Toripalimab on day 3, and the control group, which received chemotherapy and Toripalimab on day 1. The patients were then randomly allocated to either group. Paclitaxel and cisplatin were the components of the chemotherapy regimen. Four to six weeks following the second course of chemoimmunotherapy, surgery was undertaken. They reported that 13 participants in the control group and 11 participants in the experimental group had surgery. In each of these 24 cases, a R0 resection was done. Four patients (36%) in the experimental group and one patient (7%) in the control group both achieved pCR. Statistically non-significantly increased pCR rates were observed in the experimental group. One patient in the control group had a PD-L1 CPS of 10, and pCR was achieved. The other 13 patients all had PD-L1 CPSs of 1, and 11 of the 13 underwent surgery, with two of the 13 patients (in the experimental group) attaining pCR. After one round of chemoimmunotherapy, two patients experienced grade 3 adverse effects, and one patient withdrew out of the research due to immune-related enteritis that was grade 3. After surgery, a second patient passed away from a severe lung infection and elevated troponin. They concluded that postponing Toripalimab until day 3 of chemotherapy could result in a greater pCR rate than that on the same day.

In a study conducted by He *et al*[60], 20 patients with locally advanced resectable ESCC were examined to determine the effectiveness of Toripalimab with paclitaxel and carboplatin as neoadjuvant treatment and the possible prognostic biomarkers. For the 16 patients who underwent surgery without a related delay in treatment, the R0 resection rate was 87.5% (14/16). The pCR rate was 18.8% (3/16), and the MPR rate was 43.8% (7/16) among the 16 patients. Following neoadjuvant treatment, the fraction of M2-type TAMs reduced, and the number of CD8+ T cells rose in surgical tissues. Responders had greater baseline C-X-C motif chemokine 5 gene expression levels and lower baseline chemokine (C-C motif) ligand 19 and uromodulin-like 1 gene expression levels. They concluded that the treatment of locally progressed resectable ESCC with the combination of Toripalimab, paclitaxel, and carboplatin is dependable, doable, and effective, indicating its promise as a neoadjuvant therapy for ESCC. An open-label, non-randomized, single-arm, single-center

phase 2 study also looked at Toripalimab for usage as neoadjuvant treatment in conjunction with Docetaxel and Cisplatin in 20 patients with locally advanced ESCC. The ORR was reported to be 70% (14/20). McKeown's MIE was performed on twelve individuals. Sixteen percent (2/12) of initial tumors achieved pCR, while 41.7% (5/12) of primary tumors achieved MPR. The median time before surgery was 33.2 d, and no patients had surgery delayed as a result of AEs from the medication. The most typical 30-day postoperative consequence (3/12, 25%) was pneumonia. Only 1 patient experienced anastomotic leakage while they were in the hospital. No patients passed away as a result of medical care or surgery. They concluded that Toripalimab was a safe and effective new neoadjuvant treatment for locally progressed ESCC when coupled with Docetaxel and Cisplatin[61]. Toripalimab combined with nab-Paclitaxel and S-1 was evaluated in a phase 2 single-center, open-label, single-arm study in 60 ESCC patients. They discovered that 55 patients (98.21%) had successful R0 resections. In 27 patients (49.09%) with MPR, pCR was attained in 16 individuals (29.09%). There were 37 (61.67%), 21 (35.00%), and 2 (3.33%) patients with PR, stable illness, and progressing disease, respectively. Following therapy, the overall staging, Stooler dysphagia scores, and daily living ability were considerably reduced. Eleven patients (18.3%) had grade 3 AEs. Patients with PD-L1-high levels exhibited a considerably greater PR ratio than those with PD-L1-low levels. In patients who had a PR, the TMB and tumor neoantigen burden considerably decreased with treatment[62].

Nivolumab

The first immunotherapy to be recommended for resectable EC is adjuvant immunotherapy, which is appropriate for both ESCC and EAC with R0 resection following NCRT but staged as ypT-positive and/or N-positive. A PD-1 inhibitor known as Nivolumab is the ICI employed in this treatment plan. With a median follow-up of 24.4 mo, Kelly *et al*[63] conducted the worldwide, randomized, double-blind, placebo-controlled phase 3 study CheckMate 577 to assess Nivolumab as adjuvant treatment in 794 patients with esophageal or GEJ cancer. In contrast to the 262 patients who received a placebo, the authors found that the median DFS for the 532 individuals who received Nivolumab was 22.4 mo, as opposed to 11.0 mo for those who received the placebo (HR for disease recurrence or death, 0.69; 96.4% CI, 0.56 to 0.86; P = 0.001). In the Nivolumab group, 71 of 532 patients (13%), and 15 of 260 (6%) patients in the placebo group, encountered grade 3 or higher AEs. The trial regimen was discontinued in 9% of participants receiving Nivolumab and 3% of patients receiving a placebo owing to adverse drug or placebo-related events. They concluded that DFS was significantly longer in patients with resectable esophageal or GEJ cancer who had received NCRT who got Nivolumab adjuvant treatment than in those who received a placebo.

SUMMARY

Neoadjuvant ICIs are often used to treat ESCC patients, whereas perioperative ICIs are frequently used to treat EAC patients from the perspective of therapeutic models. This could be a result of NCRT having a better pathological outcome for ESCC than for EAC in earlier investigations[64]. A few clinical studies have moved into phase 3, and the majority are still in phase 2. It's important to be aware of phase 2 trial limitations, such as the limited sample size and lack of a control group. Trials investigating the use of ICIs in combination with NCRT, NCT, and PCT for resectable EC are still in their early phases in general. Credibility and generality are insufficient and need to be enhanced going forward. To get over these restrictions, multicenter or international phase 3 studies should be conducted. Very few studies have revealed the specifics of the response evaluation criteria and methodologies used in the various preoperative systemic therapy trials. The full metabolic response was the criterion used in the PALACE I investigation, which made use of the positron emission tomography-computed tomography scan. To help choose the best course of therapy, it is necessary to confirm how to more accurately gauge the reaction and specify the circumstances warranting surgery. TRAEs continue to endanger the safety of ICI therapy regimens, even if an acceptable rate of them has been recorded. All the research mentioned the above-mentioned TRAEs, and in certain situations, TRAE-related surgical delay or mortality happened. The safety of the ICIs is anticipated to drastically increase.

Immunotherapy for resectable EC patients primarily focuses on blocking the PD-1 and PD-L1 pathways, which have demonstrated impressive anti-tumor effects. But for some patients, ICIs treatment was ineffective, and in certain instances, medication resistance developed. PD-L1 expression in ESCC and microsatellite instability-high (MSI-H) or microsatellite instability-deficiency in mismatch repair are two indicators for successful ICIs treatment[65]. Unfortunately, PD-L1-positive ESCC did not significantly improve objective response to ICI treatment in a clinical investigation[66]. Furthermore, only 7% of EAC patients have MSI-H. Their predictive values are constrained. A critical issue that has to be solved is how to more effectively identify the EC patients who will benefit from PD-1 blocking treatment. A number of processes are involved in the PD-1/PD-L1 pathway's signaling process, such as chromosomal changes, mechanisms controlling molecule expression during and after transcription, and post-translational modification of molecules[67,68]. Scientists have identified various immune-related genes and chromosomal alterations unique to EC through the ongoing improvement of gene analysis techniques[69-71]. These are all possible biomarkers for EC patients who react to PD-1/PD-L1 blocking drugs. Furthermore, an immune-related long non-coding RNA signature of this pathway in ESCC was discovered, and it has the potential to be exploited as a stand-alone predictor of ESCC immunotherapy prognosis. PD-L1 expression inside the EC TME is now linked to immune infiltration frequency, which is what makes PD-1/PD-L1 blocking therapy beneficial. The frequencies of TILs, TAMs, CD8+ T cells, and Tregs are among the factors that are connected to clinical outcomes[72].

There aren't many active clinical studies of anti-CTLA-4 immunotherapy for resectable EC, in contrast to PD-1/PD-L1 blocking treatment. Severe TRAEs, the bulk of which were immune-related AEs (irAEs), limited the efficacy of CTLA-4 inhibiting therapy for a number of solid tumors. A deadly autoimmune illness is prevented by the intrinsic immune

checkpoint CTLA-4 on Tregs. Inhibiting CTLA-4 increases anti-cancer immunity but also upregulates auto-immune responses, leading to irAEs. For a safer and more effective immune checkpoint treatment, several scientists concluded that the CTLA-4 checkpoint should be preserved rather than blocked. Some people overcome this obstacle by creating anti-CTLA-4 antibodies that are specific to TMEs. There is an ongoing debate about whether CTLA-4-targeted immunotherapy should be permitted[73,74].

The immunosuppressive mechanism of LAG-3 is a recent area of study that has drawn a lot of interest. According to a recent study, MHC-II-independent fibrinogen-like protein 1 (FGL1) was a significant LAG-3 functional ligand. A poor prognosis and resistance to anti-PD-1 therapy are associated with high levels of FGL1 expression in the plasma of cancer patients, and it has been abundantly produced by human cancer cells[75,76]. The results of the clinical trial employing the Nivolumab and Relatlimab combination treatment for resectable EC may provide some insight into the riddle. Certainly, the positive results of immunotherapy for resectable EC in recent times give reason for optimism, but there are still many shortcomings to be overcome. The ICIs therapy for resectable EC will be ideal with a dependable effect and high safety in the next few days if these problems are promptly addressed.

CONCLUSION

Although systemic immunotherapy has produced encouraging preliminary findings in resectable EC in some clinical studies, and although preoperative immunotherapy may potentially be more beneficial than adjuvant therapy, more confirmation of the feasibility, safety, and effectiveness of neoadjuvant immunotherapy in sizable randomized clinical trials is still needed. Additionally, before neoadjuvant ICI techniques can be extensively used as the standard of treatment, a number of unsolved concerns must be overcome. To choose the right populations, it will be essential to identify predictive biomarkers, and the function of adjuvant treatment must be clearly understood. In order to assess any delayed toxicity and ascertain the long-term consequences, long-term follow-up is also required. Systemic immunotherapy will undoubtedly enter a new phase soon.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Exploring the regulatory mechanism of tRNA-derived fragments 36 in acute pancreatitis based on small RNA sequencing and experiments

Xi-Rui Fan, Yun Huang, Yu Su, Si-Jin Chen, Yu-Lu Zhang, Wei-Kang Huang, Hui Wang

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Abstract

BACKGROUND

Acute pancreatitis (AP) is a disease featuring acute inflammation of the pancreas and histological destruction of acinar cells. Approximately 20% of AP patients progress to moderately severe or severe pancreatitis, with a case fatality rate of up to 30%. However, a single indicator that can serve as the gold standard for prognostic prediction has not been discovered. Therefore, gaining deeper insights into the underlying mechanism of AP progression and the evolution of the disease and exploring effective biomarkers are important for early diagnosis, progression evaluation, and precise treatment of AP.

AIM

To determine the regulatory mechanisms of tRNA-derived fragments (tRFs) in AP based on small RNA sequencing and experiments.

METHODS

Small RNA sequencing and functional enrichment analyses were performed to identify key tRFs and the potential mechanisms in AP. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted to determine tRF expression. AP cell and mouse models were created to investigate the role of tRF36 in AP progression. Lipase, amylase, and cytokine levels were assayed to examine AP progression. Ferritin expression, reactive oxygen species, malondialdehyde, and ferric ion levels were assayed to evaluate cellular ferroptosis. RNA pull down assays and methylated RNA immunoprecipitation were performed to explore the molecular mechanisms.

RESULTS

RT-qPCR results showed that tRF36 was significantly upregulated in the serum of AP patients, compared to healthy controls. Functional enrichment analysis indicated that target genes of tRF36 were involved in ferroptosis-related pathways, including the Hippo signaling pathway and ion transport. Moreover, the occurrence of pancreatic cell ferroptosis was detected in AP cells and mouse models. The results of interference experiments and AP cell models suggested that tRF-36 could promote AP progression through the regulation of ferroptosis. Furthermore, ferroptosis gene microarray, database prediction, and immunoprecipitation suggested that tRF-36 accelerated the progression of AP by recruiting insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) to the p53 mRNA m6A modification site by binding to IGF2BP3, which enhanced p53 mRNA stability and promoted the ferroptosis of pancreatic follicle cells.

CONCLUSION

In conclusion, regulation of nuclear pre-mRNA domain-containing protein 1B promoted AP development by regulating the ferroptosis of pancreatic cells, thereby acting as a prospective therapeutic target for AP. In addition, this study provided a basis for understanding the regulatory mechanisms of tRFs in AP.

Key Words: Acute pancreatitis; tRNA-derived fragments; tRNA-derived fragments 36; Mouse models; Ferroptosis; Reverse transcription quantitative polymerase chain reaction

Core Tip: Based on reverse transcription quantitative polymerase chain reaction and bioinformatic analysis of small RNA sequencing data extracted from three patients and three healthy controls, and validated by 20 acute pancreatitis (AP) patients and 20 healthy controls, we found that tRNA-derived fragments 36 (tRF36) was significantly upregulated in AP. Furthermore, the results of the cell model of the MCP-83 cell line and knockdown of tRF36 suggested that tRF36 contributed to AP progression by promoting cell death.

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INTRODUCTION

As a common acute and critical condition of the digestive system, acute pancreatitis (AP) refers to local inflammation of the pancreas and even organ dysfunction due to self-digestion of the pancreas and surrounding organs after abnormal activation of pancreatic enzymes. The prevalence of AP is rising annually, with a global incidence of 4.9-73.4 per 100000 person-years[1]. Most patients display mild symptoms, recover gradually with treatment and have a good prognosis, however, approximately 20% of patients progress to moderately severe or severe pancreatitis, with a case fatality rate (CFR) of up to 30%[2]. Organ failure and pancreatic infection necrosis are believed to be the common causes of mortality in AP patients, and prompt and effective early intervention can improve the prognosis[3]. Accordingly, the prognosis of AP must be predicted at onset. However, a single indicator that can serve as the gold standard for prognostic prediction has not been discovered. Therefore, gaining deeper insights into the underlying mechanism of AP progression and the evolution of the disease and exploring effective biomarkers are important for early diagnosis, progression evaluation, and precise treatment of AP.

Programmed death of pancreatic acinar cells is the major pathophysiological change in the early stages of AP, with the mode of pancreatic acinar cell death playing a vital role in determining AP advancement and prognosis[4,5]. Ferroptosis is a new type of cell death characterized by intracellular iron-dependent lipid peroxidation. In recent years, excessive cellular ferroptosis has been demonstrated to exert a vital role in the pathogenesis of aseptic inflammatory conditions, such as ischemia-reperfusion injury and nonalcoholic steatohepatitis[6]. According to recent studies using a mouse model of AP with knockout of pancreatic tissue glutathione peroxidase 4 (GPX4), the ferroptosis of pancreatic acinar cells exacerbates pancreatic tissue injury, leading to significantly elevated levels of relevant biomarkers and accelerated AP progression[7,8]. The core proteoglycan released from the cells that have undergone ferroptosis can trigger immune responses and the production of proinflammatory cytokines, thereby exacerbating pancreatic acinar cell death and leading to further exacerbation of AP[9]. As shown above, ferroptosis of pancreatic acinar cells promotes the aggravation of AP; however, the molecular mechanism regulating the ferroptosis of pancreatic acinar cells remains unclear.

Non-coding RNAs (ncRNAs) are gaining increasing interest for AP diagnosis and treatment and are expected to be potential biomarkers and therapeutic targets[10]. tRNA-derived fragments (tRFs) have recently been identified as ncRNAs produced from mature tRNAs or tRNA precursors through a specific mechanism of action[11]. tRFs are widespread in various organisms and are extremely conserved, structurally robust, and tissue specific, participating in

different physiological and pathological processes[12]. A sequencing study revealed that several tRFs were abnormally expressed in AP cell models, among which tRF3-Thr-AGT affected AP progression by regulating trypsinogen activation in pancreatic acini[13]. Subsequently, another study revealed that tRF3-Thr-AGT expression was significantly downregulated in the pancreatic tissues of an AP cell model and an AP animal model, while overexpression of tRF3-Thr-AGT inhibited NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)-mediated pyroptosis and the inflammatory responses of pancreatic acinar cells and alleviated AP progression[14]. Accordingly, tRF3-Thr-AGT may be deployed as a biomarker for AP diagnosis and treatment[14].

In this study, serum RNA extraction for AP patients and healthy controls and small RNA sequencing were performed. Thereafter, candidate tRFs were identified using bioinformatics and validated using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The most significantly differentially expressed tRF36 was selected for subsequent investigation. An AP cell model and an AP mouse model were constructed to probe the role and mechanism of tRF36 in regulating ferroptosis and promoting AP progression and to identify the downstream effector pathways and targets. tRF36 was found to promote AP development and progression by regulating P53 expression and ferroptosis by binding to insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3). This finding provides a theoretical foundation for gaining further insights into the pathogenesis of AP and identifying new potential therapeutic targets.

MATERIALS AND METHODS

Small RNA sequencing

Serum was collected from three patients with AP and three healthy controls. Total RNA was retrieved utilizing the TRizol method, and libraries were constructed utilizing the Multiplex Small RNA Library Prep Kit for Illumina (NEB, United States). The procedure included: (1) 3′ splice ligation; (2) Reverse primer hybridization; (3) 5′ splice ligation; (4) One-strand cDNA synthesis; (5) PCR enrichment; and (6) 8% sodium dodecyl-sulfate polyacrylamide gel electrophoresis fragment sorting (SDS-PAGE). Thereafter, 2 × 150 bp sequencing was performed on an Illumina platform (Yingbiotech, Shanghai, China). This study was approved by Medical Ethics Committee of Yan'an Hospital Affiliated To Kunming Medical University (Approval No. 2022-024-01).

Identification of the differentially expressed tRFs

The overall quality of the sequencing data was evaluated *via* Fast-QC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For tRFs, sequences that did not match the miRBase (12-23 bp, 34-43 bp) and piRNAcluster (24-33 bp) were compared to rRNA. After we filtered out sequences that could be matched to rRNA, sequences that could be matched to the GtRNA database were matched to the tRFdb and tRFMINTbase databases to obtain tRF expression profiles. Finally, differential expression analysis of tRFs was performed using the DESeq2.0 algorithm (P value < 0.05 and $|\log_2$ fold change$|$ > 1).

Functional enrichment analysis

The target genes for the tRFs were mined *via* miranda (score > 150, energy < -20) and RNAhybrid (energy < -25), and the ultimate target genes were recognized by intersecting the two algorithms. For Gene Ontology (GO) analysis, the significance level of each GO was calculated depending on the GO database (http://www.geneontology.org/) and Fisher's test. The target genes were also annotated with pathways depending on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) to obtain all pathways involving the genes. The significance level (P value) of each pathway was calculated using Fisher's test and pathways with P value < 0.05 were deemed significantly enriched.

RT-qPCR

We collected serum from 20 patients with AP and 20 healthy controls. Total RNA was retrieved utilizing TRizol (Invitrogen), and reverse transcribed according to the manufacturer's instructions (Thermo, K1622). qPCR was carried out using 2 × Master Mix (Roche). The following thermocycling program was employed for qPCR: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. The relative expression level was normalized to that of the endogenous control U6 and calculated using the $2^{-\Delta\Delta Ct}$ method[15].

Establishment of the AP cell model and mouse model

Cerulein is a cholecystokinin analogue that provokes the secretion of digestive enzymes in the pancreas of humans and rodents[16]. Cerulein-induced pancreatitis is one of the best featured animal models of pancreatitis, which was first described in 1977 and is highly reproducible and economical[16-18]. Briefly, 10 nM cerulein (MCE, HY-A0190) was administered to the mouse pancreatic acinar carcinoma cell line MPC-83 (Shanghai Bei Nuo Biotechnology Co., Ltd., China) to develop an AP cell model. Male BALB/C mice (age, 6-8 wk; weight 25 ± 3 g; Beijing SiPeiFu Biotechnology Co., Ltd., China) were intraperitoneally administered cerulein 10 times at 1-h intervals to establish the AP mouse model. This study was approved by Animal Ethics and Welfare Committee of Kunming Yan'an hospital (Approval No. 2022013) in accordance with internationally accepted principles for the use of laboratory animals.

tRF interference

According to the manufacturer's protocols, Lipofectamine™ 2000 Transfection reagent (Invitrogen, United States) was

utilized to transfect the tRF-36 inhibitor into MPC-83 cells to knock down the expression of tRF-36. qRT-PCR was conducted to determine the knockdown efficiency of tRF-36.

Cell Counting Kit-8 assay

The viability of cells was determined *via* Cell Counting Kit-8 (CCK-8) assays (Beyotime, China). Then, we seeded 10^3 cells into a 96-well plate and added 10 μL of CCK-8 per well. The absorbance was measured at 450 nm by an enzyme-linked immunosorbent assay (ELISA) microplate reader (Infinite M1000, Tecan) after 1 h of incubation.

ELISA

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, mouse amylase (AMS), and mouse lipase (R&D Systems, MN, United States) were determined using ELISA kits and the absorbance was measured at 450 nm using a microplate reader (Infinite M1000, Tecan).

Detection of cell death

Cell death was assessed using the TUNEL Kit (Sangon Biotech), as described by the manufacturer. A fluorescent microscope (Nikon, Japan) was utilized to observe the specimens.

Reactive oxygen species and malondialdehyde detection

The oxidative-sensitive fluorescent probe DCFH-DA in the reactive oxygen species (ROS) Assay Kit (Beyotime, China) was utilized to assess the generation of intracellular ROS. A laser confocal microscope (Nikon, Japan) was utilized to observe the green fluorescence intensity. Malondialdehyde (MDA) is a metabolite of lipid oxidation and is widely used as an indicator of lipid oxidation. An MDA detection kit (Beyotime, S0131) was used to measure the MDA level in cells or tissues according to the manufacturer's instructions.

Ferric ion detection

A ferric ion colorimetric assay kit (Pulley's, #E1042) was used to measure ferric ion levels in mouse pancreatic tissue, according to the manufacturer's manuals.

Western blot analysis

Cells were lysed using RIPA lysis buffer, homogenized, and centrifuged. Thereafter, the supernatant was collected. Then, the protein concentration was measured using the bicinchoninic acid assay. The proteins were separated *via* SDS-PAGE and transferred to polyvinylidene fluoride membranes. After that, the membranes were incubated at 4 °C overnight with specific primary antibodies against p53 (Proteintech, 60004-1-Lg) and ferritin (Abcam, ab75973) after blocking with 5% nonfat milk. The membrane was then incubated with secondary antibody for 60 min. After the membrane was washed with TBST solution, it was analyzed using the chemiluminescence method. The relative quantification was performed using scanning software (ImageJ grayscale).

Hematoxylin and eosin staining

The mouse lung and pancreatic tissues were washed with phosphate buffered saline and fixed for 24 h in 4% paraformaldehyde fixative solution. Thereafter, conventional paraffin embedding was conducted, and tissue sections (4 μm) were prepared for hematoxylin and eosin (HE) staining. Finally, the histopathological morphology of the sections was observed under a microscope (Olympus, Tokyo, Japan).

Immunohistochemical staining

The tissue sections were dewaxed, rehydrated with xylene, and washed with an alcohol gradient. After soaking and washing with distilled water, H_2O_2 was used to destroy endogenous peroxidase activity. After incubation with anti-ferritin antibody (Abcam, ab75973), the sections were exposed to secondary antibodies. Instillation with diaminobenzidine for 5 min was then performed. Thereafter, the sections were counterstained with hematoxylin, dehydrated with ethanol, and clarified with xylene. Observation and image capture were performed using a microscope (Olympus, Tokyo, Japan).

RNA pulldown assay

The tRF36 probe labeled by biotin was generated, and a Magnetic RNA-Protein Pull-Down Kit [Sangon Biotech (Shanghai, China)] was utilized to implement the RNA pull-down assay depending on the manufacturer's instructions. First, the tRF36 probe or control probe was incubated with streptavidin magnetic beads for 30 min at room temperature. Thereafter, the cell lysates were incubated with probe-bead complexes for 1 h at 4 °C to enable binding of the proteins to RNAs. The RNA-protein complexes were washed and eluted from beads *via* incubation at 37 °C for 30 min with agitation. The eluted proteins were finally analyzed *via* silver staining. The differential protein bands of immunoglobulin G and tRF36-IP lanes were cut and sent to the mass spectrometry platform for protein profiling.

Methylated RNA immunoprecipitation-qPCR

The methylated RNA immunoprecipitation (MeRIP) experiments were completed *via* the riboMeRIPTM m6A Transcriptome Profiling Kit (Merck Millipore) to capture the RNA modified by m6A. Briefly, the total RNA of AP model cells and control cells was extracted by TRizol (Invitrogen). After fragmentation, RNA was incubated with the magnetic

bead-m6A antibody complex for immunoprecipitation. MeRIPed p53 was then analyzed using qRT-PCR.

Statistical analysis

The experimental data were compared *via* student's *t* test to determine the group difference. If not specified above, a *P* value < 0.05 was considered statistically significant.

RESULTS

Differentially expressed tRFs in AP

Based on quality control and preprocessing of the raw small RNA sequencing data for three serum samples from three AP patients and three control samples, we found that the sequencing quality met the criteria for subsequent analysis (Supplementary Figure 1). By RNA mapping based on the BWA algorithm, tRF expression data were extracted. A total of 116 upregulated tRFs and 95 downregulated tRFs in AP were screened *via* differential expression analysis (Figures 1A and B, Supplementary Table 1). According to functional enrichment analysis, many biological processes and pathways, including the Wnt signaling pathway, Hippo signaling pathway, and ion transport, were associated with the target genes of these differentially expressed tRFs (DE-tRFs) (Supplementary Figure 2).

tRF36 is upregulated in AP and contributes to AP progression

Due to the uncertainty of high-throughput sequencing, we collected serum from 20 AP patients and 20 healthy controls and verified the five most significant DE-tRFs using RT-qPCR (Figure 1C). As shown in Figure 1C, tRF36 was downregulated in the serum of AP patients, and its differential expression was the most significant ($P < 0.05$, Figure 1C). This finding implied that tRF36 played an important role in AP progression. To test our hypothesis, we generated an AP cell model using the MCP-83 cell line. Figure 2A revealed that the expression of tRF-36 was significantly reduced after the use of inhibitor. It is well known that AMS and lipase have diagnostic value for AP[19], and the inflammatory response is associated with AP-induced injury[20]. Therefore, we measured the expression of AMS and lipase as well as inflammatory factors to determine the feasibility of the model. After MCP-83 cells were treated with cerulein, the levels of AMS and lipase and the inflammatory factors TNF-α, IL-6, and IL-1β in the cell supernatant increased significantly, indicating successful establishment of the AP cell model (Figures 2B-F). After knockdown of tRF36, these markers were further assayed in the cell supernatants. Based on the results, the levels of AMS and lipase and the inflammatory factors TNF-α, IL-6, and IL-1β in the cell supernatant were significantly reduced after knockdown of tRF36 (Figures 2B-F). Cell viability and cell death were measured by CCK-8 and TUNEL assays, respectively. Knockdown of tRF36 was found to significantly increase cell viability, and significantly reduce cell death (Figures 2G and H). These results suggest that tRF36 contributes to AP progression by promoting cell death.

Presence of cell ferroptosis in AP

We proceeded to conduct functional enrichment analysis of the target genes of tRF36 (KEGG pathway). The target genes of tRF36 were found to be involved in ferroptosis-related pathways, namely, the P53 signaling pathway and mechanistic target of rapamycin (mTOR) signaling pathway (Figure 3A). To confirm the presence of ferroptosis in AP progression, we constructed an AP mouse model. Based on HE staining of lung and pancreatic tissues, significant changes were observed in pancreatic tissue cell morphology (Figure 3B). Furthermore, the serum levels of lipase and AMS in the AP model mice were significantly higher than those in the controls (Figures 3C and D). The serum levels of the inflammatory factors TNF-α, IL-6, and IL-1β were also significantly increased in the AP model mice (Figures 3E-G). Therefore, the AP mouse model was successfully established. Subsequently, the levels of ferritin expression, ferric ions, and the lipid oxidation metabolite MDA were examined in pancreatic tissue. The expression of ferritin in the pancreatic tissues of the AP model mice was significantly reduced (Figure 3H). Correspondingly, the levels of ferric ion concentration and MDA were significantly increased (Figures 3I and J). These results highlighted the presence of cell ferroptosis in AP.

tRF36 promotes AP progression through the regulation of ferroptosis

Based on the above results, we hypothesized that tRF36 may promote AP progression by regulating cell ferroptosis. Hence, we examined the ROS levels in AP model cells. The ROS levels of cells in the model group were found to be significantly higher than those in the control group (Figure 4A). Similar results were obtained using the AP cell model (*e.g.*, decreased ferritin expression and increased MDA levels) compared to control cells, which were similar to those of the mouse model (Figures 4B and C), suggesting the presence of cell ferroptosis in the AP model. After knockdown of tRF36 in MCP-83 cells, a significant decrease in ROS levels and MDA and a significant increase in ferritin expression levels were found in cells (Figures 4A-C). Therefore, we concluded that tRF36 could promote AP progression through the regulation of ferroptosis.

Exploring the molecular mechanisms by which tRF36 promotes AP progression

To further probe the molecular mechanisms underlying the regulation of ferroptosis by tRF36, we utilized a ferroptosis gene microarray to determine the differentially expressed ferroptosis genes after tRF36 knockdown. The gene expression of p53 was the most significantly downregulated of the genes (Figure 5A). Database analysis revealed that p53 mRNA interacts with several m6A proteins, including METTL3, ALKBH5, IGF2BP3, and others (Figure 5B). The SRAMP database (http://www.cuilab.cn/sramp) predicted that p53 mRNA has m6A modification sites with a very high

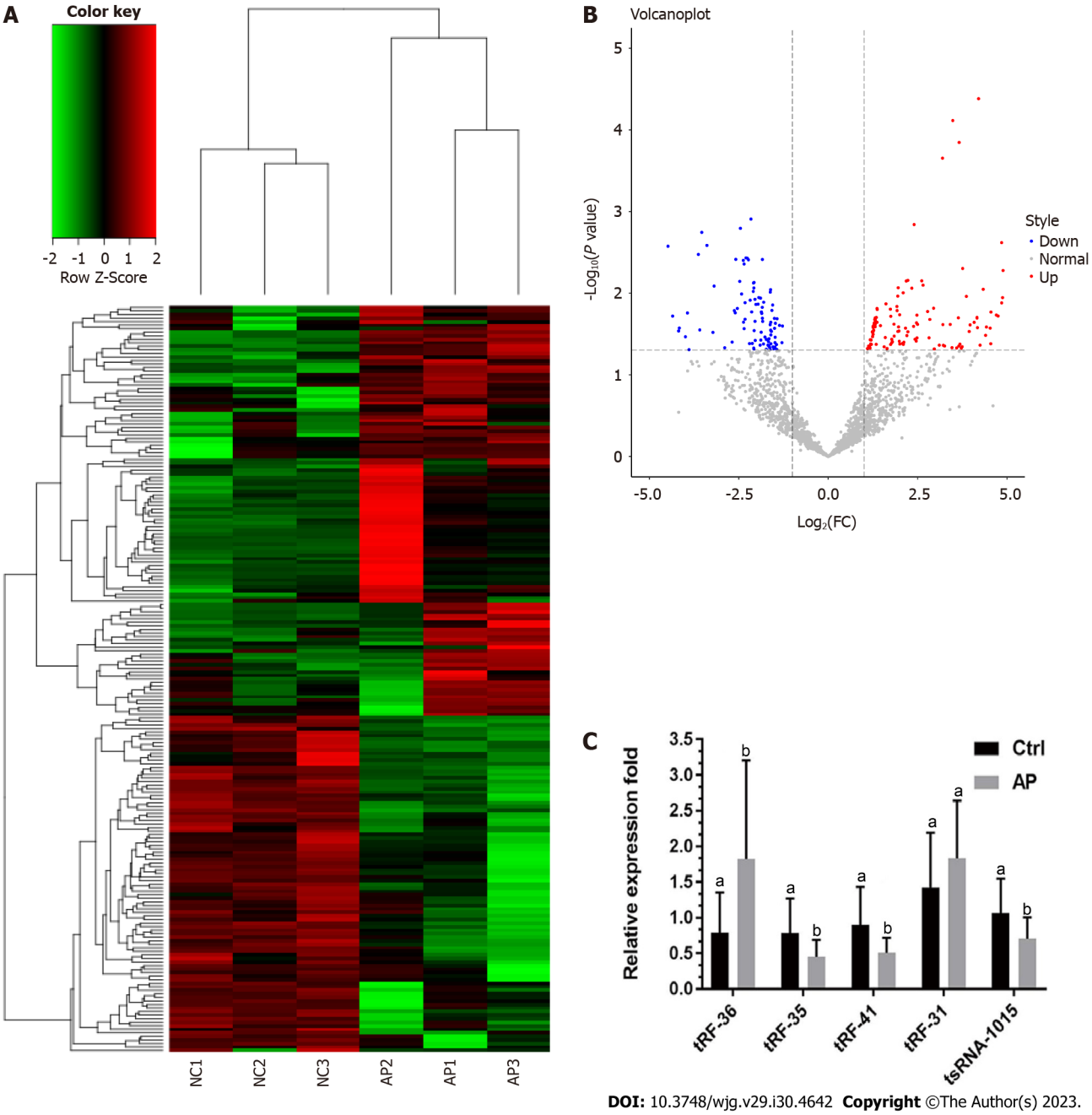

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Figure 1 Identification of 116 upregulated tRNA-derived fragments and 95 downregulated tRNA-derived fragments in acute pancreatitis by the differential expression analysis. A: Heatmap of the differentially expressed tRNA-derived fragments (DE-tRFs); B: Volcano plot for the 116 upregulated and 95 downregulated DE-tRFs; C: Gene expression of the five most significant DE-tRFs using reverse transcription quantitative polymerase chain reaction. Error bars represent the mean ± SD, a and b represent different expression levels, the same letter represents no significant difference between groups ($P > 0.05$), different letters represent significant difference between groups ($P < 0.05$). AP: Acute pancreatitis; tRF: tRNA-derived fragment.

probability of modification (Figure 5C). Therefore, we searched for proteins interacting with tRF-36 using an RNA pull down assay and mass spectrometry, which revealed that tRF-36 interacts with the m6A methylation regulator IGF2BP3 (Figures 5D and E, Supplementary Table 2). IGF2BP3 is a unique m6A reader protein that promotes stable mRNA expression and prevents mRNA degradation[21]. MeRIP-qPCR results revealed that the level of m6A modification of p53 was elevated in the AP cell model (Figure 5F). Western blot and qPCR analyses revealed that the knockdown of tRF36 in the AP cell model resulted in a significant reduction in p53 expression (Figures 5G and H). Interestingly, p53 expression was significantly restored after the overexpression of IGF2BP3 in cells with tRF36 knockdown (Figures 5G and H). Therefore, we speculated that tRF36 accelerated the progression of AP by recruiting IGF2BP3 to the p53 mRNA m6A modification site through binding to IGF2BP3, which enhanced p53 mRNA stability and promoted the ferroptosis of pancreatic follicle cells (Figure 6). The p53 expression level after the knockdown of tRF36 was determined by western blot qPCR.

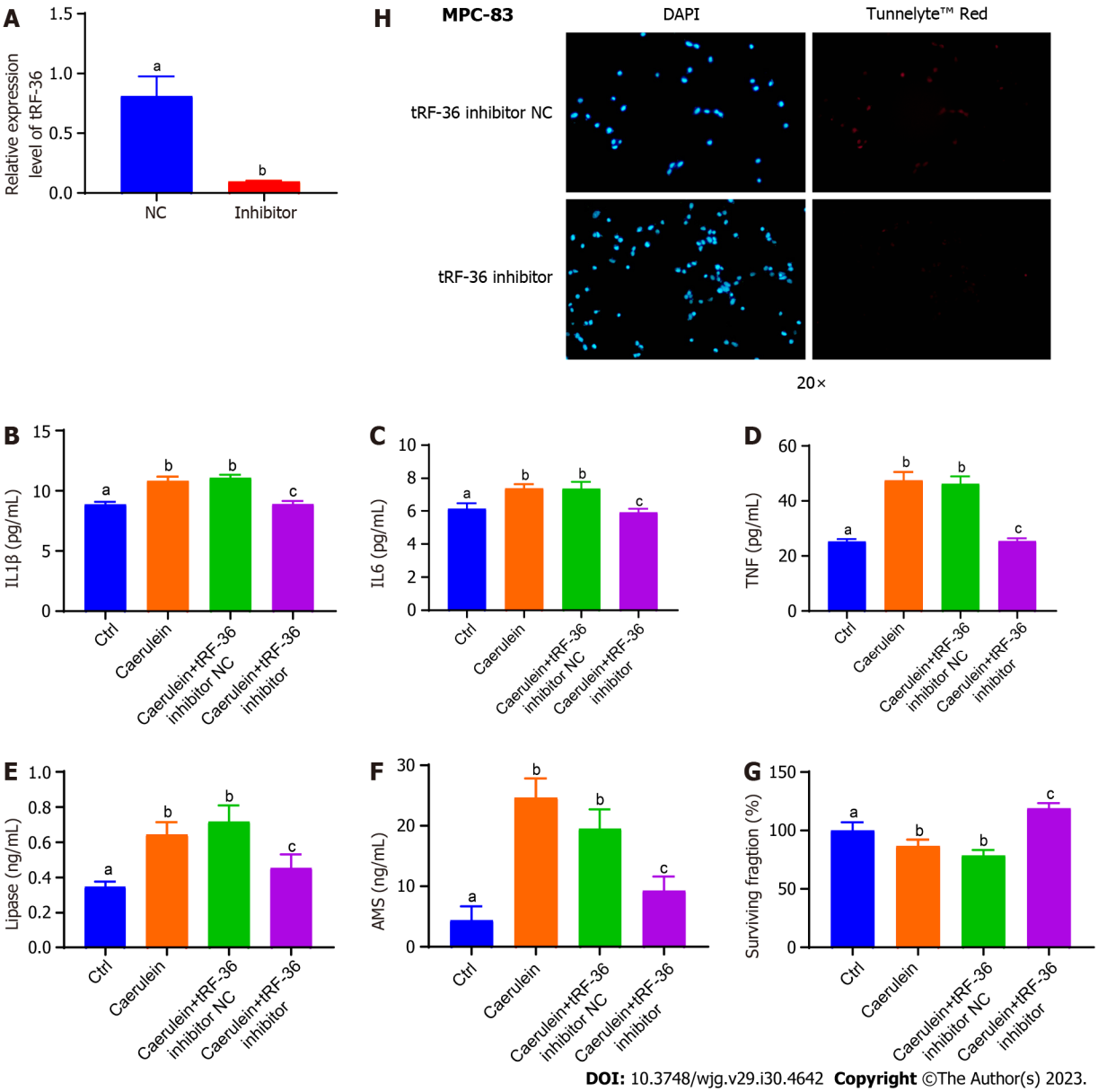

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Figure 2 Effects of tRNA-derived fragments 36 on acute pancreatitis progression in MCP-83 cells. A: Gene expression of tRNA-derived fragments 36 using reverse transcription quantitative polymerase chain reaction; B-D: The expression levels of inflammatory factors in the acute pancreatitis (AP) cell model, including interleukin-1β (B), interleukin-6 (C), and tumor necrosis factor-α (D); E: The expression levels of lipase in the AP cell model; F: The expression levels of amylase in the AP cell model; G: Cell viability examination using Cell Counting Kit-8 assays; H: Cell death examination using terminal deoxynucleotidyl transferase dUTP nick-end labeling analysis. Error bars represent the mean ± SD. a, b, and c represent different expression levels, the same letter represents no significant difference between groups ($P > 0.05$), different letters represent significant difference between groups ($P < 0.05$). tRF-36: tRNA-derived fragments 36; IL: Interleukin; TNF: Tumor necrosis factor; AMS: Amylase; NC: Normal control; DAPI: 4',6-diamidino-2-phenylindole.

DISCUSSION

The incidence of AP is increasing annually, with a global incidence of 4.9-73.4 per 100000 person-years[1]. Approximately 20% of AP cases will progress to moderate or severe pancreatitis, with a CFR of up to 30%[2]. The pathogenesis of AP is complex, and no effective clinical treatment has been developed to date. Therefore, gaining deeper insights into the underlying mechanism of AP progression and exploring effective biomarkers are important for AP diagnosis and treatment[9]. In this study, tRF36 was discovered to play an important role in AP progression; thus, its regulatory mechanism was explored.

Sequencing and bioinformatics analyses were performed using the small RNAs extracted from the serum samples of AP patients and healthy controls. Validation of tRF36, the most significantly expressed molecule, was performed using qPCR. The downstream target genes of tRF36 were then predicted and analyzed to explore their potential biological functions.

KEGG pathway enrichment analysis revealed that the target genes of tRF36 were mainly enriched in the ferroptosis-related p53 and mTOR signaling pathways. Numerous studies have confirmed that the p53 signaling pathway can regulate ferroptosis. For example, Chen *et al*[22] found that iPLA2 β-mediated lipid detoxification is vital for inhibiting

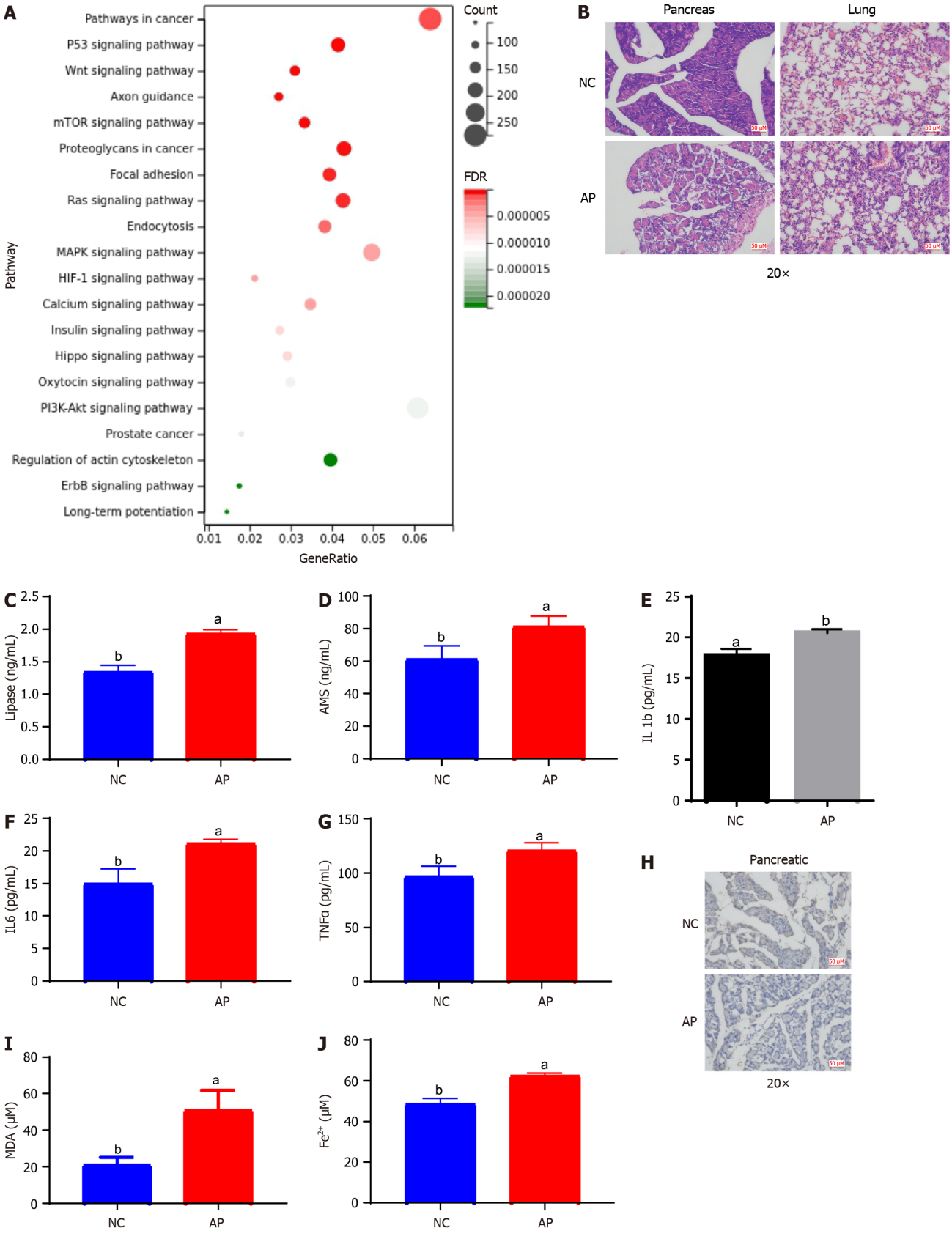

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Figure 3 Evaluation of the target genes of tRNA-derived fragments 36 for the cell ferroptosis process in acute pancreatitis. A: Functional enrichment analysis for the target genes of tRNA-derived fragments 36; B: Hematoxylin and eosin staining results of lung and pancreatic tissues in the acute pancreatitis (AP) mouse model; C and D: The serum levels of lipase and amylase in the AP mouse model; E-G: The serum levels of the inflammatory factors interleukin-1β (E), interleukin-6 (F), and tumor necrosis factor-α (G); H: The expression of ferritin in the mouse pancreatic tissues with AP; I: The levels of malondialdehyde in mouse pancreatic tissues with AP; J: The ferric ion concentration in mouse pancreatic tissues with AP. a and b represent different expression

levels, the same letter represents no significant difference between groups ($P > 0.05$), different letters represent significant difference between groups ($P < 0.05$). tRF-36: tRNA-derived fragments 36; IL: Interleukin; TNF: Tumor necrosis factor; NC: Normal control; mTOR: Mechanistic target of rapamycin; HIF-1: Hypoxia-inducible factor 1; FDR: False discovery rate; PI3K-AKT: Phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B; ErbB: Erythroblastic oncogene B; AP: Acute pancreatitis; MDA: Malondialdehyde; AMS: Amylase.

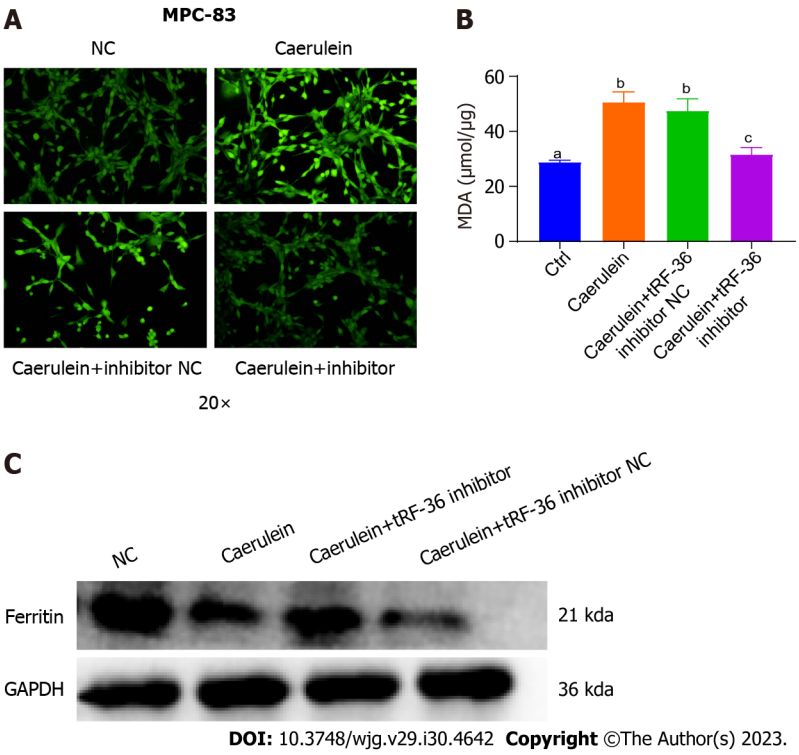

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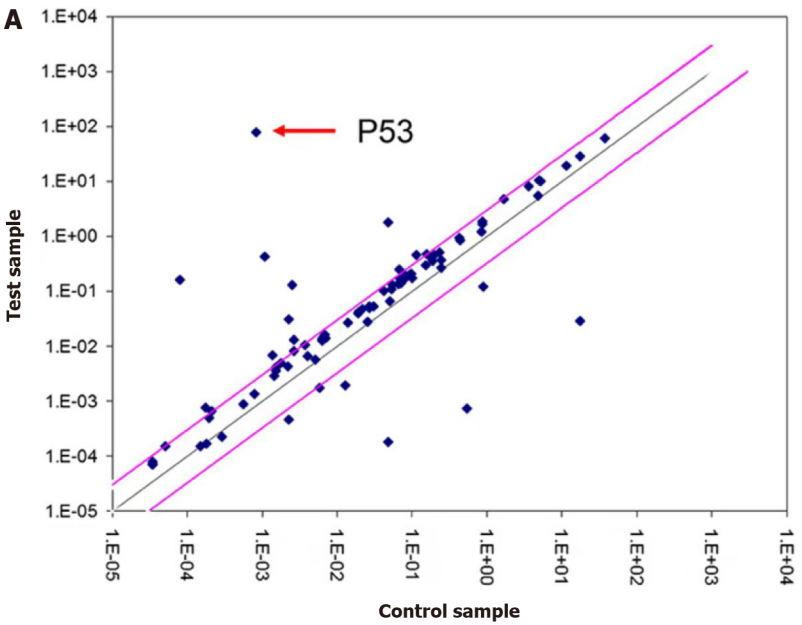
Figure 4 Effect of tRNA-derived fragments 36 on ferroptosis regulation in acute pancreatitis progression. A: The reactive oxygen species levels were detected under a laser confocal microscope; B: Exploration of the malondialdehyde levels in the acute pancreatitis cell model; C: Ferritin levels using western blot analysis. a, b, and c represent different expression levels, the same letter represents no significant difference between groups ($P > 0.05$), different letters represent significant difference between groups ($P < 0.05$). tRF-36: tRNA-derived fragments 36; NC: Normal control.

the ROS-induced ferroptosis of cancer cells. Chu *et al*[23] found an ALOX12-mediated, ACSL4-independent ferroptosis pathway that is vital for p53-dependent tumor suppression. Lei *et al*[24] found that radiotherapy (RT)-mediated p53 activation antagonizes RT-induced SLC7A11 expression and inhibits glutathione synthesis, thereby promoting RT-induced lipid peroxidation and ferroptosis. Li *et al*[25] found that ferroptosis contributes to acute lung injury induced by intestinal ischemia/reperfusion and that iASPP treatment partially inhibits ferroptosis *via* nuclear factor E2-related factor 2 (Nrf2). Jiang *et al*[26] found that p53 inhibits cystine uptake and sensitizes cells to ferroptosis by suppressing SLC7A11 expression.

Numerous studies have shown that the mTOR signaling pathway regulates ferroptosis. For example, Conlon *et al*[27] found that acute amino acid deprivation-induced proliferative arrest correlates with protection from ferroptosis in a manner independent of mTOR inhibition and GCN2/ATF4 pathway activation. Sun *et al*[28] not only supported the concept that ferroptosis is autophagy-dependent cell death but also suggested that the combined application of ferroptosis inducers and mTOR inhibitors is a promising approach to improve bladder cancer treatment options. Hsieh *et al*[29] found that ZVI-NP selectively triggered the ferroptosis of cancer cells by inhibiting the Nrf2-mediated cytoprotective program, which was attributed to ZVI-NP-induced disruption of AMPK/mTOR signaling and activation of the GSK3βSK-TrCP-dependent degradation system.

In recent years, the role of ncRNAs in AP has received increasing attention, and ncRNAs are expected to be a potential biomarker and therapeutic target. Owing to the development of sequencing technology, many AP-associated ncRNAs have been identified, such as long ncRNAs, microRNAs, and tRFs[30,31], suggesting that ncRNA dysregulation plays an important role in the development of AP. For example, Li *et al*[32] demonstrated that tRNA-derived small RNAs (tsRNAs) can be used as a potential therapeutic biomarker for bile duct cancer. The sequencing results of Yang *et al*[13] revealed that different tRFs were abnormally expressed in AP cell models, and tRF3-Thr-AGT affected AP progression by regulating trypsinogen activation in pancreatic acini. A later study further showed that tRF3-Thr-AGT expression was significantly downregulated in the pancreatic tissues of an AP cell model and an AP animal model, while overexpression of tRF3-Thr-AGT inhibited the NLRP3-mediated pyroptosis and inflammatory responses of pancreatic acinar cells, thereby alleviating AP progression[14]. However, no existing studies have reported the involvement of tRF36 in regulating the pathogenesis of AP. In the present study, tRF36 expression was upregulated in the serum of AP patients,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P53
Test sample
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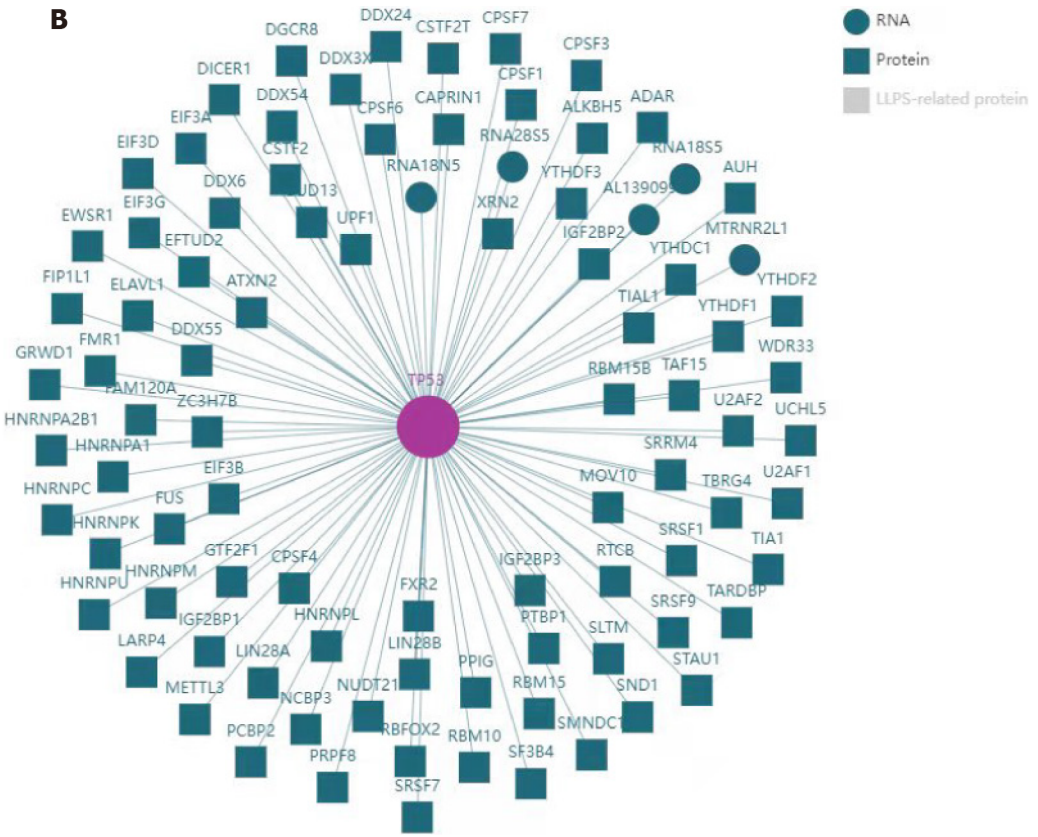
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LLPS-related protein
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EIF3B
MOV10
TBRG4
U2AF1
HNRNPC
FUS
SRSF1
HNRNPK
TIA1
GTF2F1
CPSF4
IGF2BP3
RTCB
HNRNPM
FXR2
SRSF9
TARDBP
HNRNPU
PTBP1
IGF2BP1
HNRNPL
SLTM
LARP4
LIN28A
LIN28B
STAU1
PPIG
METTL3
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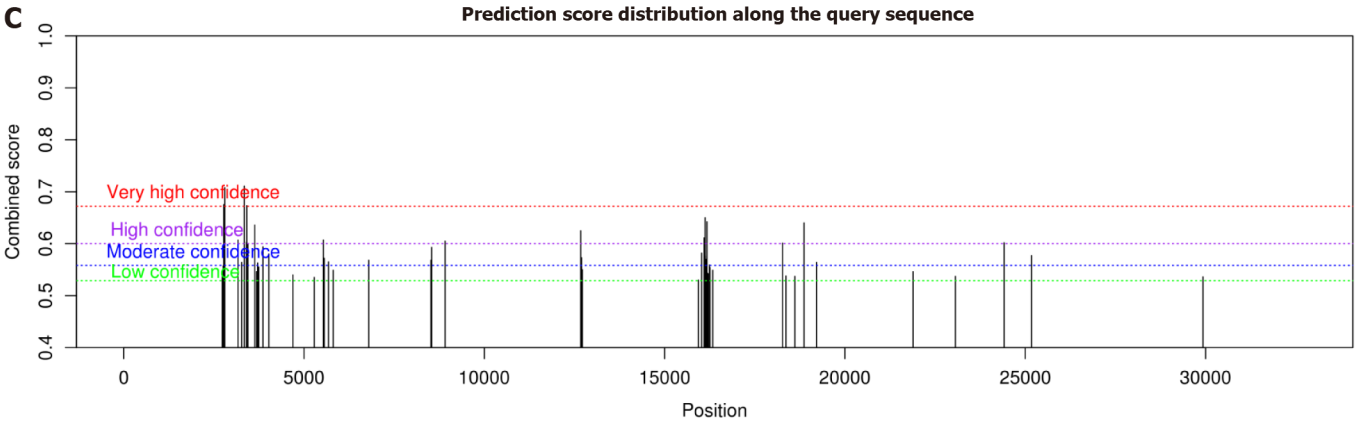
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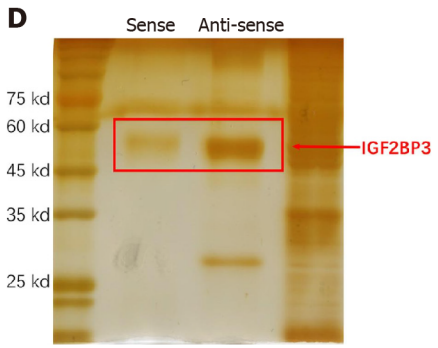
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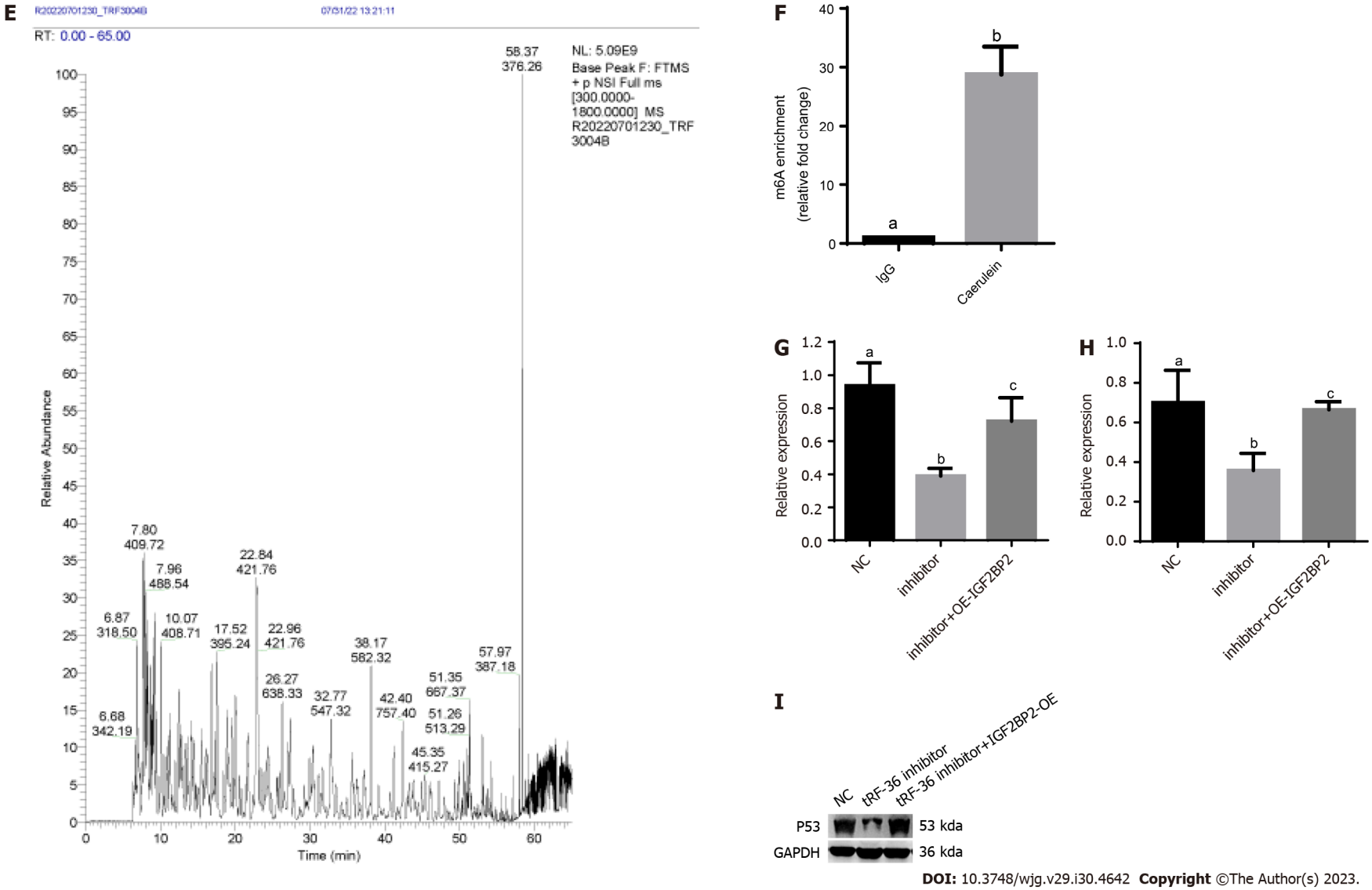

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Figure 5 Exploration of the molecular mechanisms of tRNA-derived fragments 36 in acute pancreatitis progression. A: p53 was the most significantly downregulated gene in ferroptosis gene microarray analysis after tRNA-derived fragments 36 (tRF-36) knockdown; B: The interaction network of p53 and m6A proteins; C: The m6A modification analysis for p53 mRNA by SRAMP; D: The RNA pull down assay for tRF-36 with insulin-like growth factor 2 mRNA binding

protein 3; E: Mass spectrometry results for tRF-36; F: The level of m6A modification of p53 by methylated RNA immunoprecipitation-quantitative polymerase chain reaction.; G: The p53 expression level after the knockdown of tRF-36 by reverse transcription quantitative polymerase chain reaction; H: The p53 expression level after the knockdown of tRF-36 by western blot; I: The changes in P53 expression. a, b, and c represent different expression levels, the same letter represents no significant difference between groups ($P > 0.05$), different letters represent significant difference between groups ($P < 0.05$). tRF-36: tRNA-derived fragments 36; NC: Normal control; IgG: Immunoglobulin G; OE: Overexpression; IGF2BP3: Insulin-like growth factor 2 mRNA binding protein 3.

and knockdown of tRF36 resulted in a significant decrease in AMS and lipase levels in cell supernatants and a reduction in pancreatic acinar cell death. Therefore, tRF36 exacerbates AP by promoting pancreatic acinar cell death and may be a diagnostic biomarker in clinical treatment.

In AP mouse models in which GPX4 was knocked out in pancreatic tissues, the ferroptosis of pancreatic acinar cells exacerbated pancreatic tissue damage, leading to a significant increase in the levels of relevant biomarkers and accelerated progression of AP[7,8]. The core proteoglycan released from cells that have undergone ferroptosis can trigger immune responses and the production of proinflammatory cytokines, thereby exacerbating pancreatic acinar cell death and leading to further exacerbation of AP[9]. In mice and cell models of AP, the expression of ferritin decreased and MDA level increased. Hou *et al*[33] suggested that ferritin was a major intracellular iron storage protein complex, and its increased expression would limit ferroptosis. On the contrary, there was a low ferritin expression level during ferroptosis. As shown above, ferroptosis of pancreatic acinar cells promotes the aggravation of AP.

In the present study, we constructed an AP cell model in which tRF36 was knocked down in the pancreatic acinar cells. A ferroptosis gene microarray was performed to identify the key genes involved in ferroptosis, among which P53 was demonstrated to exhibit the most significant expression. Database-based prediction revealed that p53 mRNA has an m6A modification site where p53 mRNA interacts with some m6A-reader proteins. Goodarzi *et al*[34] found that tsRNA can inhibit the stability of multiple proto-oncogene transcripts in breast cancer cells by competitively binding to the RNA-binding protein YBX1. Thus, tRFs can regulate the stability of downstream gene transcripts *via* RNA-binding proteins. Accordingly, an RNA pulldown assay combined with mass spectrometry was performed in the present study, which revealed that tRF36 interacted with IGF2BP3. Finally, based on gene interference, overexpression, MeRIP-PCR, RNA pulldown assays, western blotting, and rescue assays, IGF2BP3 was confirmed to regulate the ferroptosis of pancreatic acinar cells by regulating P53 expression by enhancing P53 mRNA stability. Taken together, the above findings suggest that the acceleration of AP progression by tRF36 may involve the process of tRF36 recruiting IGF2BP3 to the m6A modification site of P53 mRNA by binding to IGF2BP3, which enhances P53 mRNA stability, ultimately promoting the ferroptosis of pancreatic acinar cells.

To our knowledge, this is the first study to identify a tRF, tRF36, that promotes AP progression by regulating the ferroptosis of pancreatic acinar cells and to elucidate the molecular mechanism whereby tRF36 is involved in AP progression (*e.g.*, tRF36 regulates the ferroptosis of pancreatic acinar cells by regulating P53 expression through binding to IGF2BP3). The findings fill the knowledge gap by revealing how tRFs are involved in AP progression *via* the regulation of ferroptosis and provide a new direction for further studies on AP development.

The present study had some limitations. The sample size of the study cohort was small. Future studies should employ a larger sample size to further verify the expression of tRF36 in AP. The expression levels of ROS and MDA were determined only by knocking down tRF36. Accordingly, their expression should also be examined by overexpressing tRF36.

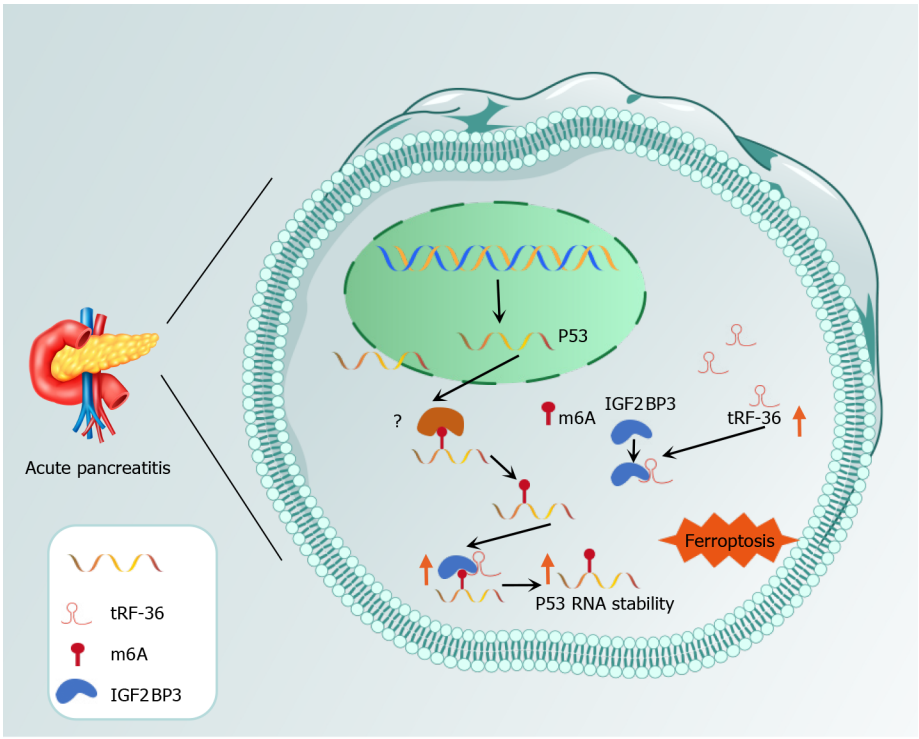

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Figure 6 A schematic of a putative mechanism of tRNA-derived fragments 36 in the progression of acute pancreatitis. In this model, tRNA-derived fragments 36 might recruit insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) to the p53 mRNA m6A modification site by binding to IGF2BP3 to enhance p53 mRNA stability and promote the ferroptosis of pancreatic follicle cells. tRF-36: tRNA-derived fragments 36; IGF2BP3: Insulin-like growth factor 2 mRNA binding protein 3.

CONCLUSION

In this study, the most significantly differentially expressed candidate, tRF36, was identified using bioinformatics and validated using qRT-PCR based on serum RNA extraction and small RNA sequencing data from AP patients and healthy controls. The AP cell model and the AP mouse model were constructed to explore the role and mechanism of tRF36 in regulating ferroptosis and promoting AP progression, indicating that tRF36 might recruit IGF2BP3 to the p53 mRNA m6A modification site by binding to IGF2BP3 to enhance p53 mRNA stability and promote the ferroptosis of pancreatic follicle cells. This finding provides a theoretical basis for gaining further insights into the pathogenesis of AP and identifying new potential therapeutic targets.

ARTICLE HIGHLIGHTS

Research background

Acute pancreatitis (AP), also known as acute inflammation of the pancreas, is an inflammatory injury resulting from the activation of pancreatic enzymes caused by a variety of pathogenic factors, leading to self-digestion of pancreatic tissue.

Research motivation

Difficult treatment, high morbidity, many complications, high cost, and poor prognosis are the current clinical status. Therefore, it is particularly important to investigate the pathogenesis of AP.

Research objectives

Screening for tRNA-derived fragments (tRFs) contribute to AP progression and exploring the molecular mechanism of its action were the main objectives of our study.

Research methods

Firstly, key tRFs and the potential mechanisms of action were explored based on the small RNA sequencing and functional enrichment analyses in AP. The role of tRF36 was investigated by constructing the AP cell and mouse models. Subsequently, the lipase, amylase, and cytokine levels were assayed to examine AP progression. Evaluation of cellular ferroptosis was implemented by analyzing the ferritin expression, reactive oxygen species, malondialdehyde, and ferric ion levels. Finally, RNA pull down assays and methylated RNA immunoprecipitation were performed to explore the

molecular mechanisms.

Research results

In total, 211 differentially expressed tRFs including 116 upregulated and 95 downregulated were identified. According to reverse transcription quantitative polymerase chain reaction, tRF36 was significantly upregulated in the serum of AP patients, compared to healthy controls. Moreover, the occurrence of pancreatic cell ferroptosis was detected in AP cells and mouse models. Furthermore, we hypothesized that tRF36 accelerated AP progression by binding to insulin-like growth factor 2 mRNA binding protein 3, which was recruited to the p53 mRNA m6A modification site, thereby enhancing the stability of p53 mRNA and promoting ferroptosis in pancreatic follicular cells.

Research conclusions

tRF36 promoted AP development by regulating the ferroptosis of pancreatic cells, which would provide a new theoretical basis for understanding the regulatory mechanism of tRF in AP, and also provide new targets for the treatment of AP.

Research perspectives

We will further validate the results of this study and continue to monitor the role of tRF36 in the development process of AP.

FOOTNOTES

Author contributions: Fan XR and Huang Y contributed equally to this work. Wang H conceived, designed, and supervised the study; Fan XR, Huang Y, and Chen SJ performed the majority of experiments and collected the data; Su Y and Huang WK performed data analysis and drafted the manuscript; and all authors have read and agreed to the published version of the manuscript.

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Institutional animal care and use committee statement: This study was approved by Animal Ethics and Welfare Committee (AEWC) of Kunming Yan'an hospital (Approval No. 2022013) in accordance with internationally accepted principles for the use of laboratory animals.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The data used during the current study are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Basic Study

Fecal microbiota transplantation alleviates experimental colitis through the Toll-like receptor 4 signaling pathway

Xin Wen, Rui Xie, Hong-Gang Wang, Min-Na Zhang, Le He, Meng-Hui Zhang, Xiao-Zhong Yang

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Abstract

BACKGROUND

Fecal microbiota transplantation (FMT) has shown promising therapeutic effects on mice with experimental colitis and patients with ulcerative colitis (UC). FMT modulates the Toll-like receptor 4 (TLR4) signaling pathway to treat some other diseases. However, it remains unknown whether this modulation is also involved in the treatment of UC.

AIM

To clarify the necessity of TLR4 signaling pathway in FMT on dextran sodium sulphate (DSS)-induced mice and explain the mechanism of FMT on UC, through association analysis of gut microbiota with colon transcriptome in mice.

METHODS

A mouse colitis model was constructed with wild-type (WT) and TLR4-knockout (KO) mice. Fecal microbiota was transplanted by gavage. Colon inflammation severity was measured by disease activity index (DAI) scoring and hematoxylin and eosin staining. Gut microbiota structure was analyzed through 16S ribosomal RNA sequencing. Gene expression in the mouse colon was obtained by transcriptome sequencing.

RESULTS

The KO (DSS + Water) and KO (DSS + FMT) groups displayed indistinguishable body weight loss, colon length, DAI score, and histology score, which showed that FMT could not inhibit the disease in KO mice. In mice treated with FMT, the relative abundance of *Akkermansia* decreased, and *Lactobacillus* became dominant. In particular, compared with those in WT mice, the scores of DAI and colon histology were clearly decreased in the KO-DSS group. Microbiota structure showed a significant difference between KO and WT mice. *Akkermansia* were the

dominant genus in healthy KO mice. The ineffectiveness of FMT in KO mice was related to the decreased abundance of *Akkermansia*. Gene Ontology enrichment analysis showed that differentially expressed genes between each group were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus. The top nine genes correlating with *Akkermansia* included Aqp4, Clca4a, Dpm^3, Fau, Mcrip1, Meis3, Nupr1 L, Pank3, and Rps13 ($|R| > 0.9$, $P < 0.01$).

CONCLUSION

FMT may ameliorate DSS-induced colitis by regulating the TLR4 signaling pathway. TLR4 modulates the composition of gut microbiota and the expression of related genes to ameliorate colitis and maintain the stability of the intestinal environment. *Akkermansia* bear great therapeutic potential for colitis.

Key Words: Toll-like receptor 4; Fecal microbiota transplantation; Colitis; *Akkermansia*; *Lactobacillus*; Aquaporin 4; Transcriptome sequencing

Core Tip: Recent studies have shown that fecal microbiota transplantation (FMT) has a therapeutic role in patients with inflammatory bowel disease. The Toll-like receptor 4 (TLR4) signaling pathway may play a critical role in intestinal injury and repair. Here, we conducted animal experiments to explore the role of TLR4 in dextran sodium sulphate-induced colitis in mice and the treatment of FMT.

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INTRODUCTION

Recent studies support that inflammatory bowel disease (IBD) can be categorized as a "microbial dysbiosis disease," because of its progression synchronizing the dysbacteriosis of gut microbiota[1]. Host physiology such as barrier function, metabolism, immune responses, and homeostasis involves microbiome-induced cell signaling, proliferation, and neurotransmitter biosynthesis[2]. In IBD patients, intestinal bacterial diversity decreases and the bacterial community structure changes[3]. In dextran sodium sulphate (DSS)-induced colitis in mice, some probiotics, including *Lactbacillus* and *Bifidobacterium*, are significantly reduced[4]. New evidence indicates that IBD is not merely a consequence of chronic inflammation, but also of disruption of the gut microbiome and destruction of the intestinal epithelial barrier[5].

Gut microbiota play a role in inflammation-related activities. Fecal microbiota transplantation (FMT) has shown high efficacy and safety in treating ulcerative colitis (UC)[6,7] due to its immunomodulatory and anti-inflammatory functions [8]. Our previous study showed that FMT can counter DSS-induced colitis in mice by increasing the relative abundance of *Lactobacillus*[9]. FMT has also shown therapeutic potential for a range of other diseases, such as hepatic disorders and metabolic syndrome[10]. Recent studies have demonstrated that Toll-like receptor 4 (TLR4) is exploited by FMT in treating many diseases such as spleen deficiency diarrhea[11], Parkinson's disease[12,13], developmental arsenic neurotoxicity[14], fluorosis[15], and acute lung injury[16]. Previous studies have indicated that FMT intervention can inhibit activation of the nuclear factor kappa B (NF-κB) signaling pathway[17], which is downstream of TLR4. However, there have been limited studies investigating the role of TLR4 in FMT for UC.

As a class of transmembrane proteins that recognize invading microbes and activate immune cells, Toll-like receptors (TLRs) regulate gene transcription and the acquired intestinal immune response[18]. In the etiology of IBD, microbes in the intestinal lumen induce abnormal immune responses, along with excessive leakage of bacterial antigens into the mucosa[19]. TLR4, an important immune activator, is highly expressed in the intestinal epithelial cells and lamina propria cells of UC patients[20]. It binds to ligands to activate cytokine signaling, recruit inflammatory cells, and damage intestinal mucosal barrier, all of which aggravate intestinal inflammatory lesions. More importantly, substantial evidence supports a pro-inflammatory role of the TLR4 signaling pathway in UC. Expression levels of TLR4 are positively correlated with disease activity indices (DAIs), endoscopy scores, and histopathological scores[21]. DSS-induced colitis deteriorates in mice with TLR4 overexpression[22,23], but is stably maintained in TLR4-deficient mice[24,25]. Multiple experiments have shown that inhibiting the TLR4 signaling pathway can prevent DSS-induced colitis[26,27]. While TLR4 plays a crucial role in intestinal injury and repair, its role in shaping colonic bacterial homeostasis and microbiota-related immunity remains poorly understood.

Our previous studies confirmed the efficacy FMT on IBD, but the mechanism has not been reported[9]. Therefore, we explored the role of TLR4 in the mechanism by which FMT treats DSS-induced colitis in the mice.

MATERIALS AND METHODS

Animals

Wild-type (WT) C57BL/10J mice and TLR4-knockout (KO) mice on the C57BL/10J background (female; 6 to 8 wk of age; weighing 18-20 g; specific pathogen-free (SPF) grade) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were reared in an SPF condition at the experimental animal center of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. Throughout the acclimatization and study periods, all mice were maintained in a 12 h-light/12 h-dark cycle (21 °C ± 2 °C with a relatively constant humidity of 45% ± 10%) and had access to food and water *ad libitum*. All mice were group-housed and reared in a standard cage, with TLR4 KO mice kept separately from C57BL/10J mice in different cages.

DSS-induced colitis

DSS (36-50 kDa) was purchased from MP Biomedicals LLC (Irvine, CA, United States) and dissolved in distilled water. Experimental colitis was induced as previously described with minor changes[9]. For different groups, the mice were administered 2.5% (w/v) DSS in drinking water for 7 d. Mice in the KO (DSS + FMT) group were fed fecal microbiota from healthy WT mice from day 8 (once every 2 d) until the end of the experiment, while mice in the KO (DSS + water) group were fed normal saline at the same time. The mice were evaluated daily by scoring *via* the disease activity index (DAI)[28]. The DAI score was calculated on a 0-4 scale as previously described[29].

Fecal preparation and transplantation

The process of FMT was performed as previously described[9]. Briefly, feces from donor mice (healthy WT mice) were collected and resuspended in sterile normal saline at 0.125 g/mL. Then 0.2 mL of this suspension was administered to mice once every 2 d by oral gavage. This process lasted 7 d.

Histopathology

Mice were euthanized by cervical dislocation, and their abdominal cavity was opened immediately. The colon tissue was dissected; colons were measured for colon length, and tissues were examined for gross macroscopic appearance and stool consistency. The distal colon segment was placed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and cut into sections 4 μm in thickness. Then the sections were stained with hematoxylin and eosin (H&E). H&E-stained sections were examined for inflammation and tissue damage by an experienced pathologist in a blinded manner. Tissue histology was scored by summing the scores of the following parameters according to a previous study[30]: Extent of inflammation, aberrant crypt foci, lymphocyte infiltration, and aberrant colon wall.

Fecal DNA extraction and 16S ribosomal RNA sequencing

Fecal DNA extraction and 16S ribosomal RNA (rRNA) sequencing were performed as previously reported[9]. The V3-V4 hypervariable region of the bacterial 16S-rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAG-GCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAA T-3′) with the ABI GeneAmp® 9700 PCR thermocycler (Applied Biosystems, Foster City, CA, United States)[9]. All PCR products were extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States)[31]. Purified amplicons were sequenced on the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, United States). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0, and merged by FLASH version 1.2.7. Operational taxonomic units (OTUs) with 97% similarity cutoff[32] were clustered using UPARSE version 7.1[33], and chimeric sequences were identified and removed. Bacterial alpha-diversity was determined by sampling-based OTU analysis. Analysis of species accumulation curves was performed to assess the rationality and efficiency of the sequencing depth. Principal component analysis (PCA) was implemented in R programming. Using the Wilcoxon rank-sum test, the bacterial taxonomic analysis was performed for comparison at the bacterial phylum, class, order, family, genus levels between two groups. Based on the matrix of normalized relative abundance, bacteria with significantly different abundances between assigned taxa were determined by linear discriminant analysis effect size (LEfSe) with the Kruskal-Wallis rank-sum test ($P < 0.05$). LDA was used to assess the effect size of each feature (LDA score [log10] = 3 as the cut-off value).

Transcriptome analysis

Total RNA was extracted from inflammatory colonic tissue. For sequencing, a 1 cm colon tissue was sampled from the site about 2 cm from the anus, regardless of whether there was visible inflammation. The tissue samples with minimum and maximum histological scores were removed. Then the colon samples from four randomly chosen animals in each group was used for sequencing. Methods for amplifying and sequencing followed those previously published[9,29]. Briefly, 2 μg RNA per sample was used to sequence on the Illumina Hiseq 4000 platform. Differential expression analysis was performed using the DESeq R package (1.10.1) according to the manufacturer's protocol. Then, to explore the potential function of the differentially expressed genes (DEGs), GOseq R package[34] and KOBAS software[35] were used to test the enrichment of DEGs in Gene Ontology (GO) functional annotations[36] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[37].

Correlation analysis for gut microbiota and transcriptome

We used Metastats software to confirm the difference in the relative abundance of microbiota among the samples ($P \leq$

0.05). We used DEG sequencing to carry out transcriptome difference analysis (the threshold was *P*adj < 0.05 & |log2FC| > 1). Finally, R psych software package was used to analyze the Spearman association between the transcriptome and intestinal microflora. Those with $|R| > 0.8$ and $P < 0.05$ (strong correlation) were screened for mapping.

Statistical analysis

Differences were analyzed using the *t*-test with Graphpad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, United States). Results are shown as the mean ± standard error of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

FMT does not improve acute colitis induced by DSS in TLR4-KO mice

In our previous experiment, we found that FMT is effective to treat colitis[9]. We further explored whether this efficacy is related to the TLR4 pathway. Acute DSS-induced colitis was induced in eight animals per group using 2.5% DSS in the drinking water. After gavage with fecal microbiota from the WT mice, mice in the KO (DSS + water) and KO (DSS + FMT) groups displayed indistinguishable body weight loss, colon length, DAI score, and histology score (Figure 1A-D). Beyond our expectation, FMT had no effect on colonic inflammation in TLR4-KO mice. We compared the expression of TLR4 gene in the intestine of WT mice before and after FMT. The transcriptome sequence data indicated that DSS increased, but FMT effectively decreased the expression of TLR4 (Figure 1E).

FMT changes the intestinal flora of TLR4-KO mice

We investigated whether FMT changed the composition of gut microbiota in the KO (DSS + water) and KO (DSS + FMT) groups. We employed LEfSe to evaluate the bacterial taxa (at genus level) in the two groups (Figure 2A). The dominating taxa in KO (DSS + FMT) group were enriched in *Lactobacillus*, which indicated that we had successfully transplanted the gut microbiota of healthy WT mice. Meanwhile, the KO (DSS + FMT) group had a lower abundance of *Akkermansia*, indicating that FMT could alter the relative abundance of *Akkermansia* in KO mice (Figure 2B).

TLR4 KO alleviates DSS-induced colitis

We used TLR4-deficient mice and WT mice to determine whether TLR4 may protect mice from DSS-induced colitis. Mice in the KO-DSS ($n = 8$) and WT-DSS ($n = 7$) groups were given distilled drinking water containing 2.5% DSS for 7 d (Figure 3A). Compared with WT mice, KO mice showed lower susceptibility to DSS, as manifested by their much smaller body weight loss (Figure 3B), lower DAI (Figure 3C), and longer colons (Figure 3D). Compared to the WT-DSS group, mice in the KO-DSS group exhibited a more intact colon structure, less severe crypt damage, and reduced inflammatory infiltration (Figure 3E). In summary, KO mice showed increased tolerance to DSS-induced colitis.

TLR4 deficiency influences the diversity and composition of gut microbiota

We further investigated whether the protection against DSS-induced colitis was due to TLR4 KO or microbiota re-composition. We detected the gut microbiota of WT and KO mice in the basal and DSS-treated states. We analyzed the beta-diversity of microbiota based on PCA. An evident clustering separation between OTUs revealed the different community structures between each two groups, suggesting that these communities are distinct in terms of their compositional structure (Figure 4A and 5A).

At the phylum level, TLR4 deficiency decreased the abundance of *Bacteroidetes* and increased the abundances of *Actinobacteria* and *Verrucomicrobia* ($P < 0.05$; Figure 4B), compared to those in WT mice. After DSS induction, a significant increase of phylum *Proteobacteria* was observed in the WT-DSS group compared to the KO-DSS group ($P < 0.05$; Figure 5B). *Verrucomicrobia* was the most abundant phylum among those with significant differences ($P < 0.05$). At the genus level, *Akkermansia* abundance was significantly higher in KO mice than in WT mice either healthy or diseased ($P < 0.05$; Figure 4C and 5C). To further investigate the potential effect of microbiota composition on DSS-induced colitis, we used the LEfSe to detect the marked differences in the dominant bacterial communities between the two groups (Figure 4D and 5D). Specifically, *Lactobacillus* and *Peptococcus* were enriched in the WT-CON group (Figure 4D), while *Escherichia Shigella* and *Anaerotruncus* were enriched in the WT-DSS group (Figure 5D). Interestingly, *Akkermansia* and *Bifidobacterium* were enriched either in healthy and diseased KO mice (Figure 4D and 5D). The collective results of our study indicated clear differences in the intestinal microbiome between WT mice and KO mice, both in healthy conditions and during illness. These findings highlight the important role of TLR4 in shaping the composition and diversity of the intestinal microbiota.

TLR4-KO-shaped microbiota affect the transcriptome in the colon of mice

To further explore whether FMT can change the gene expression related to TLR4, we investigated the DEGs between groups. Compared to those in the WT-DSS group, 1436 genes were differentially expressed in the KO-DSS group, and 309 genes in the KO (DSS + FMT) group. Furthermore, 193 DEGs were found among the KO-DSS group, WT-DSS group, and KO (DSS + FMT) group (Figure 6A). GO enrichment analysis showed that these DEGs were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus (Figure 6B). According to 16S rRNA sequencing analysis, we found that *Akkermansia* was dominant in the KO group. To characterize potential gene-microbe interactions, we computed gene-microbe correlations with Spearman correlation efficients (Figure 6C). The top nine genes correlating

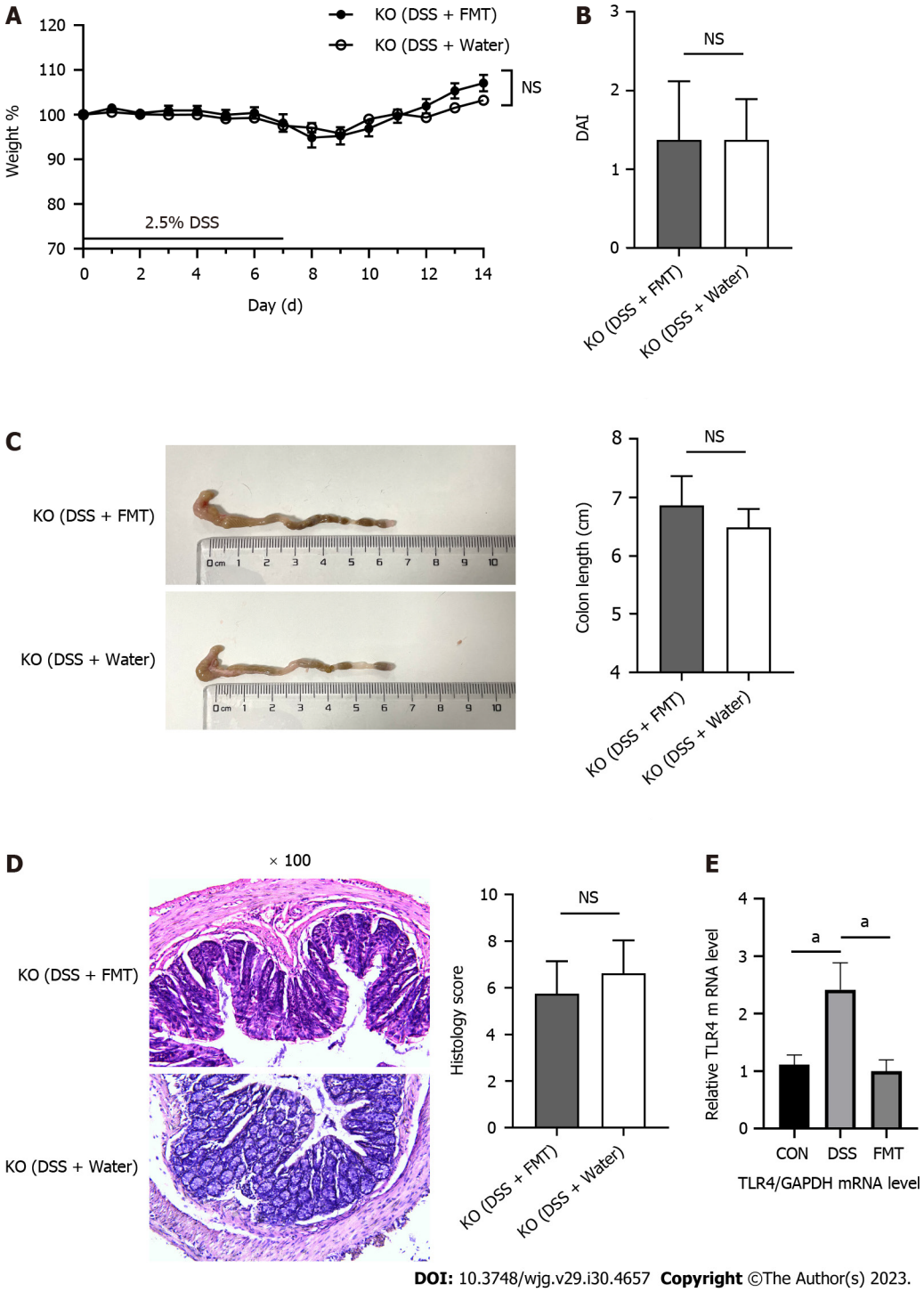

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Figure 1 Fecal microbiota transplantation did not alleviate acute colitis induced by dextran sodium sulphate in Toll-like receptor 4 knockout mice. A: Body weight of mice during the course of colitis; B: The bar chart represents the disease activity index (DAI) score of mice on day 14; C: Representative images of colons from mice (left) and statistical analysis of colon length (right); D: Representative hematoxylin and eosin staining of colon tissues, original magnification 100 ×, and histological scores (right); E: Relative quantification of the transcription level of Toll-like receptor 4 (TLR4) among groups. $^{a}P < 0.05$. DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation; KO: Knockout.

with *Akkermansia* included Aqp4, Clca4a, Dpm^{3}, Fau, Mcrip1, Meis3, Nupr1 L, Pank3, and Rps13 ($|R| > 0.9$, $P < 0.01$).

DISCUSSION

Researchers have found that patients with active UC can benefit from FMT[38]. Moreover, our previous study also verified that FMT can treat colitis in mice. In the present study, the expression of TLR4 was upregulated by DSS, and downregulated after FMT. It therefore stands to reason that, by inhibiting TLR4, a protective effect from intestinal inflam-

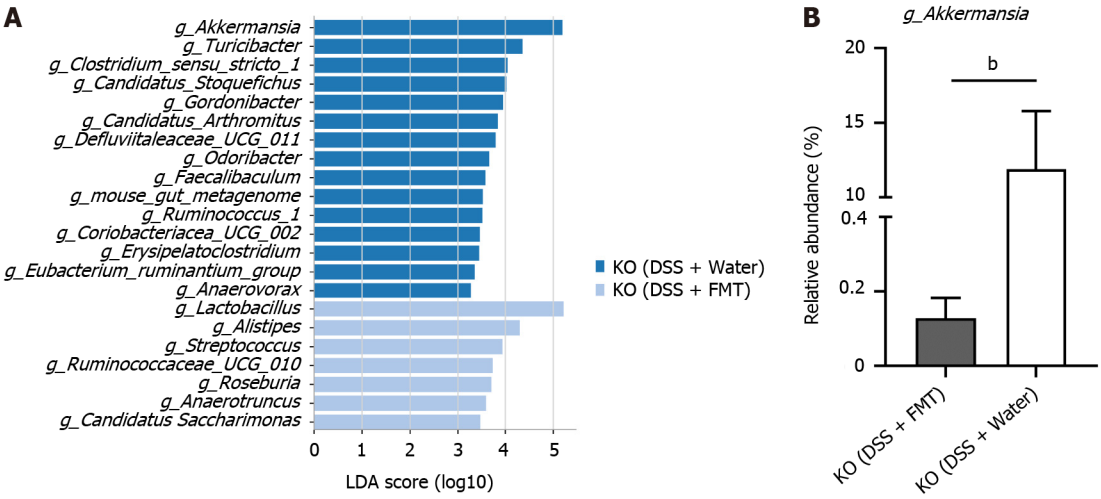

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Figure 2 Fecal microbiota transplantation changed the gut microbiota of Toll-like receptor 4 knockout mice. A: Linear discriminant analysis (LDA) effect size (LEfSe) analysis in two groups with an LDA score > 3.0; B: The relative abundance of *Akkermansia* in Toll-like receptor 4 knockout mice. [a]*P* < 0.05, [b]*P* < 0.01. DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation.

mation will be induced. Considering the ubiquitous involvement of the TLR4 signaling pathway in the activities of the mucosa, we designed this animal study to elucidate its interaction with FMT in UC. In this study, TLR4 KO significantly alleviated the clinical and histological manifestations of DSS-induced colitis. Notably, the increased relative abundance of the predominant *Akkermansia* species contributed to the heightened resistance against colon inflammation. Through further investigation, we discovered that genetic KO of TLR4 significantly impacted the structure and composition of the gut microbiota, resulting in a shift towards an anti-inflammatory configuration. This shift plays a crucial role in promoting enhanced resistance and tolerance to colitis.

At the phylum level, DSS changed the relative abundances of *Bacteroidetes*, *Actinobacteria,* and *Verrucomicrobia* in TLR4-KO mice compared to WT mice. Possibly, the high abundance of anti-inflammatory *Akkermansia* in the gut microbiota curbs the aggravation of colitis, despite the absence of TLR4 signaling. *Akkermansia* was the dominant genus in healthy KO mice, while after the treatment of FMT, their level decreased. Compared with that in the WT group, the status of colitis in the KO group was not significantly attenuated by FMT, suggesting that the therapeutic effect of FMT on colitis is closely related to the TLR4 signaling pathway and *Akkermansia*.

In the gut, the expression of TLRs changes with the composition of microbiota[39], as well as the activity of the intestinal epithelium such as inflammation[40]. In the present study, we observed the difference in microbial composition between WT-DSS and KO-DSS groups. At the phylum level, the KO-DSS group had a higher relative abundance of *Actinobacteria* and *Verrucomicrobia*, while WT-DSS had a higher relative abundance of *Proteobacteria*. In addition, *Verrucomicrobia* demonstrated the most significant difference at the phylum level. Lo Sasso *et al*[41] analyzed the composition of gut microbiota in UC patients *via* fecal microbiota whole-genome sequencing, finding increased abundance of *Proteobacteria* and decreased abundance of *Verrucomicrobia*. In addition, one study characterized the mucosal microbiome of pediatric UC patients, noting a significant decrease in the phylum *Verrucomicrobia* at the phylum level[42]. It has been reported that the abundance of *Proteobacteria* increases in UC mice[43]. Moreover, the relative abundance of *Proteobacteria* in DSS-induced mice rises remarkably, compared with that in WT mice, which can be restored to normal after Lizhong therapy[44]. Consistently, this study proves that DSS can raise the abundance of *Proteobacteria* in WT mice, rather than KO mice.

In particular, we found that the abundance of *Akkermansia* increased in the KO-DSS group, but then dropped notably after FMT, indicating its role in the effect of FMT on UC. As previously reported, the abundance of *Akkermansia* decreases in UC patients[45], but it is unclear whether this is a cause or consequence of UC. *Akkermansia* can protect intestinal barrier function and reduce the production of inflammatory cytokines[46]. On the other hand, *Akkermansia* can increase the production of short-chain fatty acids and antioxidant enzymes, indicating that *Akkermansia* may proliferate to alleviate colitis[47]. According to our experiment, the relative abundance of *Akkermansia* was negatively correlated with the severity of colitis in our animal models. *Akkermansia* bear great therapeutic potential for colitis. Studies on human and mice have revealed that the injection of beneficial bacteria such as *Lactobacillus*, *Akkermansia,* and *Bifidobacterium* can alleviate the inflammation in UC patients[48-50]. In a systematic review of three studies, the abundance of *Akkermansia* decreased in all UC patients[51]. A high abundance of *Akkermansia* can modulate host metabolism to prevent seizures [52]. Several *Akkermansia* species have demonstrated the ability to modulate immune responses and protect barrier function[53].

Despite the widely recognized beneficial properties of *Akkermansia* as a potential probiotic, it is crucial to take into account the potential occurrence of adverse effects. Patients with colorectal cancer have a higher abundance of *A. muciniphila*[3]. A prior study demonstrated that the genetic deletion of TLR4 exacerbates the severity of colon inflammation, resulting in the decreased abundance of *Akkermansia*[54]. This conflicting conclusion may be explained by various factors, such as the different mouse species and different experimental models used. When the equilibrium of the gut

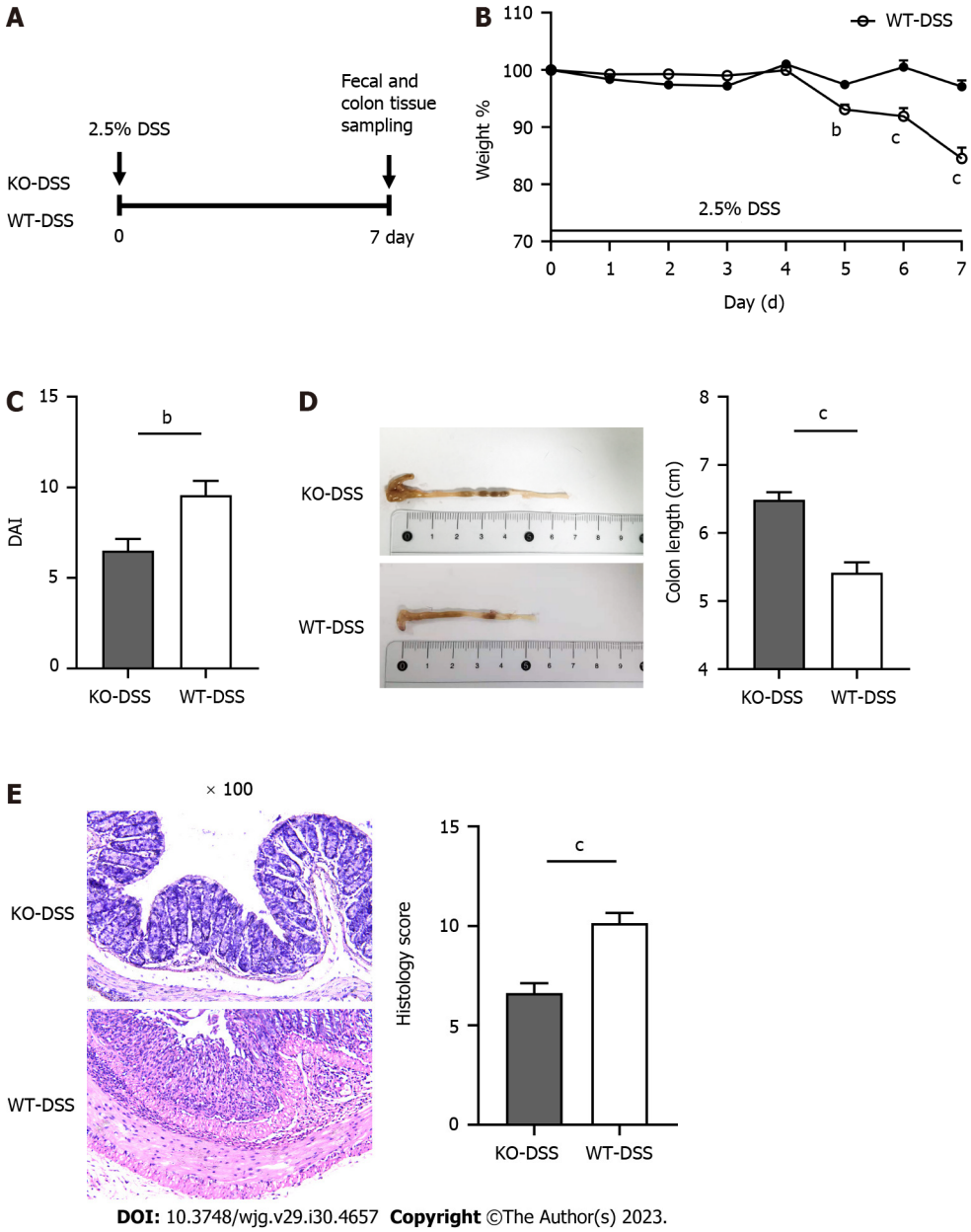

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Figure 3 Toll-like receptor 4 knockout alleviated dextran sodium sulphate-induced inflammation in the colon. A: Scheme of the animal experimental design; B: The change in body weight of mice from days 0 to 7 during the disease course (knockout-dextran sodium sulphate [KO-DSS]: $n = 8$; wild type [WT]-DSS: $n = 7$); C: The bar chart represents the disease activity index (DAI) score on day 7; D: Representative colons (left) and statistical analysis (right) of colonic length; E: Representative hematoxylin and eosin staining of colon tissues (left), original magnification 100 ×, and histological scores (right). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ were considered statistically significant. FMT: Fecal microbiota transplantation.

microbiota is disturbed, beneficial microbes have the potential to shift towards virulent species, leading to adverse effects on the host. Studies have suggested a potential link between *Akkermansia* and TLR4 signaling. A study demonstrated that the administration of anthocyanins extracted from *Lycium ruthenicum* (ACs) increases the abundance of *Akkermansia*, thus inhibiting the lipopolysaccharide/NF-κB/TLR4 pathway to improve intestinal function[55]. It has also been observed that inhibition of the TLR4 signaling pathway can increase the abundance of *Akkermansia*[56]. *Akkermansia* promotes the integrity of the intestinal barrier and regulates immune homeostasis, potentially by interacting with TLR4[57,58]. In this study, the composition and structure of gut microbiota presented a significant difference between KO-DSS mice and WT-DSS mice. Based on the above results, we advocate that *Akkermansia* can increase resistance to acute colitis in TLR4-KO mice. However, more in-depth investigations are needed to determine if *Akkermansia* negatively associated with TLR4 are a potential target of FMT in treating UC.

TLR4 is differentially expressed in patients with early and advanced UC, indicating a close correlation between TLR4 and UC[59]. Inhibition of TLR4 significantly decreases the expression of cell cycle regulatory genes. Furthermore, TLR4 signaling in colonic epithelial cells promotes the recruitment of inflammatory cells through microRNA 155-mediated posttranscriptional regulation[60]. In the current study, our results showed that FMT downregulated the expression of genes related to the TLR4/myosin light chain kinase signaling pathway in WT mice, highlighting the importance of TLR4 in the effectiveness of FMT. Functional analysis revealed that most DEGs were enriched in cytoplasmic translation and

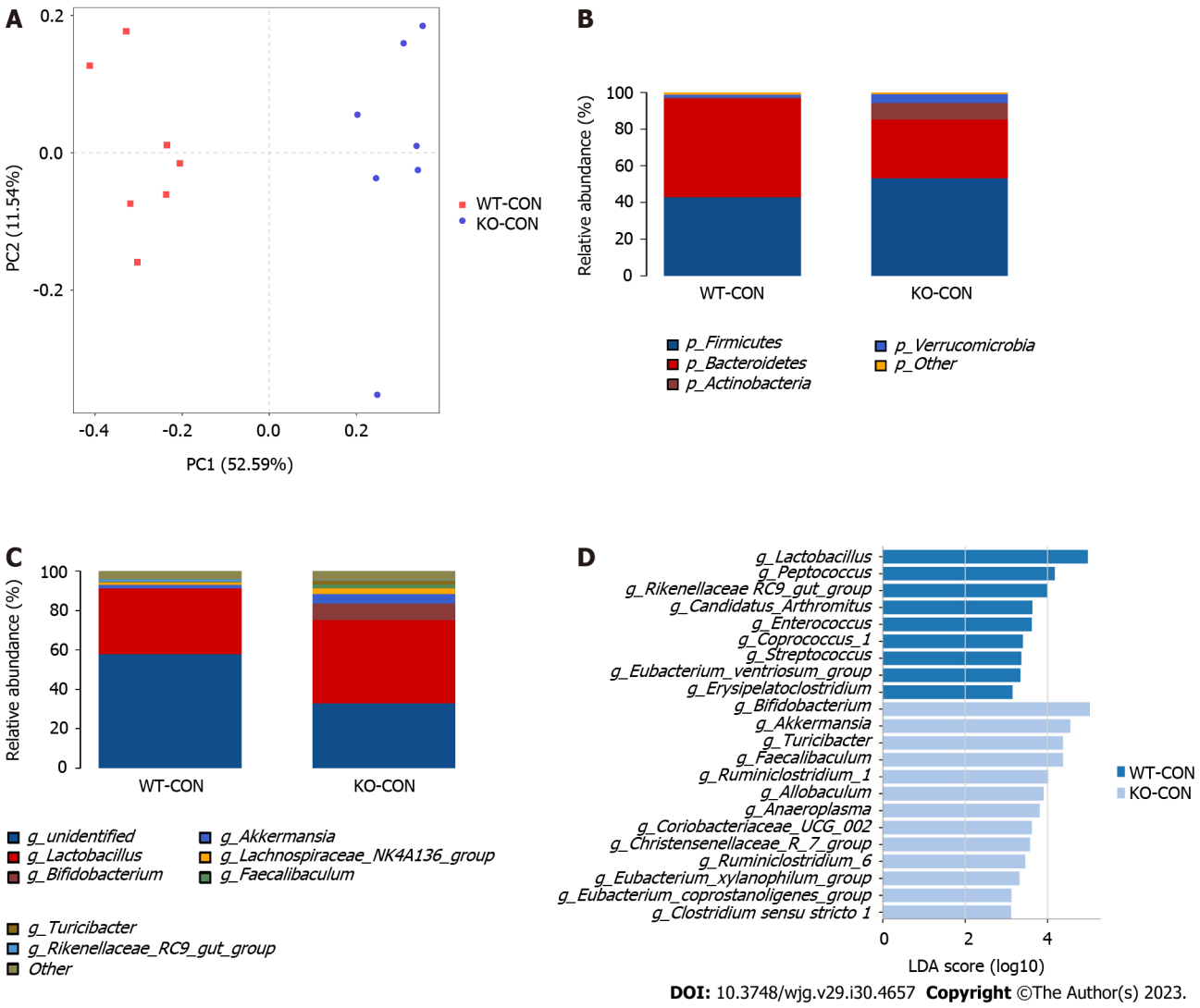

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Figure 4 Diversities and compositions of gut microbiota in knockdown-control and wild type-control groups. A: β-diversity evaluated using the weighted UniFrac-based PCA (knockdown-control [KO-CON]: *n* = 7; wild type [WT]-CON: *n* = 7); B and C: Bar graphs showing the relative abundances of different bacteria at the phylum and genus levels; D: Linear discriminant analysis (LDA) effect size analysis in groups with an LDA score > 3.0 between two groups.

cellular response to DNA damage stimulus. The top nine DEGs strongly related to *Akkermansia* were primarily associated with cell cycle regulation, transcriptional control, apoptosis, stress responses, and inflammatory responses. Their functions aligned with the main processes identified in GO analysis, indicating their involvement in crucial biological pathways. These functions highlight its potential role in modulating various cellular activities. Aquaporin 4 (AQP4), a water channel protein that facilitates transmembrane water movement, has the strongest correlation[61]. AQPs are widely distributed in mammals' secretory and absorptive epithelial cells and are responsible for transport and trafficking processes. In colonic inflammation, AQP4 is abundantly expressed in the basolateral membrane of colonic epithelial cells in humans and mice. The permeability of cell membranes is positively correlated with AQP4 expression[62]. AQP4 overexpression facilitates the entry of water into cytes, thereby contributing to cytotoxic edema[63-65]. AQP4 deficiency alleviates experimental colitis in the mice[66]. Although we did not use the same mouse KO model in the present study, the effect of AQP4 on colonic inflammation is consistent with that of TLR4. Activating the high mobility group box 1 protein/TLR4/NF-κB pathway can increase the expression of AQP4[67,68]. Furthermore, lipopolysaccharide, a potent TLR4 agonist, significantly increases the mRNA level of AQP4 expression through TLR4 signaling in the cortex and astrocytes[62]. We speculate that TLR4 deficiency can protect against colitis by increasing the abundance of *Akkermansia* and reducing the expression of AQP4. As shown by previous results, FMT can relieve colitis in WT mice[9]. However, in this study, FMT did not exert effects on colonic inflammation in TLR4-KO mice. It was intriguing to determine that the abundance of *Akkermansia*, which was dominant in TLR4-KO mice, was significantly decreased after FMT. This may be related to the decreased relative abundance of *Akkermansia*. While the DEGs mentioned above may have roles in immune regulation, inflammation, or cellular processes that can intersect with TLR4 signaling, their specific relationships with TLR4 are not extensively characterized. Notwithstanding, further studies are needed to determine whether FMT also targets *Akkermansia* to regulate the expression of related DEGs in countering colon inflammation.

In this study, we assessed the microbial diversity and composition in DSS-induced mice. The bacteria inhabited in the mucosa may play major roles in the development of IBD. So it is necessary to explore the function of microbiota in

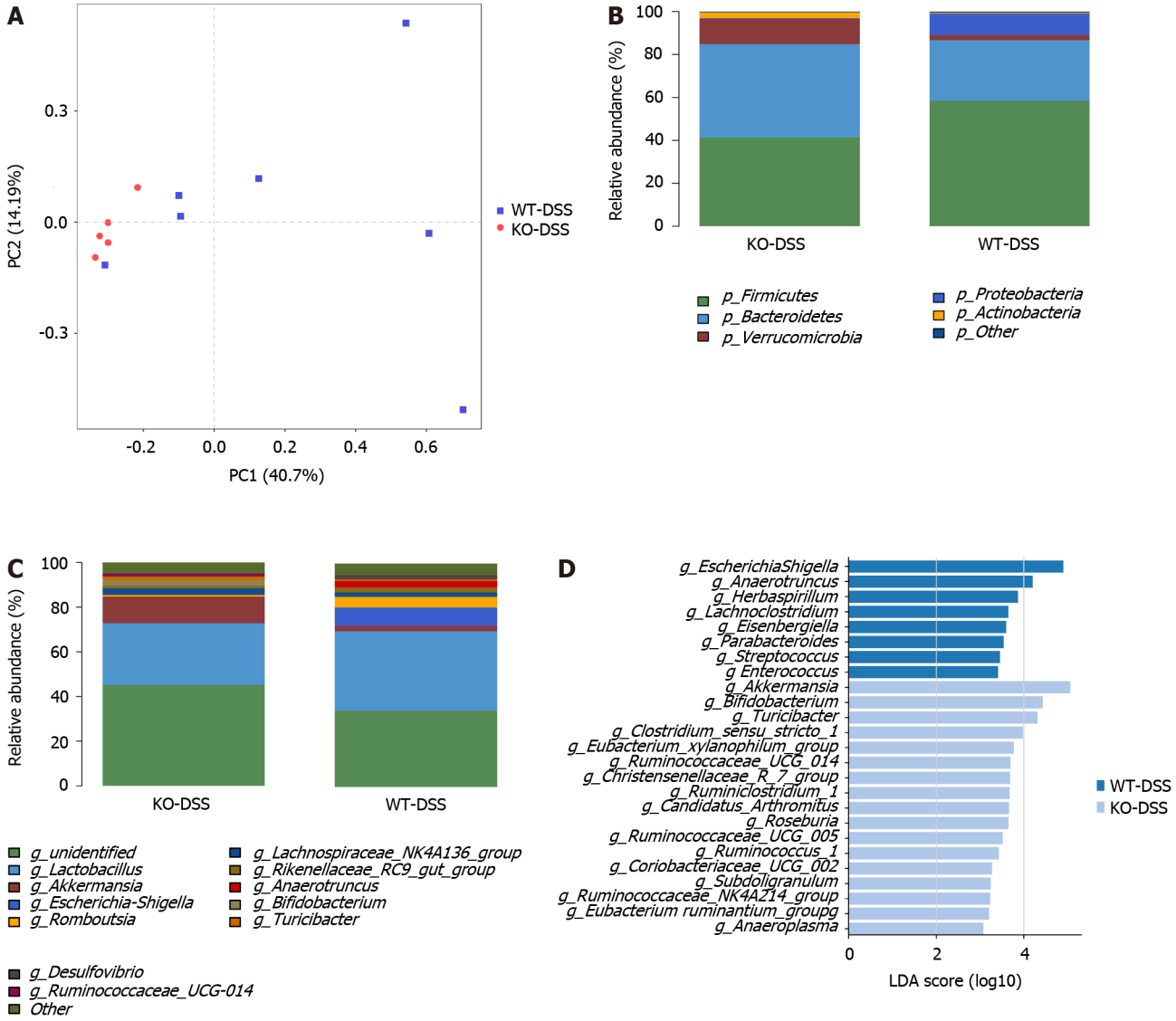

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Figure 5 Diversities and compositions of gut microbiota in knockout-dextran sodium sulphate and wild type-dextran sodium sulphate groups. A: Multiple sample principal component analysis (knockout-dextran sodium sulphate [KO-DSS]: *n* = 5; wild type [WT]-DSS: *n* = 7); B and C: Bar graphs showing the relative abundances of different bacteria at the phylum and genus levels; D: Linear discriminant analysis (LDA) effect size (LEfSe) analysis in groups with an LDA score > 3.0.

mucosal tissues in future study. However, animal studies have certain limitations in evaluating the mechanism of TLR4. Therefore, clinical studies should be designed to unveil the interplay among TLR4, gut microbiota, and UC.

CONCLUSION

TLR4 modulates the composition of gut microbiota and regulates the expression of microbiome-related genes to ameliorate colitis and maintain the stability of the intestinal environment. For the first time, we find that FMT may ameliorate DSS-induced colitis by regulating the TLR4 signaling pathway. Our findings will make the treatment of patients more targeted and is worthy of clinical trials in the future.

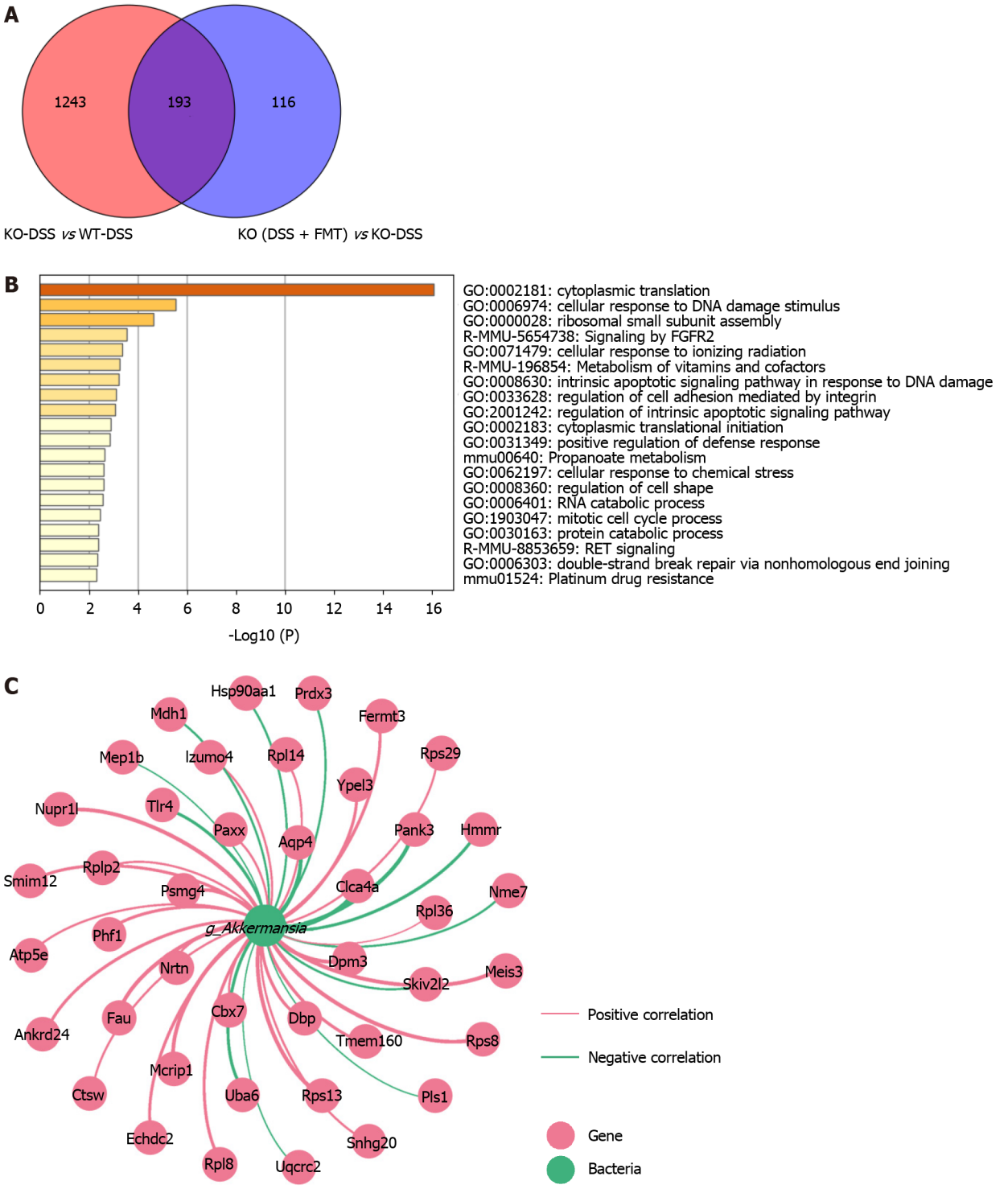

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Figure 6 Colonic transcriptome profile and gene-microbe correlation. A: Venn diagram illustrates genes regulated by fecal microbiota transplantation (FMT) and Toll-like receptor 4 knockout (KO); B: The top 20 Gene Ontology terms enriched in these 193 differentially expressed genes (DEGs); C: Network visualizing 193 DEGs associated with *Akkermansia* (|R| > 0.8, $P < 0.05$). CON: Control; DSS: Dextran Sodium Sulphate; WT: Wild type.

ARTICLE HIGHLIGHTS

Research background

It is well known that microbiota dysbiosis contributes to the occurrence of inflammatory bowel disease (IBD). Fecal microbiota transplantation (FMT) has shown promising therapeutic effects on both clinical and basic studies of ulcerative colitis (UC). Substantial evidence supports a negative pro-inflammatory role of Toll-like receptor 4 (TLR4) signaling pathway in IBD. However, it remains unknown whether this modulation is also involved in the treatment of FMT on UC.

Research motivation

FMT treats other diseases by regulating the TLR4 signaling pathway. Previous studies have shown that the expression of TLR4 is higher in the intestinal mucosa of patients with effective FMT and lower in patients with poor FMT. We speculate that the TLR4 signaling pathway may be involved in the therapeutic mechanism of FMT on IBD.

Research objectives

To clarify the necessity of TLR4 signaling pathway in FMT on regulating gut microbiota in dextran sodium sulphate (DSS)-induced colitis.

Research methods

Experimental colitis was constructed in wild-type (WT) and TLR4-knockout (KO) mice and fecal microbiota was transplanted by gavage. Colon inflammation severity in mouse model was measured by disease activity index (DAI) score and hematoxylin and eosin (H&E) staining. Gut microbiota alteration was analyzed through 16S ribosomal RNA sequencing. The difference of gene expression in mouse colon was obtained by transcriptome sequencing of colon tissue.

Research results

In KO mice treated with FMT or water, these two groups displayed indistinguishable body weight loss, colon length, DAI score, and histology score, which showed that FMT could hardly alter the disease progress in KO mice. Next, compared with WT mice, the scores of DAI and colon histology clearly decreased in the KO-DSS group. KO mice experienced enhanced resistibility to DSS-induced colitis. There was a significant difference in the microbiota structure between KO and WT mice. *Akkermansia* was the dominant genus in healthy KO mice. But unexpectedly, after treatment with FMT, the relative abundance of *Akkermansia* decreased, while the level of *Lactobacillus* in the intestine of mice was maintained. The ineffectiveness in KO mice after FMT was related to the decrease of *Akkermansia*. GO enrichment analysis showed that DEGs between each group were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus. Finally, we listed the top nine genes related to *Akkermansia*.

Research conclusions

FMT may ameliorate DSS-induced colitis by regulating the TLR4 signaling pathway.

Research perspectives

This study provides new insights into the underlying mechanisms of FMT as a treatment for UC, which greatly helps to optimize FMT treatment in the future.

FOOTNOTES

Author contributions: Wen X, Xie R, and Wang HG contributed equally to this work; Wen X, Xie R, and Wang HG conceived and designed this work, and drafted and revised the manuscript; Wen X, Zhang MN, He L, and Zhang MH performed the experiments, collected samples, and analyzed the data; Yang XZ and Wang HG worked on the concept and guidance of this study; Yang XZ and Wang HG provided the funding support and project administration; All authors have read and approved the final manuscript.

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Institutional animal care and use committee statement: The animal experimental protocol was approved by experimental animal ethics committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University (Approval No. DW-P-2018-008-01).

Conflict-of-interest statement: The authors have no conflicts interest to declare.

Data sharing statement: The data presented in the study are available in article. The datasets analysed during the current study are available in the NCBI Sequence Read Archive (SRA) database, submission number: SUB11829874.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Observational Study

Incidence, prevalence, and comorbidities of chronic pancreatitis: A 7-year population-based study

Qiu-Yu Cai, Kun Tan, Xue-Li Zhang, Xu Han, Jing-Ping Pan, Zhi-Yin Huang, Cheng-Wei Tang, Jing Li

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Abstract

BACKGROUND

Chronic pancreatitis (CP) is a fibroinflammatory syndrome leading to reduced quality of life and shortened life expectancy. Population-based estimates of the incidence, prevalence, and comorbidities of CP in China are scarce.

AIM

To characterize the incidence, prevalence, and comorbidities of CP in Sichuan Province, China, with population-based data.

METHODS

Data on CP from 2015 to 2021 were obtained from the Health Information Center of Sichuan Province. During the study period, a total of 38090 individuals were diagnosed with CP in Sichuan Province. The yearly incidence rate and point prevalence rate (December 31, 2021) of CP were calculated. The prevalence of comorbid conditions in CP patients was estimated. The annual number of CP-related hospitalizations, hospital length of stay, and hospitalization costs for CP were evaluated. Yearly incidence rates were standardized for age by the direct method using the permanent population of Sichuan Province in the 2020 census as the standard population. An analysis of variance test for the linearity of scaled variables and the Cochran-Armitage trend test for categorical data were performed to investigate the yearly trends, and a two-sided test with $P < 0.05$ was considered statistically significant.

RESULTS

The 38090 CP patients comprised 23280 males and 14810 females. The mean age of patients at CP diagnosis was 57.83 years, with male patients (55.87 years) being younger than female patients (60.11 years) ($P < 0.001$). The mean incidence rate of

CP during the study period was 6.81 per 100000 person-years, and the incidence of CP increased each year, from 4.03 per 100000 person-years in 2015 to 8.27 per 100000 person-years in 2021 ($P < 0.001$). The point prevalence rate of CP in 2021 was 45.52 per 100000 individuals for the total population, with rates of 55.04 per 100000 individuals for men and 35.78 per 100000 individuals for women ($P < 0.001$). Individuals aged 65 years or older had the highest prevalence of CP (113.38 per 100000 individuals) ($P < 0.001$). Diabetes (26.32%) was the most common comorbidity in CP patients. The number of CP-related hospitalizations increased from 3739 in 2015 to 11009 in 2021. The total costs for CP-related hospitalizations for CP patients over the study period were 667.96 million yuan, with an average of 17538 yuan per patient.

CONCLUSION

The yearly incidence of CP is increasing, and the overall CP hospitalization cost has increased by 1.4 times during the last 7 years, indicating that CP remains a heavy health burden.

Key Words: Chronic pancreatitis; Epidemiology; Incidence; Prevalence; Comorbidities; Disease burden

Core Tip: Chronic pancreatitis (CP) remains a heavy health burden worldwide. However, available epidemiological data on CP in China are scarce. We conducted a population-based study on the incidence, prevalence, comorbidities, and disease burden of CP in Sichuan Province, China, from 2015 to 2021. We analyzed a total of 38090 patients, which represents the largest series of CP patients ever reported in China. We observed an increasing incidence and rising costs for CP-related hospitalization. The point prevalence rate of CP was 45.52 per 100000 individuals in 2021. Metabolic-related diseases and pancreatic tumors were among the most common comorbidities among CP patients.

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INTRODUCTION

Chronic pancreatitis (CP) is a fibroinflammatory syndrome characterized by chronic upper abdominal pain and exocrine and endocrine pancreatic insufficiency[1]. Although some cases of CP may begin with one or recurrent bouts of pancreatitis, approximately 50% of CP patients have no history of acute pancreatitis[2,3]. Therefore, for a considerable proportion of patients, the development of CP might be attributable to different pathogeneses. As CP progresses, diabetes mellitus, malnutrition, and pancreatic cancer may develop[3,4], leading to reduced quality of life (QoL) and shortened life expectancy. The current strategies for CP include relieving symptoms, preventing disease progression, and managing complications[5]. CP causes a heavy health burden worldwide, consuming many medical resources due to less effective curative treatments for reversing the course of the disease[1,6,7]. Nevertheless, available epidemiological studies on CP are mainly from registry-based analyses and questionnaire-based surveys in European countries and the United States. Reportedly, the prevalence of CP ranges from 13.5 to 163 cases per 100000 individuals, and the incidence of CP ranges from 5 to 31.7 new cases per 100000 person-years[8-14]. The wide CP prevalence and incidence ranges may be related to the differences in case definitions, settings, patient cohorts, and statistical methods. China is one of the most populous countries in the world. However, only two epidemiological studies on CP in the eastern part of China have been conducted[15,16]. These data were mainly based on one or several databases from hospitals with small sample sizes. Population-based epidemiological data from other regions may provide a better picture of the incidence and prevalence of CP in China[1].

In addition, comorbidities, defined as the cooccurrence of two or more chronic medical conditions in one person[17], usually alter the disease management implications. Multiple comorbidities may reduce QoL and increase the risk of death and the consumption of medical resources[18]. Therefore, comorbidities have become an important part of epidemiological studies for chronic diseases[19]. However, data on the comorbidities of CP are still insufficient[20]. This epidemiological study aimed to characterize the incidence, prevalence, and comorbidities of CP in Sichuan Province in Southwest China from 2015 to 2021 with population-based data.

MATERIALS AND METHODS

Study design and population

Data on CP from 2015 to 2021 were obtained from the Health Information Center of Sichuan Province (HIC-SC), which collects electronic hospitalization summary reports (HSRs) from the electronic medical record systems of all hospitals in Sichuan Province. These records contain encrypted patient identification numbers and data on age, sex, admission and discharge dates, International Classification of Diseases, Tenth Revision (ICD-10) codes, and relevant discharge diagnoses. The HIC-SC has provided data for many scientific studies[21-23], verifying the reliability of the database. The data of permanent residents in Sichuan Province over the study years were obtained from the Sichuan Provincial Bureau of Statistics. This study was approved by the biomedical ethics review committee of West China Hospital, Sichuan Univer -sity on February 8, 2022 (IRB approval number: 2022-296), and the requirement for informed consent was waived.

Diagnosis and classification of CP

In China, the definition of CP as a continuing inflammatory disease of the pancreas, characterized by irreversible morphological changes and pain and/or permanent loss of function, has been widely accepted[7,24]. In clinical practice, computed tomography (CT) or magnetic resonance imaging (MRI) are usually recommended as first-line tests for patients with clinical symptoms of an inflammatory disorder of the pancreas (such as a previous episode of acute pancreatitis, characteristic pain, and/or maldigestion) because they are universally available and reproducible. The major imaging characteristics of CP include pancreatic atrophy, fibrosis, duct distortion and strictures, stones in pancreatic ducts or multiple calcifications distributed throughout the entire pancreas, *etc*. Endoscopic ultrasound, because of its invasiveness, is used only if the diagnosis is in question after CT or MRI. The diagnosis of CP in some patients is established by histopathological examination after surgery due to unexplained pancreatic masses. Therefore, patients who had a discharge diagnosis of CP in their HSR provided by the HIC-SC with the following ICD-10 codes were included in this study: K86.102 for CP, K86.852 for pancreatic atrophy, K86.811 for pancreatic calcifications, K86.809 for stones in pancreatic ducts, K86.806/807 for duct distortion and strictures, K86.154 for pancreatic fibrosis, K86.201 for pancreatic cysts, K86.804 for pancreatemphraxis, and K86.901/902 for pancreatic masses. Furthermore, this study classified CP according to the following ICD-10 codes: K86.051 for alcoholic CP, K86.153 for autoimmune pancreatitis (AIP), and K86.151 for biliary pancreatitis. Patients with conflicting information, *i.e.*, different birth dates at each admission, were excluded.

Identification of comorbidities

The comorbidities of CP in this study were selected by referring to previous studies[4,13,25]. Data on comorbidities were extracted from the electronic database by identifying their corresponding ICD-10 codes (Table 1).

Epidemiological outcomes

The major outcomes were the incidence and prevalence of CP. Incident cases were defined as patients with a first-time diagnosis of CP. Patients with a diagnosis of CP before the calculated year were excluded from the incidence estimate calculation. Prevalent cases were defined as patients with a diagnosis at some timepoint before the end of 2021. Crude incidence rates were calculated for each year between 2015 and 2021 as the number of incident CP cases divided by the average permanent population in a year (per 100000 person-years). Yearly incidence rates were standardized for age by the direct method using the permanent population of Sichuan Province in the 2020 census as the standard population. The point prevalence rate (December 31, 2021) was calculated as the number of patients who were diagnosed with CP before the end of 2021 divided by the permanent population of Sichuan Province in 2021. Other outcomes were the number of hospitalizations, the hospital length of stay (LoS), and the hospitalization costs. LoS was defined as the total length of hospital stay of the patient within one year. Costs for hospitalization, including total costs and the mean costs per patient, were calculated. Costs were adjusted by the consumer price index for each year to 2021 costs.

Statistical analysis

Data management and computations were performed using Oracle version 11.2.0 (https://www.oracle.com) and R version 4.1.3 (R Foundation for Statistical Computing; https://www.R-project.org/). Continuous variables are shown as the means ± SD. Since this was a population-based study including the entire permanent population of Sichuan Province during the observation period, no confidence intervals are provided for the estimates of the incidence or prevalence rates. Statistical analysis, such as the student's *t*-test for continuous variables and the chi-squared test for categorical variables, was used for the comparison of statistics between sexes and age groups. To investigate yearly trends, we performed an analysis of variance tests for the linearity of scaled variables and the Cochran-Armitage trend test for categorical data. A two-sided test with $P < 0.05$ was considered statistically significant.

RESULTS

Data collection

There were a total of 61112 electronic records (38296 individuals) with a diagnosis of CP from 2015 to 2021. Among all the records, 3180 records were excluded due to missing encrypted patient identification numbers (1518 records) and conflicting information (1662 records from 206 individuals). The exclusion of the data resulted in the loss of 5.2% of the

Table 1 The comorbidities and the corresponding International Classification of Diseases, Tenth Revision codes

Comorbidities	ICD-10 codes
Diabetes	E10, E11, E12, E13, E14
Type I diabetes	E10
Type II diabetes	E11
Secondary diabetes	E13, E14
Hypertension	I10, I11, I12, I13, I15, O10
Dyslipidemia	E78
Cholelithiasis	K80, O99.603, K56.3
Fatty liver disease	K76.0, K70.0
Alcoholic fatty liver disease	K70.0
Coronary heart disease	I21, I22, I23, I24, I25
Cerebrovascular disease	I60, I61, I62, I63, I64, I65, I66, I67, I69, G45
Ischemic cerebrovascular disease	I63, I64, I65, I66, I67, G45
Hemorrhagic cerebrovascular disease	I60, I61, I62
Pancreatic cyst	K86.2
Pancreatic pseudocyst	K86.3
Acute pancreatitis	K85
Heart failure	I50, I11.0, I13.0, I13.2
Hyperuricemia	M10, E79
Gastrointestinal bleeding	K92.0, K92.1, K92.2
Liver cirrhosis/fibrosis	K70.3, K74
Alcoholic cirrhosis	K70.3
Pancreatic tumor	C24.102, C25, D13.6, D37.752, D37.703, D37.704
Malignant tumor of the pancreas	C25, C24.102
Malnutrition	E12, E40, E41, E42, E43, E44, E45, E46
Ulcer disease	K25, K26, K27, K28
Gastric ulcer	K25
Duodenal ulcer	K26
Osteoporosis	M80, M81
Inflammatory bowel disease	K50, K51
Crohn's disease	K50
Ulcerative enteritis	K51

ICD: International Classification of Diseases.

original electronic records and a loss of 0.5% from the original patient cohort. Therefore, 57932 electronic records from 38090 unique patients were ultimately analyzed in this study (Figure 1). Data for 30756 CP patients (80.7% of the cohort) were extracted by the ICD-code K86.102. A total of 3641 CP patients (9.6%) were identified by the ICD-10 code K86.809 (pancreatic calculus). The left 9.7% of the CP patients were included by other ICD-10 codes (K86). Only 397 CP patients (1%) were classified as having alcoholic CP. Sichuan Province is one of the most populous provinces in China, with approximately 83.67 million permanent residents in 2020.

Characteristics of the patients

The demographics of the CP patients with a first diagnosis are presented in Table 2 by calendar year. The number of new cases increased each year from 2015 to 2021, with a significant upward trend ($P < 0.001$). There were 1.56-fold more male patients than female patients. The mean age of the patients at CP diagnosis was 57.83 years, with males (55.87 years) being younger than females (60.11 years) ($P < 0.001$). More than 60% of the patients diagnosed each year were aged 15-64

Table 2 Demographics of chronic pancreatitis patients with a first diagnosis by calendar year

	2015	2016	2017	2018	2019	2020	2021	*P* value
Electronic records, *n*	3739	5760	7688	9488	9865	10383	11009	< 0.001
New case, *n*	3088	4334	5299	5945	5987	6475	6962	< 0.001
Mean hospitalizations per patient, *n*	1.21	1.16	1.19	1.20	1.17	1.16	1.15	0.097
Sex, %								
Male	62.01	59.23	60.52	59.73	60.23	62.63	62.90	0.282
Female	37.99	40.77	39.48	40.27	39.77	37.37	37.10	
Age, yr, mean ± SD	57.48 ± 15.78	58.01 ± 15.31	58.28 ± 15.59	58.84 ± 15.81	58.27 ± 15.77	57.52 ± 15.99	56.57 ± 16.57	0.387
Male	55.87 ± 15.35	56.58 ± 15.19	56.85 ± 15.17	57.37 ± 15.62	56.74 ± 15.38	56.10 ± 15.58	54.98 ± 16.41	0.415
Female	60.11 ± 16.12	60.09 ± 15.25	60.48 ± 15.96	61.01 ± 15.86	60.60 ± 16.06	59.88 ± 16.38	59.27 ± 16.51	0.389
Age group, %								
0-14	0.39	0.35	0.51	0.64	0.33	0.34	0.39	0.902
15-64	64.57	63.99	63.37	60.52	61.78	63.83	64.97	0.820
≥ 65	35.04	35.65	36.12	38.84	37.88	35.83	34.65	0.807
Ethnicity, %								
Han	94.36	94.78	95.32	95.31	94.27	94.18	95.17	0.722
Yi	1.33	1.80	1.91	1.55	2.04	2.12	2.08	0.515
Zang	1.69	2.03	1.83	2.27	2.24	2.30	1.74	0.923
Qiang	0.36	0.39	0.47	0.37	0.45	0.83	0.43	0.646
Other	2.27	0.99	0.47	0.50	1.00	0.57	0.57	0.007

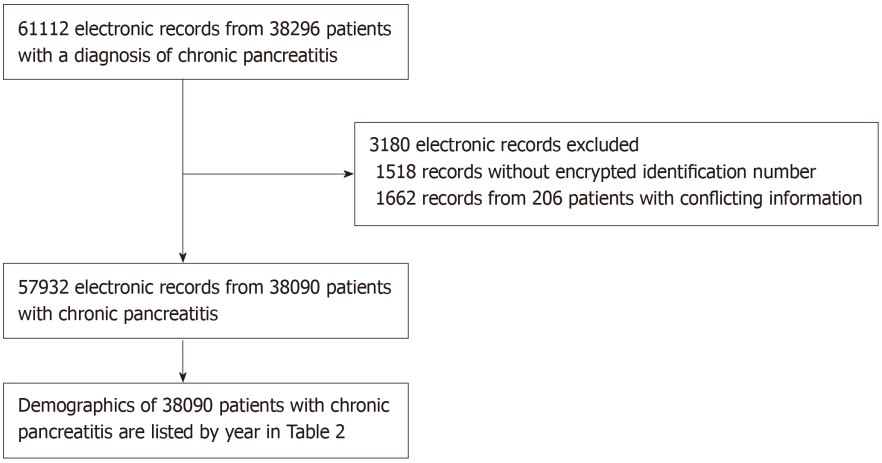

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Figure 1 Flowchart of the inclusion and exclusion processes. A total of 61112 electronic records from 38296 patients with a diagnosis of chronic pancreatitis were retrieved from the Health Information Center of Sichuan Province. A total of 3180 records were excluded due to missing encrypted patient identification numbers (1518 records) and conflicting information (1662 records from 206 individuals). A total of 57932 electronic records from 38090 unique patients with chronic pancreatitis were ultimately included in this study.

years, and the proportion of each group remained stable over time (Table 2). Similar to the population ratio, the component ratio of the Han Chinese was the highest (94.92%) in CP patients, although Sichuan is a multiethnic province.

Incidence

The mean standardized incidence of CP was 6.81 per 100000 person-years, and the incidence of CP increased yearly during the study period ($P < 0.001$), from 4.03 per 100000 person-years in 2015 to 8.27 per 100000 person-years in 2021 (Figure 2). The mean incidence of CP in males was 7.95 per 100000 person-years, which was significantly higher than that in females (5.13 per 100000 person-years) ($P < 0.001$). Both sexes presented similar upward trends ($P < 0.001$) (Figure 3A).

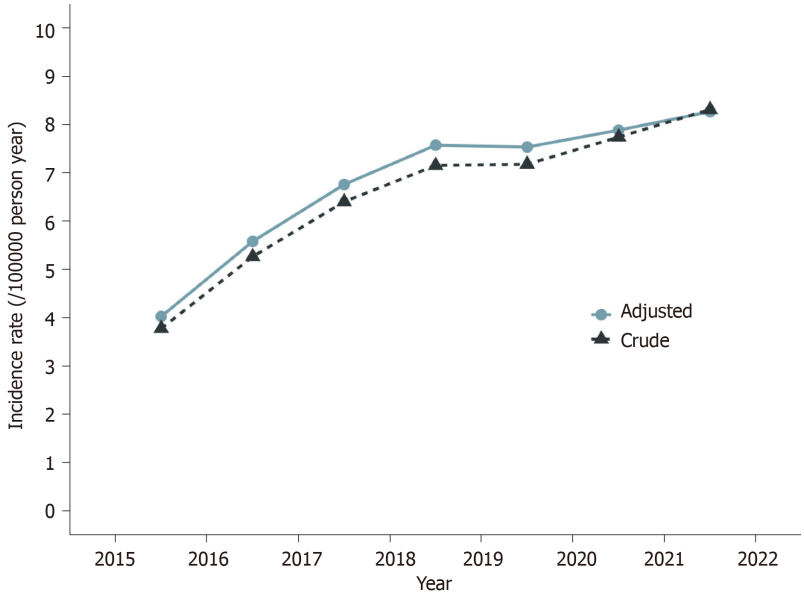

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Figure 2 Incidence trends of chronic pancreatitis. Crude and age-adjusted annual incidence trends of chronic pancreatitis from 2015 to 2021 in Sichuan Province, China (per 100000 person-years).

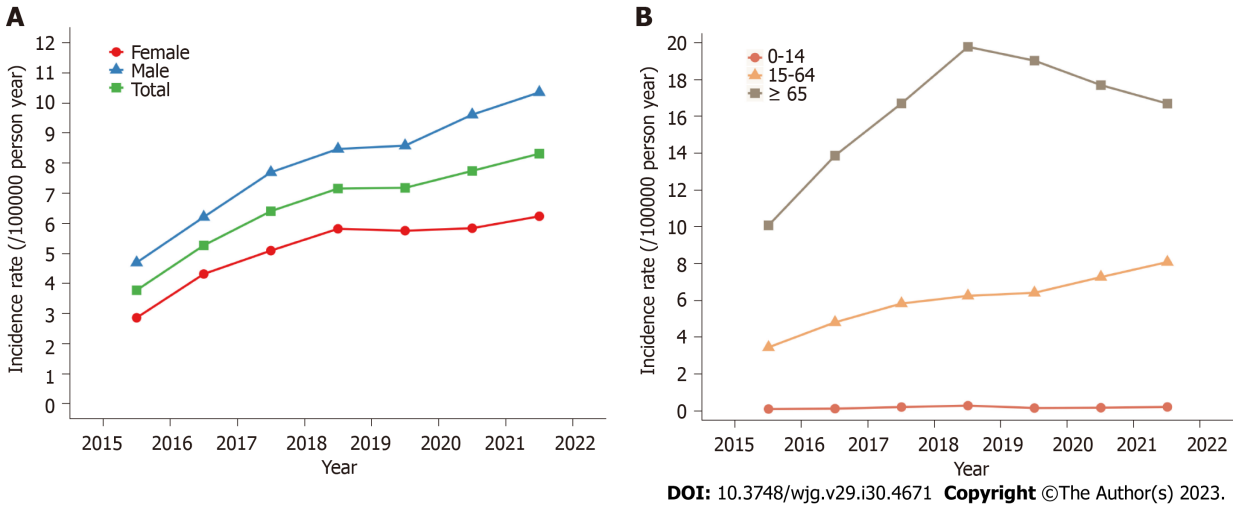

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Figure 3 Sex- and age-grouped incidence of chronic pancreatitis. A and B: The crude incidence of chronic pancreatitis in the population grouped by sex (A) and age (B) (per 100000 person-years) from 2015 to 2021.

For males, the incidence of CP was 4.70 per 100000 person-years in 2015 and 10.36 per 100000 person-years in 2021; for females, the incidence of CP was 2.86 per 100000 person-years in 2015 and 6.24 per 100000 person-years in 2021. The highest CP incidence (16.27 per 100000 person-years) was observed in patients aged ≥ 65 years, with an upward trend from 10.08 per 100000 person-years in 2015 to 16.70 per 100000 person-years in 2021 ($P < 0.001$) (Figure 3B). Individuals aged 14 years or younger showed the lowest CP incidence (0.17 per 100000 person-years), which remained stable during the 7 years ($P = 0.830$) (Figure 3B). The seven-year mean incidence for the 15-64-year age group was 6.01 per 100000 person-years, with an increase from 3.45 per 100000 person-years in 2015 to 8.08 per 100000 person-years in 2021 ($P < 0.001$) (Figure 3B).

Prevalence

A total of 38090 individuals were diagnosed with CP in Sichuan Province during the study period. The age distribution of the CP patients up to 2021 is shown in Figure 4A. Among all the patients, 23280 (61.12%) were men, and 14810 (38.88%) were women ($P < 0.001$). The point prevalence of CP was 45.52 per 100000 individuals in 2021 for the total population, 55.04 per 100000 individuals for the male population, and 35.78 per 100000 individuals for the female population ($P < 0.001$). The prevalence of CP was 1.53 times higher [95% confidence interval (CI): 1.51-1.57; $P < 0.001$] for men than for women. Individuals aged 65 years or older had the highest prevalence of CP (113.38 per 100000 individuals), with a prevalence of 127.58 per 100000 individuals for men and 100.03 per 100000 individuals for women ($P < 0.001$) (Figure 4B).

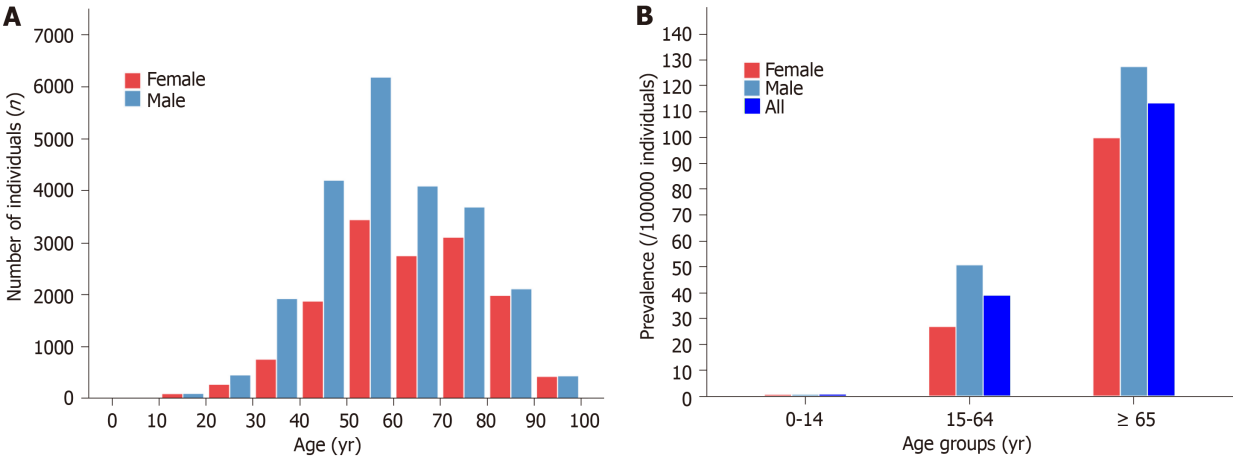

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Figure 4 The prevalence of chronic pancreatitis. A and B: The age distribution of chronic pancreatitis patients (A) and the prevalence of chronic pancreatitis by sex and age groups (B) in 2021.

For individuals aged between 15 and 64 years, the prevalence of CP was 39.11 per 100000 individuals, with a prevalence of 50.86 per 100000 individuals for men and 27.00 per 100000 individuals for women ($P < 0.001$) (Figure 4B). The prevalence of CP was 2.90 times higher (95%CI: 2.84-2.96; $P < 0.001$) for individuals aged 65 years or older than for individuals aged between 15 and 64 years. Individuals aged 14 years or younger had the lowest prevalence of CP (0.81 per 100000 individuals), with a prevalence of 0.82 per 100000 individuals for boys and 0.80 per 100000 individuals for girls ($P = 1$) (Figure 4B).

Prevalence of comorbid conditions

Diabetes (26.32%) was the most common comorbidity in CP patients, and patients with diabetes were similar in age to the overall cohort (Table 3). Metabolic-related diseases, including hypertension (21.51%), cholelithiasis (16.79%), dyslipidemia (16.70%), and fatty liver diseases (12.39%), were also common comorbidities among CP patients. The incidence of acute exacerbations of CP was 5.08% in all CP patients, with an incidence of 6.35% in males and 3.07% in females. A total of 2.65% of the CP patients were also diagnosed with pancreatic tumors, and 2.36% were diagnosed with malignant tumors of the pancreas.

Disease burden of CP

The number of CP-related hospitalizations increased approximately 2-fold, from 3739 in 2015 to 11009 in 2021 ($P < 0.001$) (Figure 5A). The LoS per CP patient decreased from 14.52 d in 2016 to 12.38 d in 2021 ($P = 0.008$) (Figure 5B). The total costs for CP-related hospitalizations for CP patients over the study period were 667.96 million yuan, with an average of 17538 yuan per patient. Total costs for CP-related hospitalizations increased from 50.60 million yuan in 2015 to 124.16 million in 2021 ($P < 0.001$) (Figure 5C), while the costs per patient decreased from 16387 yuan in 2015 to 13986 yuan in 2021 ($P = 0.024$) (Figure 5D).

DISCUSSION

We conducted a population-based study on the epidemiology of CP in Sichuan Province, China, from 2015 to 2021. This study showed that the mean incidence of CP was 6.81 per 100000 person-years in the last 7 years, and the point prevalence of CP was 45.52 per 100000 individuals in 2021 for the total population in Sichuan Province. The incidence and prevalence of CP increased with age and were significantly higher in males than in females over the study years. Metabolic-related diseases, including diabetes (26.32%), hypertension (21.51%), cholelithiasis (16.79%), dyslipidemia (16.70%), and fatty liver disease (12.39%), were common comorbidities in CP patients. In addition, 2.65% of the CP patients were diagnosed with pancreatic tumors. During the 7-year period, CP-related hospitalizations increased approximately 2-fold, and the overall hospitalization cost of CP increased by 1.4-fold. However, the LoS and the costs per CP patient decreased over the study years.

It may be valuable to improve epidemiological data of CP in the southwest area of China due to the rapid economic and societal development of the country. The first population-based epidemiological study of CP reported a mean CP incidence of 6.81 per 100000 person-years in Sichuan Province in the last 7 years. In other words, Sichuan Province has an average of 5652 new cases of CP every year. Thus, although hospitalization costs per patient decreased yearly, overall hospitalization costs still increased year by year. The overall hospitalization cost of CP increased by 1.4 times during the 7-year period. Although this study was analyzed with big data, the incidence rate may be underestimated because the data only covered inpatients with CP, and outpatients with CP were not included. A variety of databases have been used for the epidemiological analysis of CP[8,9,14,26], but it is still difficult to accurately describe the epidemic status of CP

Table 3 Prevalence of comorbidities in chronic pancreatitis patients

Diseases	Total cohort, *n* = 38090		Males, *n* = 23280		Females, *n* = 14810		*P* value
	n, %	Age, yr, mean ± SD	*n*, %	Age, yr, mean ± SD	*n*, %	Age, yr, mean ± SD	
Total	100.00	57.58 ± 15.79	100.00	56.26 ± 15.25	100.00	59.99 ± 16.44	-
Diabetes	26.32	56.57 ± 14.60	28.94	55.43 ± 14.03	22.20	59.14 ± 15.51	< 0.001
Type I diabetes	0.79	45.33 ± 15.11	0.81	47.32 ± 12.81	0.76	42.23 ± 17.70	0.591
Type II diabetes	21.84	58.04 ± 14.26	23.73	56.78 ± 13.89	18.87	60.75 ± 14.66	< 0.001
Secondary diabetes	2.05	50.04 ± 13.24	2.71	49.35 ± 12.40	1.01	53.27 ± 16.27	< 0.001
Hypertension	21.51	66.32 ± 14.41	20.23	64.27 ± 14.43	23.51	69.36 ± 13.82	< 0.001
Cholelithiasis	16.79	62.33 ± 16.14	14.95	60.23 ± 15.37	19.67	64.90 ± 16.68	< 0.001
Dyslipidemia	16.70	51.49 ± 14.28	16.76	48.57 ± 13.12	16.61	56.01 ± 14.81	0.708
Cerebrovascular disease	12.76	69.75 ± 13.34	12.19	68.52 ± 13.55	13.65	71.60 ± 12.79	< 0.001
Ischemic cerebrovascular disease	11.75	69.55 ± 13.38	11.17	68.54 ± 13.57	12.65	71.09 ± 12.92	< 0.001
Hemorrhagic cerebrovascular disease	0.34	64.05 ± 14.42	0.43	62.63 ± 14.55	0.20	69.39 ± 12.79	< 0.001
Fatty liver disease	12.39	49.53 ± 13.50	13.46	47.20 ± 12.63	10.72	54.28 ± 13.96	< 0.001
Alcoholic fatty liver disease	0.35	54.07 ± 12.45	0.53	54.32 ± 12.38	0.07	50.50 ± 13.39	< 0.001
Coronary heart disease	10.07	72.15 ± 12.77	8.37	71.13 ± 13.10	12.75	73.27 ± 12.31	< 0.001
Heart failure	5.27	74.15 ± 13.38	4.72	72.62 ± 13.98	6.14	76.06 ± 12.33	< 0.001
Acute pancreatitis	5.08	51.38 ± 15.68	6.35	52.17 ± 14.18	3.07	48.80 ± 19.57	< 0.001
Hyperuricemia	4.93	56.86 ± 17.25	6.42	55.07 ± 16.87	2.59	64.28 ± 16.85	< 0.001
Ulcer disease	4.68	60.16 ± 14.54	5.28	59.30 ± 14.06	3.73	62.22 ± 15.46	< 0.001
Gastric ulcer	2.17	60.34 ± 13.91	2.40	59.36 ± 13.34	1.80	62.60 ± 14.92	< 0.001
Duodenal ulcer	1.30	59.15 ± 14.46	1.65	58.53 ± 14.65	0.74	61.28 ± 13.62	< 0.001
Liver cirrhosis/fibrosis	3.49	58.70 ± 13.92	4.35	57.05 ± 13.12	2.13	65.09 ± 15.04	< 0.001
Alcoholic cirrhosis	0.72	53.23 ± 10.21	1.15	53.31 ± 10.19	0.05	49.60 ± 10.54	< 0.001
Pancreatic pseudocyst	3.45	52.55 ± 14.28	4.46	51.57 ± 12.92	1.85	56.38 ± 18.17	< 0.001
Malnutrition	3.36	64.04 ± 16.53	3.80	62.74 ± 15.52	2.67	66.86 ± 18.23	< 0.001
Gastrointestinal bleeding	2.93	61.76 ± 16.38	3.46	59.78 ± 15.84	2.10	67.13 ± 16.64	< 0.001
Pancreatic tumor	2.65	61.43 ± 12.13	2.97	61.27 ± 11.77	2.15	61.79 ± 12.89	< 0.001
Malignant tumor of pancreas	2.36	61.74 ± 11.99	2.64	61.50 ± 11.67	1.91	62.27 ± 12.65	< 0.001
Osteoporosis	2.03	75.28 ± 13.19	1.37	70.13 ± 13.89	3.06	78.02 ± 11.94	< 0.001
Inflammatory bowel disease	0.14	56.13 ± 15.76	0.13	56.75 ± 14.03	0.17	55.43 ± 17.76	0.388
Crohn's disease	0.03	56.40 ± 14.07	0.03	58.67 ± 10.76	0.03	53.00 ± 19.37	1
Ulcerative enteritis	0.12	56.00 ± 16.05	0.11	56.15 ± 14.56	0.14	55.83 ± 17.90	0.429

due to the limitations of various databases. There may be many CP outpatients, but if their condition is not severe enough to require hospitalization, the medical costs and the disease burden would be relatively low, and the epidemiological significance may not be very important. Reportedly, the rates of harmful drinking behaviors and smoking, which may be risk factors for CP, have increased in Chinese individuals in the last 10 years[27,28]. In contrast, the incidences of CP remained stable or even decreased yearly in recent years in the United States and Denmark, indicating that the decline in CP incidence might be due to the decrease in tobacco and alcohol consumption in both countries[8,14,29]. However, it was difficult to evaluate the factors related to the increasing incidence of CP in this study because electronic HSRs did not include risk factors associated with CP. CT and MRI have become universally available in hospitals at all levels in Sichuan Province, China. This greatly enhances the detection rate of CP and may partly contribute to the increasing CP incidence rate.

The point prevalence of CP was 45.52 per 100000 individuals in 2021 for the total population in Sichuan Province. The prevalence of CP was 39.11 and 113.38 per 100000 individuals for the population aged between 15 and 64 years and the population aged 65 years or older, respectively. The current results are consistent with those of studies in Minnesota

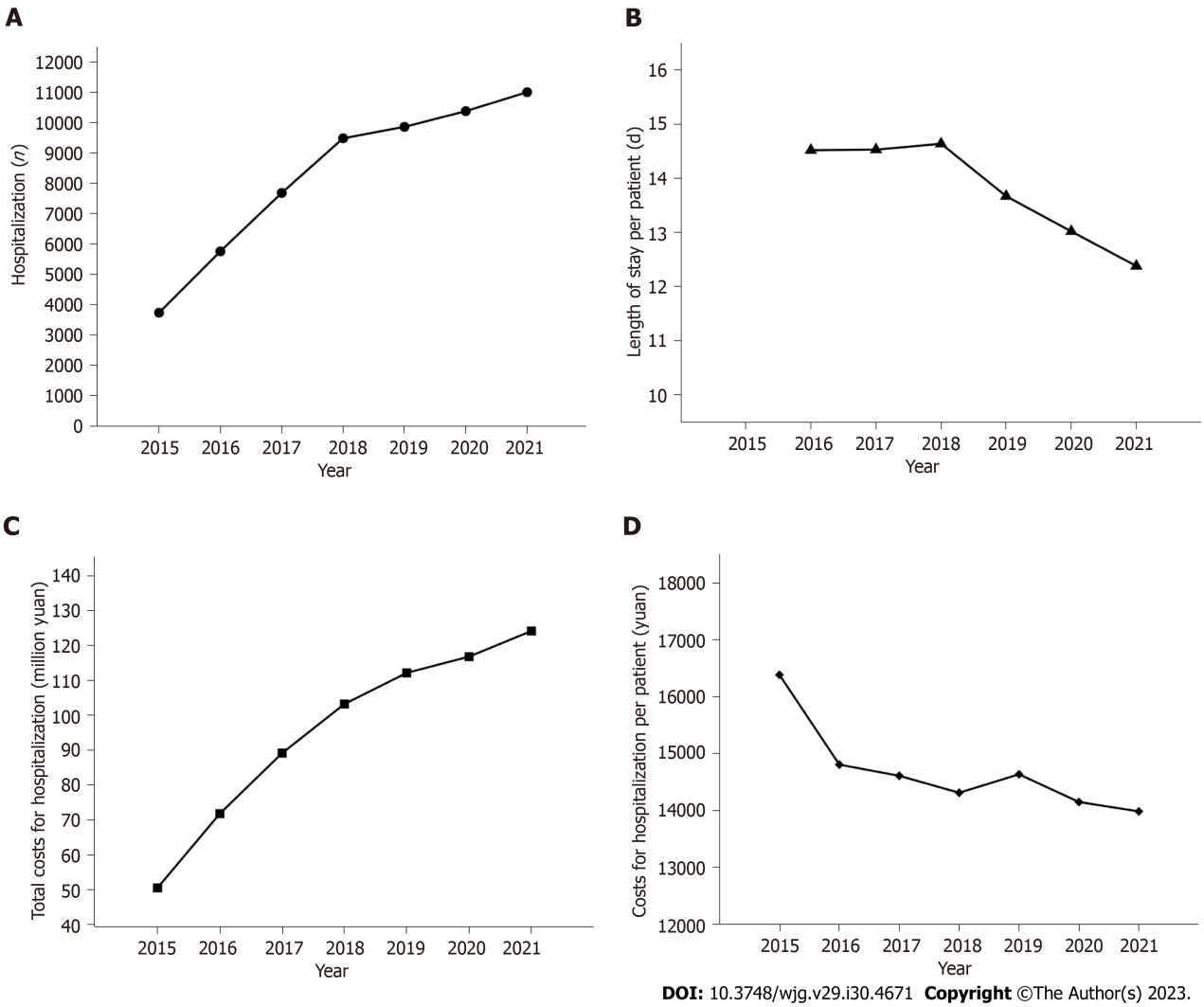

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Figure 5 Disease burden of chronic pancreatitis. A: Annual number of chronic pancreatitis-associated hospitalizations; B: Mean length of stay per patient; C: Total costs for hospitalization; D: Costs for hospitalization per patient. Costs were adjusted by the consumer price index every year to 2021 costs (yuan).

(41.76/100000 individuals), Japan (52.4/100000 individuals), and Spain (49.3/100000 individuals), which were all based on questionnaire-based surveys[10,11,30]. In contrast, the prevalence of CP was considerably higher in register-based studies covering inpatients as well as outpatients[8,9,14]. Based on insurance claims databases, Machicado *et al*[9] and Sellers *et al*[14] reported similar results for the prevalence of CP in adults in America, which was 98.7/100000 individuals (2001-2013) and 91.9/100000 individuals (2007-2014), respectively. Using nationwide health care registries over a long period (1994–2018), Olesen *et al*[8] estimated the prevalence of CP at 153.9/100000 individuals in Denmark. This discrepancy may be explained by differences in study designs or by true differences in the CP prevalence across regions. Compared to the multicenter study in 2003, our current study adds to the prevalence data on CP over the past decades and verifies the upward trend in the prevalence of CP[15]. The increase in the CP prevalence could be explained by the increasing CP incidence and improved prognosis and life expectancy of CP patients[31-33].

Both the incidence and prevalence of CP in males were significantly higher than those in females over the study years. The prevalence for men was 1.53 times higher than that for women. Of all CP patients, 61.12% were men and 38.88% were women. Both the incidence and prevalence of CP were higher among men than women in all age groups, which was consistent with most previous studies[8,11,15,34,35]. This difference may be due to higher alcohol and tobacco exposure and increased genetic susceptibility to alcoholic CP among men[36,37]. The male hemizygous CLDN2 genotype and the female homozygous CLDN2 genotype are known to confer an amplified risk of pancreatitis together with alcohol use, and the frequency of the male hemizygous CLDN2 genotype (0.26) is much higher than that of the female homozygous CLDN2 genotype (0.07); thus, males have a higher genetic susceptibility to alcoholic CP than females[37]. In addition, the prevalence and incidence of CP were the highest among elderly individuals compared with young and middle-aged people in our study. These results are also similar to those of previous studies, suggesting that the incidence and prevalence of CP increases with aging[8,14]. Although the results might represent the prevalence of CP, it is undeniable that there was bias. In terms of the inpatient database, elderly people had the highest rate of hospitalization, which was accompanied by a higher detection rate of CP. In comparison, the detection rates of CP might be lower in other age groups, leading to an underestimation of the incidence and prevalence of CP.

Table 4 Available studies on comorbidities of chronic pancreatitis

Region	Study design	Study period	Total CP patients	Frequent comorbidities compared with controls	Ref.
Denmark	Nationwide retrospective cohort study	1995-2010	11972	Cerebrovascular disease, chronic pulmonary disease, ulcer disease, diabetes, chronic renal disease, and pancreatic cancer	[4]
United States and Denmark	Cross-sectional, multicenter prospective study	Not available	171	Anxiety and depression	[40]
United States	Retrospective cohort study	2015-2020	63230	Hypertension, diabetes, and myocardial Infarction	[41]
United States	Ongoing longitudinal cohort study	2017-2022	488	Depression, anxiety, sleep disturbance, and physical disability	[42]
United States	Cohort study (NAPS2 cohort)	2000-2014	1024	Gallstones, diabetes, heart disease/heart attack/stroke, liver disease, renal disease, or prior history of cancer	[25]
United Kingdom	Cohort Study (the United Kingdom BioBank cohort)	2000-2020	1027	Gallstones, hyperlipidemia, diabetes, hypertriglyceridemia, hypercalcemia, pancreatic cancer, celiac, and ulcerative colitis	[13]
Canada	Retrospective study	2007-2014	75744	Anxiety and depression	[43]
China	Population-based cohort study	2000-2011	15848	Urolithiasis	[44]
China	Population-based cohort study	2000-2011	17810	Subsequent pyogenic liver abscess	[45]
China	Population-based cohort study	2000-2011	16672	Cerebrovascular disease	[46]
China	Population-based cohort study	2000-2011	17778	Deep vein thrombosis and pulmonary embolism	[20]
China	Population-based cohort study	2000-2011	17796	Inflammatory bowel disease	[47]
China	Prospective observational study	2019-2021	720	Anxiety and depression	[48]

As comorbidity, diabetes was reported in 26.32% of CP patients in this study. The prevalence of diabetes in CP patients reported in the previous literature was 15.2%-41.5%[4,13,25]. The high comorbidity of CP with diabetes suggests the importance of the identification of post-pancreatitis diabetes mellitus, which might be greatly different from type 2 diabetes mellitus in its manifestation and treatment. In addition, hypertension (21.51%), cholelithiasis (16.79%), and fatty liver disease (12.39%) were also common comorbidities in CP patients. A cohort study of the United Kingdom Biobank reported an even higher prevalence of gallstones (32.4%), hyperlipidemia (34.7%), and pancreatic cancer (4.7%) in CP patients and a strong association between CP and essential hypertension[13]. In the same cohort (United Kingdom Biobank), a case-control study reported that CP was associated with a significantly higher risk of pancreatic cancer in patients within the first 3 years[38]. These comorbidities may be explained by shared etiologies resulting from general exposure to risk factors such as tobacco use and alcohol consumption among CP patients. However, it is also possible that some of these comorbidities are involved in the pathogenesis of CP development, and some comorbidities may be complications caused by CP. Prospective studies, including longitudinal cohort studies and randomized control trials, may provide more definitive evidence on potential associations with multimorbidity. As shown in Table 4, different comorbidities were reported to be associated with CP in European, American, and East Asian countries. This may be related to different regions, times, and types of research. In addition, the pattern of comorbidities of CP is still under exploration, and the comorbidities reported by the abovementioned studies are similar to our findings. Smaller sample sizes and more accurate and complete diagnoses of diseases in cohort studies as well as different study inclusion criteria could explain the discrepancy between cohort and register-based studies.

AIP represents < 5%-10% of pancreatitis cases and has a smaller prevalence of approximately 1-2/100000 individuals [39]. In the design of this study, AIP was classified as a type of CP. However, data on AIP were not extracted from the electronic HSRs because there is no special ICD-10 code for AIP. Moreover, only 1% of CP patients were classified as having alcoholic CP, which might be underestimated because doctors may not have paid enough attention to alcoholic CP when they filled out the electronic HSRs. This study suggests some improvements for the HIC-SC to enhance the quality of electronic HSR completion.

CONCLUSION

The yearly incidence of CP increased, along with the absolute number of CP patients and associated costs for hospitalization in Sichuan Province over the study period. The current work shows that CP remains a heavy burden on patients and the healthcare system. The implication of the high prevalence of diabetes (26.32%) in CP patients may vary with different people.

ARTICLE HIGHLIGHTS

Research background

Chronic pancreatitis (CP) is a fibroinflammatory syndrome leading to reduced quality of life and shortened life expectancy. Population-based estimates of the prevalence and incidence of CP in China are scarce.

Research motivation

Accurate epidemiological estimates of CP are vital in shaping health resource allocation and medical provider training priorities.

Research objectives

To evaluate the prevalence and time trends of the incidence of CP and describe the comorbidities and disease burden of CP in Sichuan Province, China.

Research methods

Data on CP from 2015 to 2021 were obtained from the Health Information Center of Sichuan Province. During the study period, a total of 38090 individuals were diagnosed with CP in Sichuan Province. The yearly incidence rate and point prevalence rate (December 31, 2021) of CP were calculated. The prevalence of comorbid conditions in CP patients was estimated. The annual number of CP-related hospitalizations, hospital length of stay, and costs for hospitalization for CP were evaluated. Yearly incidence rates were standardized for age by the direct method using the permanent population of Sichuan Province in the 2020 census as the standard population. An analysis of variance test for the linearity of scaled variables and the Cochran-Armitage trend test for categorical data were performed to investigate the yearly trends, and a two-sided test with $P < 0.05$ was considered statistically significant.

Research results

The 38090 CP patients comprised 23280 males and 14810 females. The mean age of patients at CP diagnosis was 57.83 years, with males (55.87 years) being younger than females (60.11 years) ($P < 0.001$). The mean incidence rate of CP during the study period was 6.81 per 100000 person-years, and the incidence of CP increased each year, from 4.03 per 100000 person-years in 2015 to 8.27 per 100000 person-years in 2021 ($P < 0.001$). The point prevalence rate of CP in 2021 was 45.52 per 100000 individuals for the total population, with rates of 55.04 per 100000 individuals for men and 35.78 per 100000 individuals for women ($P < 0.001$). Individuals aged 65 years or older had the highest prevalence of CP (113.38 per 100000 individuals) ($P < 0.001$). Diabetes (26.32%) was the most common comorbidity in CP patients. The number of CP-related hospitalizations increased from 3739 in 2015 to 11009 in 2021. The total costs for CP-related hospitalizations for CP patients over the study period were 667.96 million yuan, with an average of 17538 yuan per patient.

Research conclusions

The yearly incidence of CP increased in Sichuan Province, along with the absolute number of CP patients and associated costs for hospitalization between 2015 and 2021. The point prevalence rate of CP in 2021 was 45.52/100000 individuals for the total population. Diabetes (26.32%), other metabolic-related diseases, and pancreatic cancer are among the common comorbidities in CP patients. The current work shows that CP continues to place a heavy burden on patients and the healthcare system.

Research perspectives

Further studies are needed to identify CP and its comorbidities earlier, triggering potentially preventive mana-gement to relieve the disease burden.

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FOOTNOTES

Author contributions: Cai QY analyzed the data and wrote the manuscript; Tan K and Zhang XL collected the data; Han X established the database and cleaned the data; Pan JP managed the data; Huang ZY analyzed the data; Tang CW designed the study and revised the manuscript; Li J designed the study and revised the manuscript.

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META-ANALYSIS

Diagnostic value of conventional endoscopic ultrasound for lymph node metastasis in upper gastrointestinal neoplasia: A meta-analysis

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Abstract

BACKGROUND

Upper gastrointestinal neoplasia mainly includes esophageal cancer and gastric cancer, both of which have high morbidity and mortality. Lymph node metastasis (LNM), as the most common metastasis mode of both diseases, is an important factor affecting tumor stage, treatment strategy and clinical prognosis. As a new fusion technology, endoscopic ultrasound (EUS) is becoming increasingly used in the diagnosis and treatment of digestive system diseases, but its use in detecting LNM in clinical practice remains limited.

AIM

To evaluate the diagnostic value of conventional EUS for LNM in upper gastrointestinal neoplasia.

METHODS

Using the search mode of "MeSH + Entry Terms" and according to the predetermined inclusion and exclusion criteria, we conducted a comprehensive search and screening of the PubMed, EMBASE and Cochrane Library databases from January 1, 2000 to October 1, 2022. Study data were extracted according to the predetermined data extraction form. The quality of the included studies was assessed using the Quality Assessment of Diagnostic Accuracy Studies tool, and the results of the quality assessment were presented using Review Manager 5.3.5 software. Finally, Stata14.0 software was used for a series of statistical analyses.

RESULTS

A total of 22 studies were included in our study, including 2986 patients. The pooled sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, diagnostic score and diagnostic odds ratio of conventional EUS in the diagnosis of upper gastrointestinal neoplasia LNM were 0.62 [95% confidence interval (CI): 0.50-0.73], 0.80 (95%CI: 0.73-0.86), 3.15 (95%CI: 2.46-4.03), 0.47 (95%CI: 0.36-0.61), 1.90 (95%CI: 1.51-2.29) and 6.67 (95%CI: 4.52-9.84), respectively. The area under the summary receiver operating characteristic curve was 0.80 (95%CI: 0.76-0.83). Sensitivity analysis indicated that the results of the meta-analysis were stable. There was considerable heterogeneity among the included studies, and the threshold effect was an important source of heterogeneity. Univariable meta-regression and subgroup analysis showed that tumor type, sample size and EUS diagnostic criteria were significant sources of heterogeneity in specificity ($P < 0.05$). No significant publication bias was found.

CONCLUSION
Conventional EUS has certain clinical value and can assist in the detection of LNM in upper gastrointestinal neoplasia, but it cannot be used as a confirmatory or exclusionary test.

Key Words: Endosonography; Esophageal neoplasms; Stomach neoplasms; Lymphatic metastasis; Diagnosis; Meta-analysis

Core Tip: This meta-analysis examined the diagnostic value of conventional endoscopic ultrasound (EUS) for lymph node metastasis (LNM) in upper gastrointestinal neoplasia. The pooled analyses of 2986 patients from 22 studies performed herein show that conventional EUS has certain clinical value and can assist in the detection of LNM in upper gastrointestinal neoplasia, but it cannot be used as a confirmatory or exclusionary test. More high-quality studies are needed to further verify the diagnostic value of EUS and determine the best diagnostic criteria.

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INTRODUCTION

Upper gastrointestinal neoplasia mainly includes esophageal cancer and gastric cancer, and their morbidity and mortality have long been among the top ten of the global cancer list, bringing great pain and burden to countries all over the world, and they are major global public health problems[1-4]. The onset of esophageal cancer and gastric cancer is hidden, and the best time for treatment has often been passed by the time they are clinically diagnosed. Lymph node metastasis (LNM), as the most common metastasis mode of both diseases, is an important basis for tumor staging, which largely determines the treatment plan and clinical prognosis of patients[5-8]. For patients with early tumor stages and no LNM, we can attempt endoscopic minimally invasive treatment, but for patients with LNM or advanced tumor stages, it is often necessary to consider comprehensive treatment, including radiotherapy, chemotherapy or surgery[9-11]. One study showed that when esophageal cancer has 0, 1-2 or more than 2 malignant lymph nodes, the median patient survival time is 66 mo, 14.5 mo or 6.5 mo, respectively[12]. Therefore, it is very important to accurately predict LNM.

Endoscopic ultrasound (EUS) combines the advantages of endoscopic technology and ultrasound technology; that is, it can evaluate the mucous membrane of the digestive tract with the naked eye, and it can also be used to detect the hierarchical structure and surrounding tissues of the digestive tract wall with ultrasound wave. EUS has the advantages of close observation distance, high resolution, low price and few adverse events. Since the 1980s, EUS has been gradually used in the diagnosis and treatment of many digestive system diseases, including the staging of gastrointestinal tumors, the identification of submucosal tumors, and the study of pancreatic or biliary tract diseases[13,14]. Conventional EUS uses grayscale imaging technology for analysis, which can clearly display the status of lymph nodes near upper gastrointestinal neoplasia and identify the nature of lymph nodes according to the imaging features. When the endosonographic characteristics of lymph nodes are hypoechoic, round in shape, with a clear boundary and a size greater than 1 cm, the accuracy of conventional EUS in predicting malignant lymph nodes is more than 80%[15]. Some studies have shown that the ability of conventional EUS to detect LNM in upper gastrointestinal neoplasia is better than that of computed tomography and positron emission tomography, but some scholars believe that the diagnostic performance of conventional EUS is poor, and study results have varied widely[16-20]. The purpose of this meta-analysis was to explore the diagnostic value of conventional EUS for LNM in upper gastrointestinal neoplasia to guide clinical practice more effectively.

MATERIALS AND METHODS

This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Review and Meta-Analysis statement[21,22]. The study protocol was registered in the PROSPERO database with the number CRD42022372170.

Literature search

We used the "MeSH + Entry Terms" search mode to conduct a comprehensive search of the PubMed, EMBASE and Cochrane Library databases before October 1, 2022. The specific search terms were as follows: ("esophageal neoplasms" OR "stomach neoplasms" OR "duodenal neoplasms") AND ("lymphatic metastasis" OR "lymph nodes") AND "endosonography" AND "diagnostic test search strategy". We also manually searched the references of related studies.

Study selection

We imported all the retrieved articles into EndNote software (Version X9.1; Clarivate Analytics; Philadelphia, United States). Two researchers independently conducted study selection according to the predetermined inclusion and exclusion criteria with the process of identification, screening, eligibility and inclusion. To ensure consistency, we conducted exercises and tests before the formal selection, and the data were verified for internal consistency with the Kappa test during the selection process. If there was any disagreement, the decision was made by the two researchers together through consultation.

The study inclusion criteria were as follows: (1) Patients older than 18 who had recently been diagnosed with upper gastrointestinal neoplasia such as esophageal cancer, gastric cancer, and duodenal cancer; (2) LNM detected by conventional EUS; and (3) Diagnostic testing.

The exclusion criteria were as follows: (1) Studies published before 2000; (2) Case reports, conference abstracts, reviews, comments, letters, meta-analyses and systematic reviews; (3) Animal or *in vitro* models used as the objects of the study; (4) Sample size less than ten cases; (5) Inclusion of only stage cN0 patients; (6) Patients with other malignant tumors; (7) Patients who received or may have received preoperative neoadjuvant therapy; (8) Use of assistive technologies such as fine needle aspiration (FNA); (9) LNM diagnosis not made with postoperative pathological examination as the gold standard or radical surgery not performed for all patients; (10) Per patient not used as the analysis unit; (11) Inability to extract 2 × 2 tables of true positives (TP), false positives (FP), false negatives (FN), and true negatives (TN); (12) Repeated publication of the same data; and (13) Full text of English literature not found.

Data extraction and quality assessment

Two researchers independently extracted the study data using the predetermined data extraction form, and when they faced disagreement, a third researcher was consulted. Extracted data included: (1) Study characteristics such as first author, publication year, study country, study design and participating center; (2) Diagnostic test characteristics such as EUS model, EUS scan type, EUS examination method, EUS scan frequency, EUS diagnostic criteria, type and number of image interpretation experts, blinding, interval between EUS and surgery, gold standard and analysis unit; (3) Patient/tumor characteristics such as tumor type, tumor location, tumor stage, tumor histological type, neoadjuvant therapy, location of metastatic lymph nodes, age, sex and sample size; and (4) Statistical indicators such as TP, FP, FN, and TN. If the data were not reported directly, the sensitivity, specificity, accuracy and other indicators were used for reverse calculation.

Two researchers independently assessed the quality of the included studies using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool[23]. Disagreements were resolved through consultation. The results of the quality assessment were presented using Review Manager software (Version 5.3.5; Nordic Cochrane Centre; Copenhagen, Denmark).

Statistical analysis

All data evaluation and picture generation were completed by Stata software (Version 14.0; StataCorp LP; Texas, United States) using the MIDAS module of the bivariable mixed effects model. This model not only considers factors such as heterogeneity between studies, threshold effect and study size but also enables the bivariate nature of the original data to remain unchanged throughout the analysis process, thereby generating reliable statistical indicators. The pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic score (DS) and diagnostic odds ratio (DOR) were calculated by drawing forest plots. The higher the values of DS and DOR were, the better the diagnostic effect of conventional EUS. The area under the curve (AUC) was obtained by drawing a summary receiver operating characteristic (SROC) curve, and the diagnostic performance was considered low, moderate, and high for AUCs of 0.5-0.7, 0.7-0.9 and 0.9-1.0, respectively. Fagan's nomogram was used to reveal changes in the posttest probabilities. Likelihood ratio scatter diagram was used to evaluate the diagnostic performance of conventional EUS. Sensitivity analysis was used to assess the influence of individual studies on heterogeneity and observe the stability of the summary statistics. The threshold effect was determined according to whether the ROC plane showed a "shoulder-arm" point distribution. The Q statistical test was applied to assess the heterogeneity among the included studies, and heterogeneity was considered statistically significant when $P < 0.05$. The degree of heterogeneity was estimated based on the I^2 statistic, where $I^2 < 25\%$, 25%-50%, 50%-75%, and ≥ 75% were considered low, moderate, substantial, and considerable heterogeneity, respectively. If the heterogeneity was high, meta-regression and subgroup analysis were used to explore the most significant source of heterogeneity. Publication bias was assessed with Deeks' funnel plot, and $P < 0.05$ indicated statistical significance. The statistical methods of this study were reviewed by Professor Yao Zhang from the Department of Epidemiology, College of Preventive Medicine, Army Medical University of China.

RESULTS

Study selection

The study selection process is shown in Figure 1. A total of 729 articles were retrieved from three databases, and 22 articles were included in the manual search. The complete retrieval strategy of each database and manual search literature catalog can be found in Supplementary Table 1. Among them, 99 repeated articles were excluded after checking duplicates with EndNote software, 525 obviously irrelevant articles were excluded after reading the publication year, title and abstract, 8 articles were not published in English, 97 articles that did not meet the requirements were excluded after full-text reading, and 22 articles were included in the analysis according to the screening criteria[24-45]. In addition, the Kappa coefficient of the consistency test of the final selection results of the two researchers was 0.810 (P = 0.000).

Characteristics and quality of the included studies

This meta-analysis included 22 studies with 2986 patients. The basic information of the studies is shown in Table 1, and the detailed information is shown in Supplementary Table 2. Among them, the vast majority of studies were retrospective studies (21/22, 95.5%) and single center studies (20/22, 90.9%); ten studies were conducted in eastern countries, and twelve studies were conducted in western countries; the objects of twelve studies and ten studies were esophageal cancer and gastric cancer, respectively; none of the patients received neoadjuvant therapy before EUS and surgery, and the gold standard for the diagnosis of LNM in all studies was postoperative pathology. The results of the quality assessment based on the QUADAS-2 tool are shown in Figure 2, and detailed quality assessment information is shown in Supplementary Table 3.

Meta-analysis outcomes

Primary outcomes: The pooled sensitivity and specificity of conventional EUS in the diagnosis of upper gastrointestinal neoplasia LNM were 0.62 [95% confidence interval (CI): 0.50-0.73, I^2 = 91.50%] and 0.80 (95%CI: 0.73-0.86, I^2 = 86.10%), respectively, as shown in Figure 3A. According to the SROC curve, the AUC was 0.80 (95%CI: 0.76-0.83), as shown in Figure 4.

Secondary outcomes: The pooled PLR, NLR, DS, and DOR of conventional EUS in the diagnosis of upper gastrointestinal neoplasia LNM were 3.15 (95%CI: 2.46-4.03, I^2 = 61.17%), 0.47 (95%CI: 0.36-0.61, I^2 = 92.21%), 1.90 (95%CI: 1.51-2.29, I^2 = 60.94%) and 6.67 (95%CI: 4.52-9.84, I^2 = 99.99%), respectively, as shown in Figures 3B and C. The likelihood ratio scatter diagram showed that the summary PLR and NLR for the index test were in the fourth quadrant, suggesting that conventional EUS cannot be used as a confirmatory or exclusionary test, as shown in Figure 5. According to Fagan's nomogram, when the EUS results were positive, the probability of diagnosing LNM increased from 50% to 76%; when the EUS results were negative, the probability of diagnosing LNM decreased from 50% to 32%, as shown in Figure 6.

Validation of meta-analysis results

Sensitivity analysis: We conducted sensitivity analysis by eliminating studies one by one, and the results showed that the pooled sensitivity change rate was ≤ 4.84% (I^2 change rate ≤ 2.75%), and the pooled specificity change rate was ≤ 2.50% (I^2 change rate ≤ 5.04%), indicating that the results of the meta-analysis were stable. Detailed data from the sensitivity analysis are shown in Supplementary Table 4.

Heterogeneity: Based on the Q statistical test and I^2 statistic, considerable heterogeneity was observed in the analysis for diagnostic sensitivity and specificity of conventional EUS. The ROC plane showed that the sensitivity was positively correlated with (1 - specificity), resulting in a "shoulder-arm" point distribution and indicating the existence of a threshold effect, as shown in Figure 7. According to the calculations from Stata software, the proportion of heterogeneity likely due to the threshold effect was 0.54. Because of the obvious heterogeneity among studies, we included five covariates: Tumor type (esophageal cancer or gastric cancer), study area (eastern country or western country), publication year (2010-2018 or 2000-2009), sample size (≥ 100 cases or < 100 cases) and EUS diagnostic criteria (criteria 1 or criteria 2). Univariable meta-regression and subgroup analysis were performed to identify the significant sources of heterogeneity. The results showed that tumor type, sample size and EUS diagnostic criteria were significant sources of heterogeneity in specificity (P < 0.05), as shown in Figure 8. The covariable assignment instructions are shown in Supplementary Table 5. The indicators for evaluating the diagnostic value of conventional EUS in each subgroup are shown in Table 2.

Publication bias

Deeks' funnel plot showed that the distribution of all studies was relatively symmetrical; the asymmetry was not statistically significant (P = 0.654), indicating that there was no significant publication bias among the 22 studies, as shown in Figure 9.

DISCUSSION

In this meta-analysis, upper gastrointestinal neoplasia was considered as a whole, and the diagnostic value of conventional EUS for LNM was analysed. To ensure the reliability of the research results, we excluded studies that were old, had incomplete data, had small sample sizes and were published in languages other than English. The effects of incomplete surgical resection, neoadjuvant therapy, animal experiments, assistive technologies and other malignant tumors on the

Table 1 Characteristics of the included studies

Ref.	Country	Study design	Center	EUS scan type	EUS scan frequency (MHz)	EUS diagnostic criteria[1]	Gold standard	Tumor type	Age[2] (yr)	Sample size (cases)	TP	FP	FN	TN
Jeong *et al* [24], 2018	Korea	Retrospective	1	Radial	12/20	Criteria 1	Postoperative pathology	Esophageal cancer	64	435	57	31	80	267
Shi *et al* [25], 2017	China	Retrospective	1	Radial	-	Criteria 2	Postoperative pathology	Esophageal cancer	59	86	28	5	8	45
Shan *et al* [26], 2015	China	Prospective	1	Radial	7.5	Criteria 1	Postoperative pathology	Esophageal cancer	≥ 44	94	11	5	23	55
Lee *et al* [27], 2014	Korea	Retrospective	1	Radial	7.5/12/20	Criteria 2	Postoperative pathology	Esophageal cancer	69	12	2	0	3	7
Meister *et al* [28], 2013	Germany	Retrospective	5	Radial	20	Criteria 1	Postoperative pathology	Esophageal cancer	≥ 34	93	39	12	12	30
Yen *et al* [29], 2012	China	Retrospective	1	Radial	12/20	Criteria 2	Postoperative pathology	Esophageal cancer	≥ 43	27	5	12	0	10
Pech *et al* [30], 2010	Germany	Retrospective	1	Radial	7.5-10	Criteria 1	Postoperative pathology	Esophageal cancer	64	179	48	29	20	82
Machlenkin *et al*[31], 2009	Israel	Retrospective	1	Radial	7.5-12	Criteria 1	Postoperative pathology	Esophageal cancer	≥ 28	13	2	0	2	9
Mennigen *et al*[32], 2008	Germany	Retrospective	1	-	7.5/15	Criteria 1	Postoperative pathology	Esophageal cancer	65	97	49	15	10	23
Shimpi *et al* [33], 2007	United States	Retrospective	1	Radial	20	Criteria 1	Postoperative pathology	Esophageal cancer	-	37	9	1	3	24
Shinkai *et al* [34], 2000	Japan	Retrospective	1	Radial	7.5/12/ 15/20	Criteria 1	Postoperative pathology	Esophageal cancer	≥ 42	102	41	20	13	28
Richards *et al*[35], 2000	United Kingdom	Retrospective	1	Radial	7.5/12	Criteria 1	Postoperative pathology	Esophageal cancer	≥ 35	69	19	9	23	18
Li *et al*[36], 2017	China	Retrospective	1	Radial	5/7.5/ 10/12	Criteria 2	Postoperative pathology	Gastric cancer	57	81	48	4	3	26
Serrano *et al*[37], 2016	United States	Retrospective	1	Radial	7.5/10	Criteria 2	Postoperative pathology	Gastric cancer	≥ 42	46	8	6	8	24
Spolverato *et al*[38], 2015	United States	Retrospective	7	-	-	Criteria 2	Postoperative pathology	Gastric cancer	-	144	34	12	36	62
Fairweather *et al*[39], 2015	United States	Retrospective	1	Radial	5-10	Criteria 2	Postoperative pathology	Gastric cancer	67	49	2	3	25	19
Feng *et al* [40], 2013	China	Retrospective	1	-	5/7.5/12/ 15/20	Criteria 2	Postoperative pathology	Gastric cancer	57	610	307	45	118	140
Kutup *et al* [41], 2012	Germany	Retrospective	1	Radial	7.5/10/12	Criteria 2	Postoperative pathology	Gastric cancer	61	123	64	18	17	24
Zheng *et al* [42], 2011	China	Retrospective	1	Radial	7.5/12	Criteria 2	Postoperative pathology	Gastric cancer	58	162	48	20	49	45
Bohle *et al* [43], 2011	Germany	Retrospective	1	Radial	5-20	Criteria 1	Postoperative pathology	Gastric cancer	63	62	30	5	9	18
Hwang *et al* [44], 2010	Korea	Retrospective	1	Radial	5/7.5/ 12/20	Criteria 2	Postoperative pathology	Gastric cancer	≥ 49	247	16	6	67	158
Bentrem *et al*[45], 2007	United States	Retrospective	1	-	7.5-12	Criteria 2	Postoperative pathology	Gastric cancer	-	218	81	39	27	71

[1]Criteria 1 (hypoechoic, round, well-defined margin, diameter ≥ 10 mm), Criteria 2 (others).

[2]Mean age or youngest age.

EUS: Endoscopic ultrasound; TP: True positives; FP: False positives; FN: False negatives; TN: True negatives.

Table 2 Subgroup analysis of the diagnostic value of conventional endoscopic ultrasound for lymph node metastasis in upper gastrointestinal neoplasia

Subgroup		Studies	Sensitivity (95%CI)	Specificity (95%CI)	PLR (95%CI)	NLR (95%CI)	DS (95%CI)	DOR (95%CI)	AUC (95%CI)
	All studies	22	0.62 (0.50-0.73)	0.80 (0.73-0.86)	3.15 (2.46-4.03)	0.47 (0.36-0.61)	1.90 (1.51-2.29)	6.67 (4.52-9.84)	0.80 (0.76-0.83)
Tumor type	Esophageal cancer	12	0.64 (0.51-0.76)	0.81 (0.70-0.88)	3.33 (2.27-4.87)	0.44 (0.33-0.59)	2.02 (1.53-2.50)	7.52 (4.64-12.18)	0.79 (0.75-0.83)
	Gastric cancer	10	0.59 (0.38-0.76)	0.80 (0.71-0.87)	2.95 (2.09-4.17)	0.52 (0.34-0.79)	1.74 (1.09-2.40)	5.71 (2.96-10.99)	0.79 (0.75-0.82)
Study area	Eastern country	10	0.63 (0.42-0.80)	0.84 (0.72-0.91)	3.83 (2.46-5.95)	0.44 (0.28-0.71)	2.15 (1.48-2.83)	8.62 (4.38-16.94)	0.82 (0.79-0.85)
	Western country	12	0.61 (0.48-0.73)	0.76 (0.69-0.82)	2.57 (2.13-3.11)	0.51 (0.38-0.67)	1.62 (1.26-1.99)	5.07 (3.53-7.30)	0.77 (0.73-0.80)
Publication year	2010-2018	16	0.60 (0.44-0.74)	0.82 (0.75-0.88)	3.36 (2.58-4.38)	0.48 (0.35-0.68)	1.94 (1.47-2.41)	6.93 (4.34-11.08)	0.81 (0.77-0.84)
	2000-2009	6	0.70 (0.56-0.81)	0.75 (0.56-0.88)	2.79 (1.50-5.18)	0.41 (0.27-0.62)	1.93 (1.01-2.85)	6.87 (2.74-17.22)	0.78 (0.74-0.81)
Sample size	≥ 100 cases	9	0.60 (0.46-0.73)	0.78 (0.67-0.87)	2.79 (2.14-3.65)	0.51 (0.40-0.65)	1.71 (1.45-1.97)	5.52 (4.24-7.18)	0.76 (0.72-0.80)
	< 100 cases	13	0.65 (0.61-0.90)	0.83 (0.72-0.89)	3.80 (2.45-5.89)	0.42 (0.26-0.69)	2.20 (1.42-2.99)	9.04 (4.13-19.81)	0.83 (0.80-0.86)
EUS diagnostic criteria[1]	Criteria 1	10	0.62 (0.50-0.73)	0.79 (0.71-0.86)	3.04 (2.38-3.88)	0.47 (0.37-0.60)	1.86 (1.58-2.14)	6.43 (4.84-8.54)	0.78 (0.74-0.82)
	Criteria 2	12	0.61 (0.41-0.79)	0.81 (0.70-0.88)	3.17 (2.19-4.60)	0.48 (0.30-0.75)	1.89 (1.22-2.57)	6.63 (3.37-13.05)	0.80 (0.76-0.83)

[1]Criteria 1 (hypoechoic, round, well-defined margin, diameter ≥ 10mm), Criteria 2 (others).

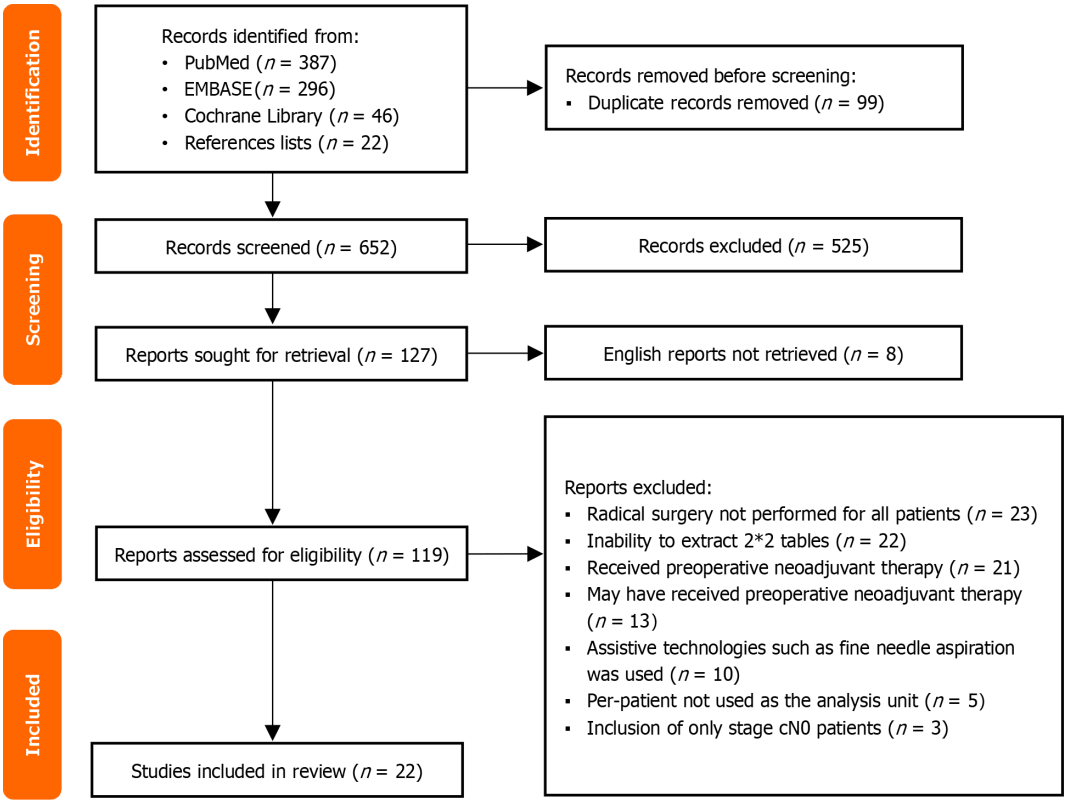
PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; DS: Diagnostic score; DOR: Diagnostic odds ratio; AUC: Area under the curve.

statistical results were excluded (which is also the reason for the small number of studies included in this meta-analysis). The results of conventional EUS were compared with postoperative pathology, and the data of 2986 patients in 22 studies were analyzed in detail.

The results of the quality assessment showed that many studies had a risk of bias, mainly because the proportion of retrospective studies was too high, and selective bias may have been present in the patient inclusion process. Four studies did not clearly describe the diagnostic criteria in the use of EUS, five studies unreasonably excluded some tumor patients (two studies limited the tumor location, two studies defined the location of metastatic lymph nodes, and one study only included esophageal cancer of ≤ pT2 stage), and fifteen studies did not specify the interval between EUS and surgery. However, we believe that since both esophageal cancer and gastric cancer are malignancies, examination and surgery should be arranged as soon as possible after clinical diagnosis. Although many studies did not specify the interval, it should not have had a significant impact on the research results. Regarding concerns regarding applicability, we think that the main reasons were the difference in diagnostic criteria of EUS and bias in patient selection. Therefore, caution should be taken in interpreting the results of the meta-analysis.

Due to the significant heterogeneity among the included studies, we used the bivariate mixed effect model to calculate statistics on various diagnostic evaluation indicators. The results showed that the pooled sensitivity and specificity of conventional EUS in diagnosing LNM in upper gastrointestinal neoplasia were 0.62 and 0.80, respectively, and the AUC of the SROC curve was 0.80, which indicated that the diagnostic value of conventional EUS was moderate. When EUS indicated positive or negative results, the posttest probability could be adjusted from the previous 50% to 76% and 32%, respectively. This result is meaningful for noninvasive examinations, indicating that conventional EUS has certain clinical value. However, it is undeniable that because the PLR < 10 and NLR > 0.1 in conventional EUS diagnosis and the DS and DOR were relatively small, this examination cannot be used to confirm or exclude LNM, which is consistent with the results of previous studies[46-49]. It is not difficult to understand that, as with other imaging examinations, it is difficult for conventional EUS to reach such a high diagnostic level without obtaining lymph node tissue.

We explored the sources of heterogeneity among the included studies. First, we believe that the threshold effect could lead to heterogeneity because the 22 studies adopted a variety of EUS diagnostic criteria, and the "shoulder-arm" point distribution in the ROC plane also confirmed our view; the threshold effect might contribute 54% of the heterogeneity. Then, in view of the differences among the various studies, we included five covariables that could be easily grouped according to the collected data for meta-regression and subgroup analysis. Considering the limited number of studies, it would have been difficult to guarantee the accuracy of the statistical results of the simultaneous inclusion of five covariates, so we included individual covariates one by one for analysis. Although we were unable to identify significant

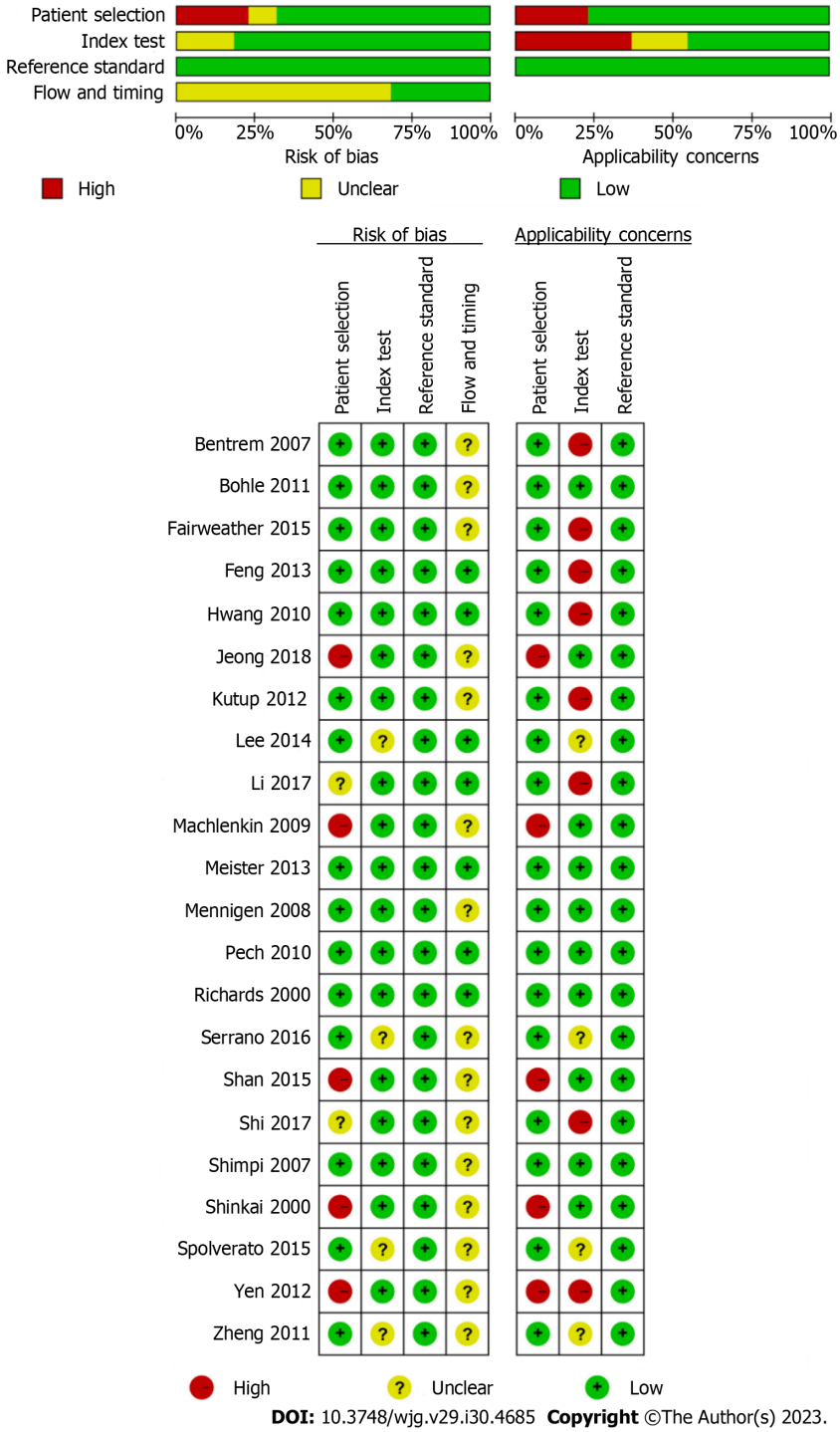


DOI: 10.3748/wjg.v29.i30.4685

Figure 1 Flow chart for study selection.

sources of heterogeneity in sensitivity, we found that the significant sources of heterogeneity in specificity included tumor type, sample size, and EUS diagnostic criteria. However, after excluding the influence of the above factors, the heterogeneity within each subgroup was still obvious. Therefore, we have reason to believe that the heterogeneity was caused by a combination of factors. Many unincluded factors may also have been sources of heterogeneity; examples and the reasons they were not analyzed in detail included the study design, participating center and EUS scan type (because of the proportion imbalance within the group), the qualifications of the endoscopists, the EUS model and scan frequency (because of the complexity of the data), and the tumor stage, tumor location and location of metastatic lymph nodes (because these could not be accurately distinguished). We also found that the study area was not a significant source of heterogeneity, indicating that the diagnostic performance of conventional EUS for LNM in patients with upper gastrointestinal neoplasia in eastern and western countries is comparable. Publication year was also not a significant source of heterogeneity, indicating that the diagnostic performance of conventional EUS has not changed significantly in the past 20 years and that there may be technical barriers in conventional EUS that limit opportunities to significantly improve the ability of conventional EUS to identify malignant lymph nodes by relying solely on the diagnostic criteria of size, shape, boundary and echo.

Although the performance of conventional EUS in diagnosing LNM in upper gastrointestinal neoplasia remains nonideal, the diagnostic ability can be greatly improved with the assistance of EUS-guided FNA (EUS-FNA), EUS elastography (EUS-E) and contrast-enhanced EUS (CE-EUS)[50-52]. EUS-FNA uses a slender biopsy needle to perform puncture biopsy for suspicious lesions under the guidance of EUS, which can provide histopathological information and is an accurate method to distinguish between benign and malignant lymph nodes. The sensitivity and accuracy of EUS-FNA in the diagnosis of regional LNM of upper gastrointestinal neoplasia are higher than those of conventional EUS. Chen *et al*[53] included 26 studies with 2753 patients for meta-analysis and found that the pooled sensitivity and specificity of EUS-FNA in differentiating benign and malignant lymph nodes were 87% and 100%, respectively, and the AUC was as high as 0.9912. EUS-E uses different colors to distinguish tissue hardness and displays different color images according to the elastic difference between lymph nodes and surrounding tissues, which can more clearly identify metastatic lymph nodes, improve the diagnostic performance of conventional EUS, and reduce unnecessary biopsies. Xu *et al*[54] included seven studies with 368 patients for meta-analysis and showed that the pooled sensitivity, specificity and AUC of EUS-E in the diagnosis of LNM were 88%, 85% and 0.9456, respectively. CE-EUS obtains enhanced images by using contrast agents, which can provide more information about the lesion tissue and can be used to identify metastatic lymph nodes. Lisotti *et al*[55] included four studies with 336 patients in their meta-analysis and indicated that the pooled sensitivity and specificity of CE-EUS in diagnosing LNM were 82.1% and 90.7%, respectively. However, our study only analyzed the diagnostic value of conventional EUS for LNM of upper gastrointestinal neoplasia, without considering the role of the above assistive technologies, which may underestimate the diagnostic value of EUS and affect the choice of clinicians. Therefore, we can carry out relevant studies in the next stage to evaluate the diagnostic value of various EUS assistive technologies in detail.

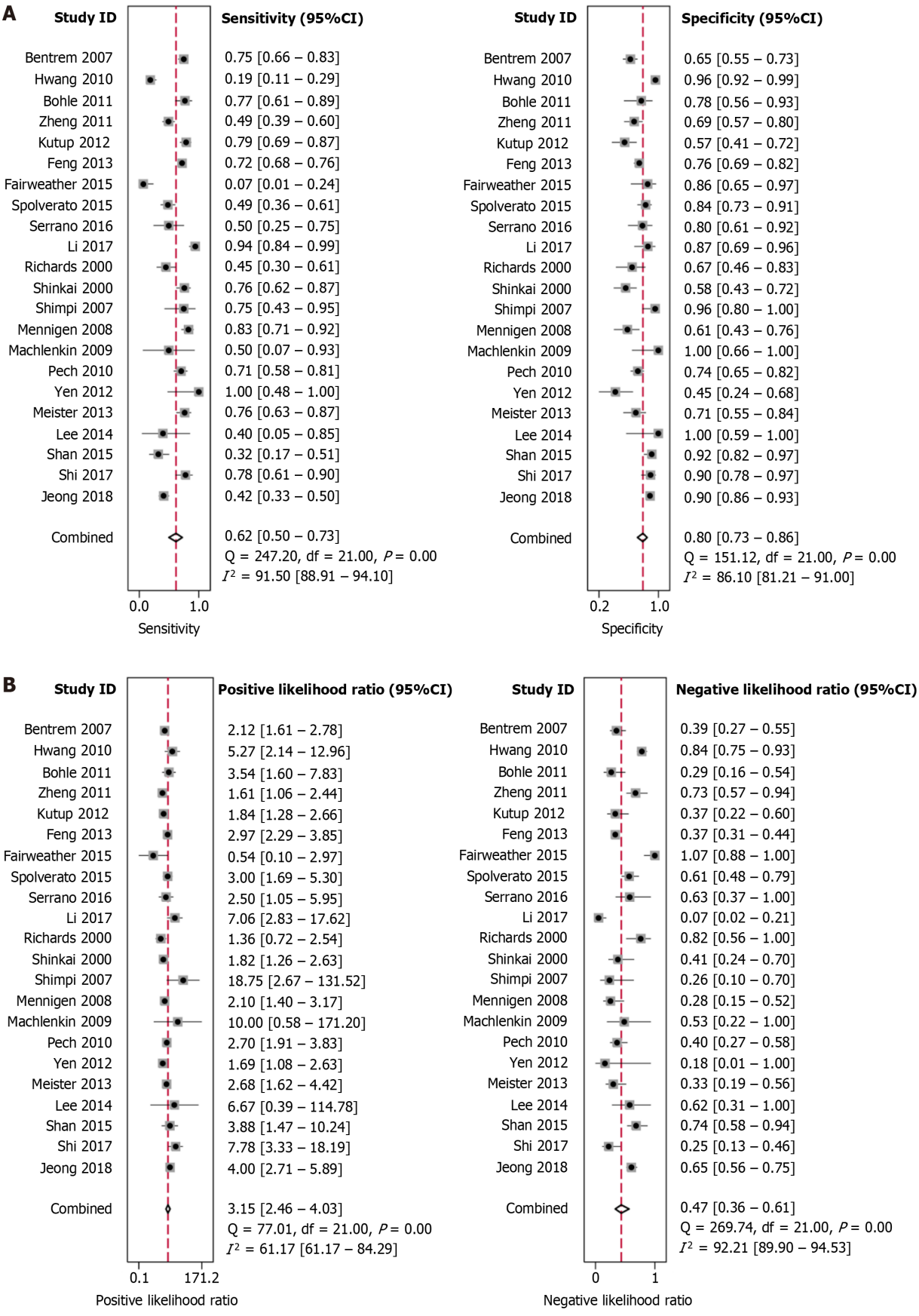


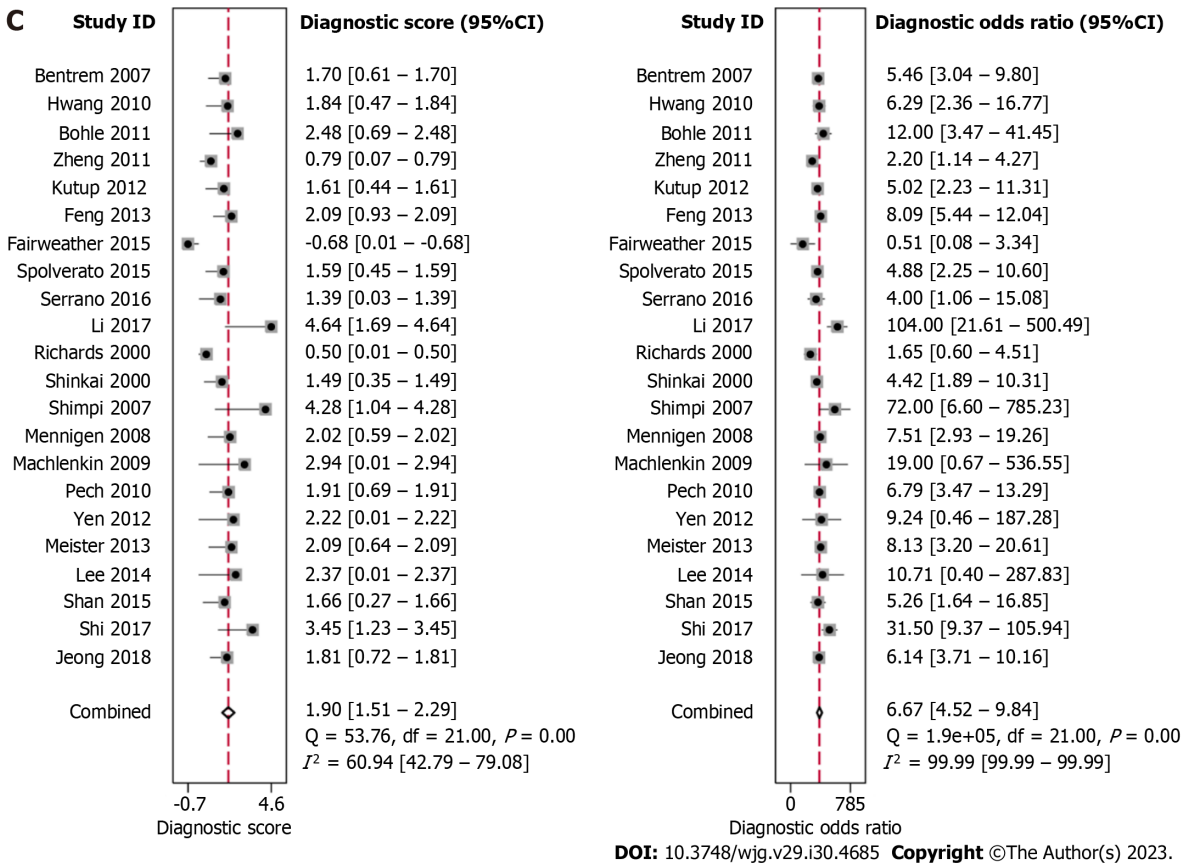
DOI: 10.3748/wjg.v29.i30.4685

Figure 2 Quality assessment of included studies based on the Quality Assessment of Diagnostic Accuracy Studies tool criteria.

Our study only included patients who underwent radical surgery and did not receive preoperative neoadjuvant therapy, which inevitably led to case selection bias and excluded some patients with early tumors suitable for endoscopic treatment or patients with advanced tumors not suitable for surgical treatment. In addition, because preoperative neoadjuvant chemoradiotherapy can improve the treatment effect and prolong the survival time of some patients with upper gastrointestinal neoplasia, some patients with positive LNM may not have received the best treatment in this study. However, it is difficult to know the exact situation of LNM without obtaining complete pathological tissue, and preoperative neoadjuvant therapy will cause necrosis, fibrosis or inflammation of lymph nodes, which will affect the diagnostic effect of conventional EUS and the manifestations of postoperative histopathology. Therefore, to provide a reliable reference standard, we had to abandon the above cases in the study design stage.

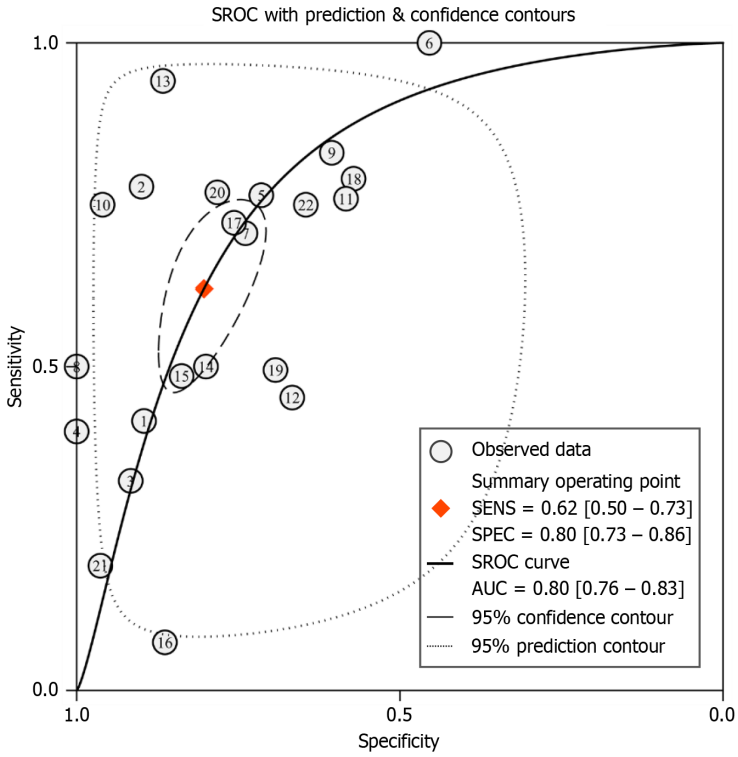
Our study also has the following limitations. First, there were many retrospective studies with a long time span and use of different technologies and tools, which may have led to selection bias. Second, only studies published in English were included, which may have led to information bias. Third, there were differences in the study designs and implementation processes, which may have led to confounding bias. In addition, the significant heterogeneity may have


A
Study ID
Sensitivity (95%CI)
Bentrem 2007 0.75 [0.66 – 0.83]
Hwang 2010 0.19 [0.11 – 0.29]
Bohle 2011 0.77 [0.61 – 0.89]
Zheng 2011 0.49 [0.39 – 0.60]
Kutup 2012 0.79 [0.69 – 0.87]
Feng 2013 0.72 [0.68 – 0.76]
Fairweather 2015 0.07 [0.01 – 0.24]
Spolverato 2015 0.49 [0.36 – 0.61]
Serrano 2016 0.50 [0.25 – 0.75]
Li 2017 0.94 [0.84 – 0.99]
Richards 2000 0.45 [0.30 – 0.61]
Shinkai 2000 0.76 [0.62 – 0.87]
Shimpi 2007 0.75 [0.43 – 0.95]
Mennigen 2008 0.83 [0.71 – 0.92]
Machlenkin 2009 0.50 [0.07 – 0.93]
Pech 2010 0.71 [0.58 – 0.81]
Yen 2012 1.00 [0.48 – 1.00]
Meister 2013 0.76 [0.63 – 0.87]
Lee 2014 0.40 [0.05 – 0.85]
Shan 2015 0.32 [0.17 – 0.51]
Shi 2017 0.78 [0.61 – 0.90]
Jeong 2018 0.42 [0.33 – 0.50]
Combined 0.62 [0.50 – 0.73]
Q = 247.20, df = 21.00, P = 0.00
I² = 91.50 [88.91 – 94.10]
0.0 1.0
Sensitivity
Study ID
Specificity (95%CI)
Bentrem 2007 0.65 [0.55 – 0.73]
Hwang 2010 0.96 [0.92 – 0.99]
Bohle 2011 0.78 [0.56 – 0.93]
Zheng 2011 0.69 [0.57 – 0.80]
Kutup 2012 0.57 [0.41 – 0.72]
Feng 2013 0.76 [0.69 – 0.82]
Fairweather 2015 0.86 [0.65 – 0.97]
Spolverato 2015 0.84 [0.73 – 0.91]
Serrano 2016 0.80 [0.61 – 0.92]
Li 2017 0.87 [0.69 – 0.96]
Richards 2000 0.67 [0.46 – 0.83]
Shinkai 2000 0.58 [0.43 – 0.72]
Shimpi 2007 0.96 [0.80 – 1.00]
Mennigen 2008 0.61 [0.43 – 0.76]
Machlenkin 2009 1.00 [0.66 – 1.00]
Pech 2010 0.74 [0.65 – 0.82]
Yen 2012 0.45 [0.24 – 0.68]
Meister 2013 0.71 [0.55 – 0.84]
Lee 2014 1.00 [0.59 – 1.00]
Shan 2015 0.92 [0.82 – 0.97]
Shi 2017 0.90 [0.78 – 0.97]
Jeong 2018 0.90 [0.86 – 0.93]
Combined 0.80 [0.73 – 0.86]
Q = 151.12, df = 21.00, P = 0.00
I² = 86.10 [81.21 – 91.00]
0.2 1.0
Specificity
B
Study ID
Positive likelihood ratio (95%CI)
Bentrem 2007 2.12 [1.61 – 2.78]
Hwang 2010 5.27 [2.14 – 12.96]
Bohle 2011 3.54 [1.60 – 7.83]
Zheng 2011 1.61 [1.06 – 2.44]
Kutup 2012 1.84 [1.28 – 2.66]
Feng 2013 2.97 [2.29 – 3.85]
Fairweather 2015 0.54 [0.10 – 2.97]
Spolverato 2015 3.00 [1.69 – 5.30]
Serrano 2016 2.50 [1.05 – 5.95]
Li 2017 7.06 [2.83 – 17.62]
Richards 2000 1.36 [0.72 – 2.54]
Shinkai 2000 1.82 [1.26 – 2.63]
Shimpi 2007 18.75 [2.67 – 131.52]
Mennigen 2008 2.10 [1.40 – 3.17]
Machlenkin 2009 10.00 [0.58 – 171.20]
Pech 2010 2.70 [1.91 – 3.83]
Yen 2012 1.69 [1.08 – 2.63]
Meister 2013 2.68 [1.62 – 4.42]
Lee 2014 6.67 [0.39 – 114.78]
Shan 2015 3.88 [1.47 – 10.24]
Shi 2017 7.78 [3.33 – 18.19]
Jeong 2018 4.00 [2.71 – 5.89]
Combined 3.15 [2.46 – 4.03]
Q = 77.01, df = 21.00, P = 0.00
I² = 61.17 [61.17 – 84.29]
0.1 171.2
Positive likelihood ratio
Study ID
Negative likelihood ratio (95%CI)
Bentrem 2007 0.39 [0.27 – 0.55]
Hwang 2010 0.84 [0.75 – 0.93]
Bohle 2011 0.29 [0.16 – 0.54]
Zheng 2011 0.73 [0.57 – 0.94]
Kutup 2012 0.37 [0.22 – 0.60]
Feng 2013 0.37 [0.31 – 0.44]
Fairweather 2015 1.07 [0.88 – 1.00]
Spolverato 2015 0.61 [0.48 – 0.79]
Serrano 2016 0.63 [0.37 – 1.00]
Li 2017 0.07 [0.02 – 0.21]
Richards 2000 0.82 [0.56 – 1.00]
Shinkai 2000 0.41 [0.24 – 0.70]
Shimpi 2007 0.26 [0.10 – 0.70]
Mennigen 2008 0.28 [0.15 – 0.52]
Machlenkin 2009 0.53 [0.22 – 1.00]
Pech 2010 0.40 [0.27 – 0.58]
Yen 2012 0.18 [0.01 – 1.00]
Meister 2013 0.33 [0.19 – 0.56]
Lee 2014 0.62 [0.31 – 1.00]
Shan 2015 0.74 [0.58 – 0.94]
Shi 2017 0.25 [0.13 – 0.46]
Jeong 2018 0.65 [0.56 – 0.75]
Combined 0.47 [0.36 – 0.61]
Q = 269.74, df = 21.00, P = 0.00
I² = 92.21 [89.90 – 94.53]
0 1
Negative likelihood ratio



DOI: 10.3748/wjg.v29.i30.4685

Figure 3 Forest plots showing the pooled evaluation indicators and heterogeneity test results. A: Pooled sensitivity and specificity; B: Pooled positive likelihood ratio and negative likelihood ratio; C: Pooled diagnostic score and diagnostic odds ratio. CI: Confidence interval.



DOI: 10.3748/wjg.v29.i30.4685

Figure 4 Summary receiver operating characteristic curve for evaluating the diagnostic performance of conventional endoscopic ultrasound. SROC: Summary receiver operating characteristic; SENS: Sensitivity; SPEC: Specificity; AUC: Area under the curve.

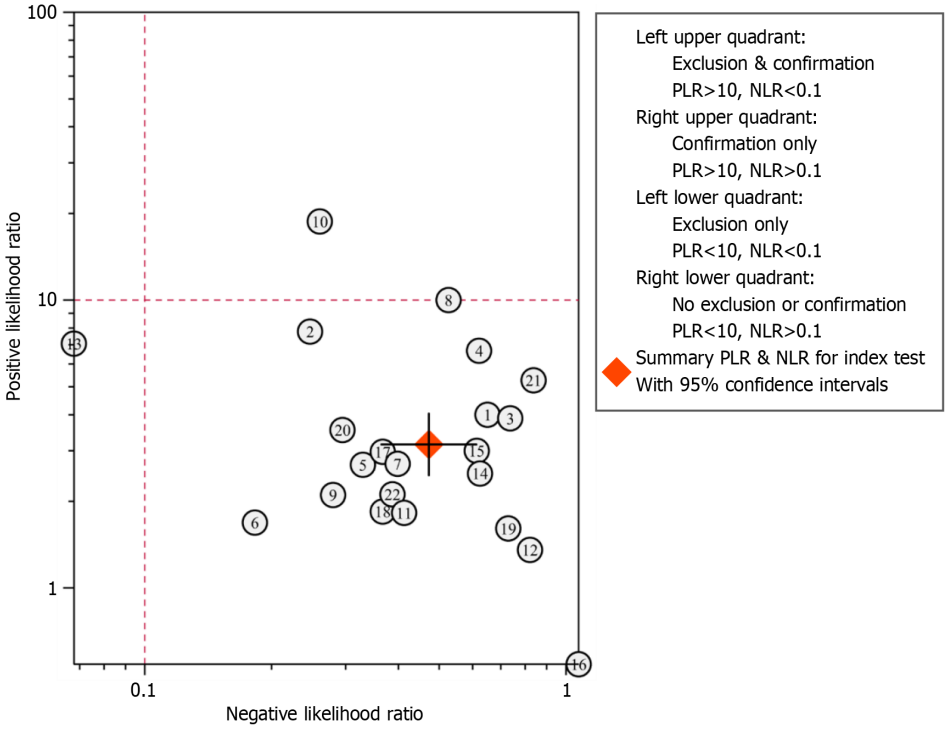


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Figure 5 Likelihood ratio scatter diagram for evaluating the diagnostic performance of conventional endoscopic ultrasound. PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.

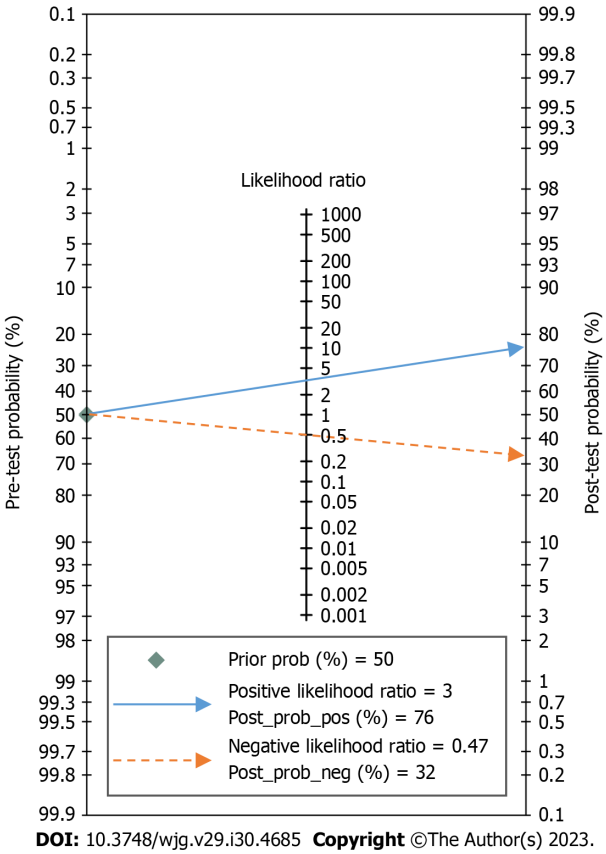

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Figure 6 Fagan's nomogram for the diagnosis of lymph node metastasis with conventional endoscopic ultrasound.

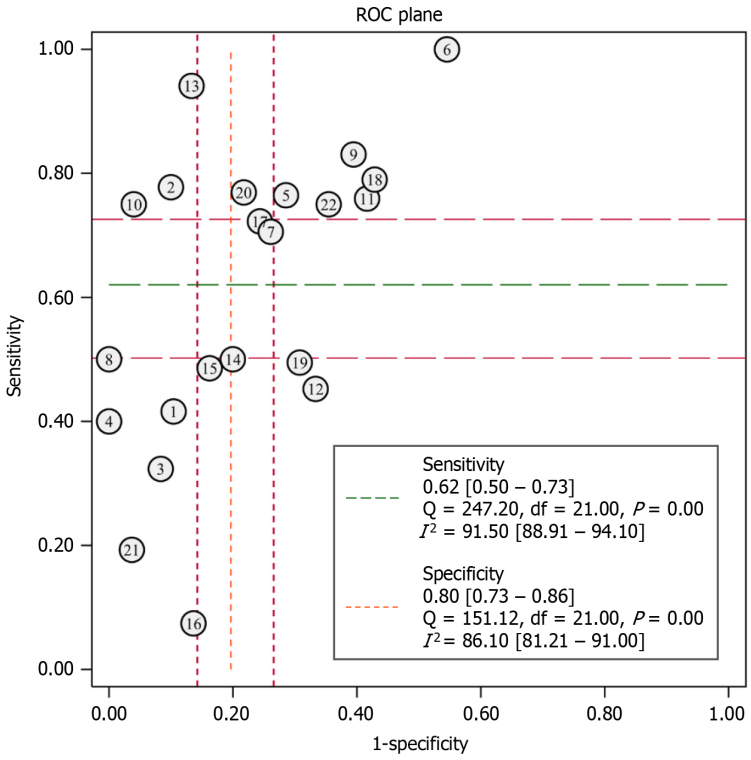

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Figure 7 Receiver operating characteristic plane for testing the threshold effect. ROC: Receiver operating characteristic.

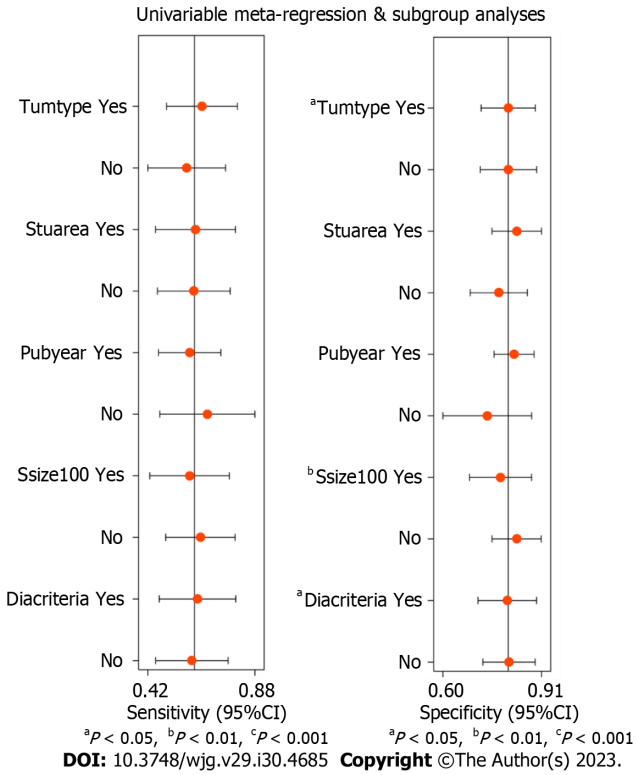

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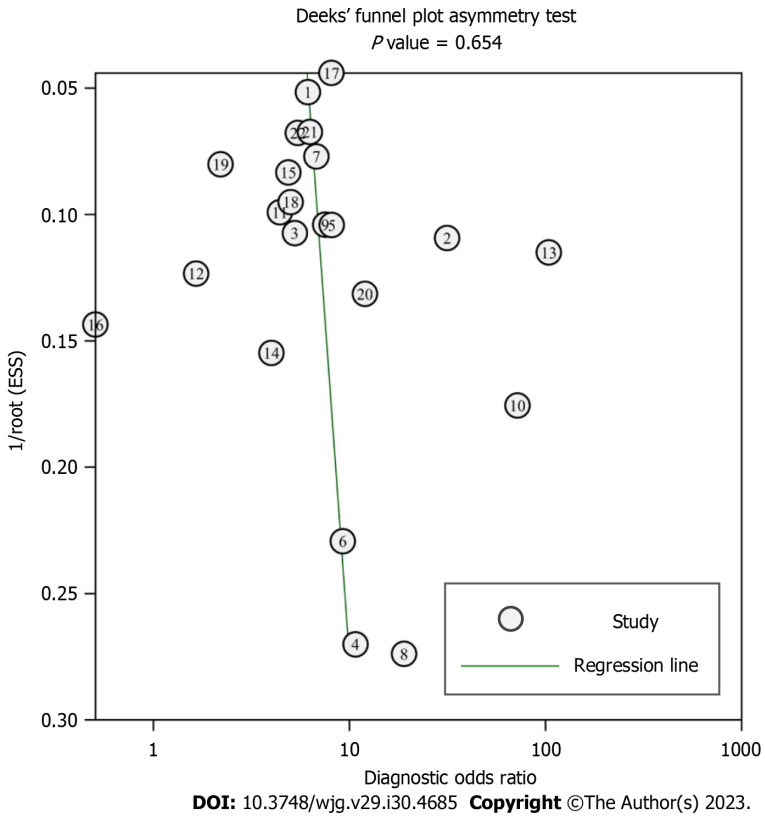
Figure 8 Univariable meta-regression and subgroup analyses for finding sources of heterogeneity. Tumtype: Tumor type (Yes: Esophageal cancer; No: Gastric cancer); Stuarea: Study area (Yes: Eastern countries; No: Western countries); Pubyear: Publication year (Yes: 2010-2018; No: 2000-2009); Ssize: Sample size (Yes: At least 100 cases; No: Less than 100 cases); Diacriteria: Diagnostic criteria (Yes: Hypoechoic, round, well-defined margin, diameter ≥ 10mm; No: Others). CI: Confidence interval.



DOI: 10.3748/wjg.v29.i30.4685

Figure 9 Deeks' funnel plot for assessing publication bias of the included studies.

affected the reliability and repeatability of the analysis results.

CONCLUSION

In conclusion, conventional EUS has certain clinical value and can assist in the detection of LNM in upper gastrointestinal neoplasia, but it cannot be used as a confirmatory or exclusionary test. There was great heterogeneity among the included studies, and more high-quality studies are needed to further verify the diagnostic value of EUS and determine its best diagnostic criteria. However, with the popularization of EUS technology, the use of assistive technologies such as EUS-FNA, EUS-E or CE-EUS, and the training of high-quality endoscopists, we believe that EUS will be increasingly valuable in the diagnosis of LNM in upper gastrointestinal neoplasia.

ARTICLE HIGHLIGHTS

Research background

Upper gastrointestinal neoplasia, mainly including esophageal cancer and gastric cancer, is a common cancer with high mortality. Accurate prediction of lymph node metastasis (LNM) is of great significance for guiding clinical treatment and improving the prognosis of patients. In recent years, endoscopic ultrasound (EUS) has become increasingly used in the diagnosis and treatment of gastrointestinal diseases, but its application in the detection of LNM remains limited.

Research motivation

Although previous studies have reported the diagnostic value of conventional EUS for LNM in upper gastrointestinal neoplasia, the relevant research conclusions were controversial, and the research results have varied widely. Therefore, we intend to further carry out this research through meta-analysis.

Research objectives

This study aimed to systematically search the literature and examine the diagnostic value of conventional EUS for LNM in upper gastrointestinal neoplasia by summarizing and analyzing the data.

Research methods

We conducted a comprehensive search and screening of the PubMed, EMBASE and Cochrane Library databases from January 1, 2000 to October 1, 2022. Then, relevant study data were extracted, and the quality of the included studies was assessed based on the Quality Assessment of Diagnostic Accuracy Studies tool. Afterward, a meta-analysis was performed using the statistical software Stata 14.0.

Research results

A total of 2986 patients in 22 studies were included. The results showed that the pooled sensitivity, specificity and area under the summary receiver operating characteristic curve of conventional EUS in the diagnosis of upper gastrointestinal neoplasia LNM were acceptable, which were 0.62 [95% confidence interval (CI): 0.50-0.73], 0.80 (95%CI: 0.73-0.86) and 0.80 (95%CI: 0.76-0.83), respectively. However, the pooled positive likelihood ratio and negative likelihood ratio were relatively poor, at 3.15 (95%CI: 2.46-4.03) and 0.47 (95%CI: 0.36-0.61), respectively. The pooled diagnostic score and diagnostic odds ratio were relatively small, at 1.90 (95%CI: 1.51-2.29) and 6.67 (95%CI: 4.52-9.84), respectively.

Research conclusions

Conventional EUS has certain clinical value and can assist in the detection of LNM in upper gastrointestinal neoplasia, but it cannot be used as a confirmatory or exclusionary test. More high-quality studies are needed to further verify the diagnostic value of EUS and determine the best diagnostic criteria.

Research perspectives

In the future, further clinical studies should be carried out to evaluate the diagnostic value of various EUS assistive technologies for LNM in upper gastrointestinal neoplasia and to evaluate the influence of neoadjuvant therapy on the diagnostic value of EUS for LNM in upper gastrointestinal neoplasia.

FOOTNOTES

Author contributions: Chen C and Chen L conceived and designed the study; Chen C and Song YL contributed to the literature search, study selection and drafted the manuscript; Chen C, Wu ZY, and Chen L extracted the data; Chen C and Chen J contributed to the quality assessment; Chen C, Song YL, and Zhang Y analyzed the data; Chen C, Wu ZY, and Chen J interpreted the data; Wu ZY edited the manuscript; Chen J revised the manuscript; Chen L and Zhang Y contributed to the critical revision; and all authors have read and approved the final manuscript.

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P-Editor: Zhao S

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LETTER TO THE EDITOR

Pitfalls and promises of bile duct alternatives: There is plenty of room in the regenerative surgery

Ilya D Klabukov, Denis S Baranovskii, Peter V Shegay, Andrey D Kaprin

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Abstract

Current abdominal surgery has several approaches for biliary reconstruction. However, the creation of functional and clinically applicable bile duct substitutes still represents an unmet need. In the paper by Miyazawa and colleagues, approaches to the creation of bile duct alternatives were summarized, and the reasons for the lack of development in this area were explained. The history of bile duct surgery since the nineteenth century was also traced, leading to the conclusion that the use of bioabsorbable materials holds promise for the creation of bile duct substitutes in the future. We suggest three ideas that may stimulate progress in the field of bile duct substitute creation. First, a systematic analysis of the causative factors leading to failure or success in the creation of bile duct substitutes may help to develop more effective approaches. Second, the regeneration of a bile duct is delicately balanced between epithelialization and subsequent submucosal maturation within limited time frames, which may be more apparent when using quantitative models to estimate outcomes. Third, the utilization of the organism's endogenous regeneration abilities may enhance the creation of bile duct substitutes. We are convinced that an interdisciplinary approach, including quantitative methods, machine learning, and deep retrospective analysis of the causes that led to success and failure in studies on the creation of bile duct substitutes, holds great value. Additionally, more attention should be directed towards the balance of epithelialization and submucosal maturation rates, as well as induced angiogenesis. These ideas deserve further investigation to pave the way for bile duct restoration with physiologically relevant outcomes.

Key Words: Bile duct alternative; Bile duct substitute; Regenerative medicine; Regene-

rative surgery; Theoretical surgery; Quantitative human physiology

Core Tip: Progress towards the development of clinically applicable bile duct substitutes can be achieved by applying an interdisciplinary approach. This approach should include the utilization of quantitative mathematical methods, principles of cross-tissue interactions for epithelial and submucosal tissues, as well as deep retrospective data analysis of the causes of success and failure in studies on the creation of bile duct substitutes.

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TO THE EDITOR

We read with great interest the paper by Miyazawa *et al*[1], who analyzed the drawbacks and advantages of various alternatives for bile ducts. The authors described the current approaches for substituting the bile duct and acknowledged that a suitable graft has not yet been developed due to limited understanding of the mechanisms involved in the healing and regeneration of bile duct tissue. They are convinced that the use of bioabsorbable materials may facilitate the creation of bile duct substitutes in the future.

The authors have fairly mentioned that there is a 100-year history of attempts to create satisfactory bile duct substitutes [2]. However, despite significant advancements in material and life sciences, the development of engineered bile ducts has not been successful. None of the grafts currently available are reliable enough for use in clinical practice. Additionally, due to the variant anatomy of bile ducts (Figure 1A)[3,4], it is nearly impossible to completely prevent iatrogenic injuries.

One of the most valuable features of the paper by Miyazawa *et al*[1] is the systematization and analysis of the early attempts to create bile duct alternatives, as application of a systematic approach. The authors traced the history of reconstructive hepatobiliary surgery since the 1880s and discussed many intriguing studies. However, we noticed that the causes of negative or positive results are not usually analyzed or systematized in these studies. For example, in the study by Doillon *et al*[5], it was shown that pre-exposure of a venous graft in glycerol improves surgical outcomes, but the fundamental reasons for such improvement were not investigated in this study, as well as in many other papers.

First, we want to emphasize that the analysis of the causes of negative and positive results is just as important as the results themselves. Additionally, studies that describe the unsuccessful use of rubber tubes[6], polyvinyl chloride[7], silastic[8], lyophilized and siliconized dura mater[9] in both humans and animals are valuable as they shed light on the various causes of postoperative surprises. We believe that a systematic analysis of the factors that contribute to failure or success in creating bile duct substitutes could help develop more effective approaches. It is important to note that such a systematic review has never been conducted before.

Secondly, quantitative methods of analysis can also highlight interesting patterns. Specifically, the studies reviewed by the authors revealed the limitations of bile duct epithelialization, which was found to be approximately 3 cm long and not more than 1-2 mm per week[10-12]. Additionally, the required time for submucosal maturation was estimated to be around 6-12 mo[10,13]. These findings provide support for the hypothesis that there are several deterministic mechanisms of bile duct regeneration that have yet to be discovered and completely understood. Furthermore, this suggests that the regeneration of the bile duct is delicately balanced between epithelialization and subsequent submucosal maturation within limited time frames (Figure 1B).

Thirdly, the utilization of the organism's endogenous regeneration abilities may enhance the effectiveness of bile duct substitutes. Cross-tissue interactions play a crucial role in the process of regeneration, as they determine the normal and pathological proliferation and maturation of tissues[14]. Moreover, in interconnected tissues, not only interactions but also division of labor and competition between different tissues become relevant[15]. This phenomenon has gained significant importance, particularly in relation to the biliary microbiota's role in inflammatory diseases of the biliary tract [16]. Therefore, the correct surgical approach with incompatible tissues, or the reconstruction of biliary tissues with incompatible materials, may lead to the development of chronic disorders and require additional supplementation. For example, some methods that have been underestimated include the stimulation of not only blood vessel growth but also lymphatic vessel growth in the interstitium, as well as the specific chemotaxis of mesenchymal stromal cells to the site of injury (Figure 1C).

Miyazawa *et al*[1] have demonstrated a connection between vascularization/angiogenesis and the maturation of biliary glands, as well as the stabilization of inflammation[17]. Considering the recently discovered crucial role played by vascular endothelial growth factor in biliary tree development and cell cycle regulation[18], inducing angiogenesis could be a promising avenue for future research. It is worth noting that the use of grafts may yield varying outcomes, mainly due to differences in their structure, immunogenicity, and vascular density, regardless of whether they are autologous or

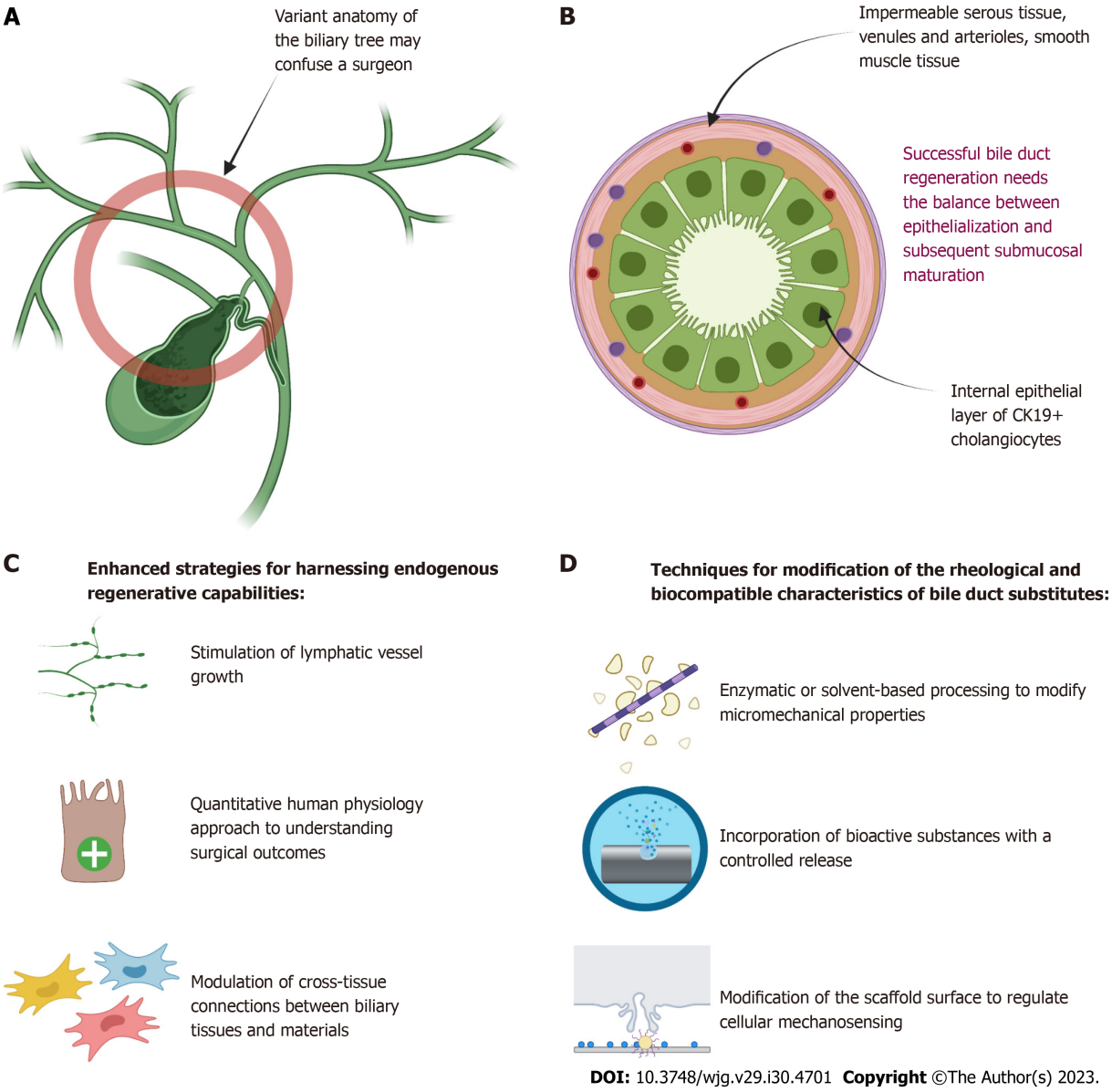

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Figure 1 Bioengineer's Bile duct wallet cards. A: Variant anatomy of the biliary tree may confuse the surgeon; B: Successful bile duct regeneration needs the balance between epithelialization and subsequent submucosal maturation; C: Advanced techniques for the use of endogenous regeneration abilities; D: Techniques for modification of the rheological and biocompatible characteristics of bile duct substitutes. Created with BioRender.com.

allogeneic. Therefore, the mechanical (rheological) and cyto compatible properties of the substitute material are important (Figure 1D).

The functional and biomimetic properties of the bile duct substitute are expected to promote the growth of resident cells and facilitate tissue regeneration[19]. However, significant advancements in regenerative medicine necessitate innovative ideas from interdisciplinary fields. The application of mathematical logic to human physiology has the potential to accelerate progress in tissue engineering[20]. For example, the phase space method, well known in physics, can provide new insights into physiological relevance in bioengineering[21]. This promising and novel concept relies on the application of mathematical logic and machine learning to forecast surgical outcomes, commonly referred to as theoretical surgery[22].

We assume that the views of the surgeon and the biophysicist on the same facts and problems differ, particularly in terms of abstract concepts. This highlights the necessity for collaborative work among specialists from various fields (Table 1). We firmly believe that the integration of quantitative methods and retrospective analysis in bioengineering will open up new avenues for developing models of inter-tissue interaction, ultimately yielding groundbreaking outcomes in bile duct engineering.

Table 1 Differences in understanding of the clinical evidences between a surgeon and a physiologist

Clinical evidences	Understanding of a surgeon	Understanding of a physiologist
There are many negative results of the use of materials for bile duct repair	Materials associated with complications are not suitable for clinical practice	The causes of failure are what we need to determine. Can we cluster these causes to understand the underlying mechanisms? If we only consider successful cases, we will commit survival bias and be unable to determine the reasons for successful outcomes
Bile duct epithelialization never exceed the following limits: About 3 cm long and growth not more than 1-2 mm per week	We must avoid implanting grafts (autografts) longer than 3 cm. This is an interesting fact. My experience supports these values, so I am aware of the potential outcomes that may be achieved after a surgical procedure	The presence of stable values indicates the conservatism of the underlying regenerative mechanisms, which are not dependent on the surgeon's skills or the quality of the materials
The normal human bile is not sterile and contains both living cholangiocytes and normal biliary microbiota	These are interesting facts, but we still lack the necessary tools to support endogenous regeneration in routine clinical practice. The application of cells remains unproven	The presence of living cells in bile may support the existence of unknown ways for the migration of bile duct cells. These methods need to be discovered and applied for bile duct regeneration

FOOTNOTES

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